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Influence of Structurally Distinct Cationic Biocides on Antimicrobial Resistance Development and Virulence in *Pseudomonas aeruginosa*

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By

Germán G. Vargas-Cuebas
B.S., University of Puerto Rico – Mayagüez, 2013

Advisor: William M. Wuest, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Microbiology and Molecular Genetics

2024

Abstract

Influence of Structurally Distinct Cationic Biocides on Antimicrobial Resistance Development and Virulence in *Pseudomonas aeruginosa*

By Germán G. Vargas-Cuebas

Cationic biocides are an essential component in our arsenal against infectious agents. Their presence is ubiquitous in our lives, from biocides used in the food industry as preservatives, to those used for disinfection of biotic and abiotic surfaces in households and clinical settings. My dissertation work focused on two classes of cationic biocide: quaternary ammonium compounds (QACs) and quaternary phosphonium compounds (QPCs).

QACs are a class of cationic biocide commonly found as active ingredients in disinfectants and antiseptics. However, despite their widespread use and abundant production, commercially available QACs lack structural diversity, leading to concerns of ineffectiveness and resistance development. Over the years, the Wuest and Minbiole groups have developed a wide array of QACs and QPCs with diverse structures to address this gap. In my dissertation, I first explored how an intrinsic physicochemical property of QACs can be correlated with their effectiveness as bactericidal agents to improve screening of numerous for disinfectants. Then, we investigated why cationic biocides with distinct chemical structures showed dramatic differences in effectiveness against *P. aeruginosa*, an important pathogen that displays high levels of intrinsic resistance to disinfectants. These investigations led us to uncover how these chemical differences can dictate the cellular target of these compounds in gram-negative pathogens, ultimately leading to differences cellular responses and genetic adaptations. Finally, we show that these strains not only have defined resistant determinants associated with adaptations to these different cationic biocides, but also affect *P. aeruginosa* virulence factor production and pathogenesis differently.

In summary, these studies highlight how cationic biocides can influence virulence in pathogens and highlight the importance of evaluating the intended effects of antimicrobials without overlooking the unintended effects they might have in bacterial physiology and pathogenesis.

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Acknowledgements

I would like to start by thanking my advisor Dr. William M. Wuest for the opportunity to perform research in his lab and allowing me to work with such an amazing team. To my fellow Wuestie Boys, thank you for making me feel so welcomed in the research group, having the patience for my most basic questions about chemistry, and indulging me when a "bio" topic presented itself. My perspective about science improved so much thanks to those small but impactful interactions.

To my Bio Team, thank you for making our research such an enjoyable and collaborative effort. Dr. Christian A. Sanchez, thank you for being a great research partner during these past 2 years, I learned a lot from you! To ARCS Scholar Elise L. Bezold, thank you for bringing that amazing energy to our group, and for dropping in from time to time into my office to chat about random stuff when was most needed.

To the rest of the Team, both present and future, I just have to say I feel honored to have worked alongside a group of such talented and amazing individuals! Ben, Martina, Wanli, Asantha, Sim, LJ, Andy, Carter, Ricardo, Minhua, Amelia, Shehreen, and any other member I forgot to mention, thank you for all the positive vibes and contagious passion for science you bring to lab, for random competition brackets, and for lunch time.

To my previous and current dissertation committee members, thank you for taking the time to discuss science and provide invaluable suggestions. Phil, thank you for agreeing to stay on my committee, and taking on additional responsibilities during certain periods of my graduate career.

Para mi familia…

Primero le quiero agradecer a mis padres, Germán Vargas Irizarry y Evelyn Cuebas Avilés por brindarme el regalo de la vida, por perdonar mis errores y por siempre hacerme sentir bienvenido en casa, aún en mis peores momentos. Además, les quiero agradecer porque nunca falto nada en casa, incluso cuando la situación no era la mejor. Gracias por proveernos a mí y a mis hermanos lo necesario, incluso cuando eso conllevó muchos sacrificios. A mis dos hermanos, Omar Alexander y Axel Omir, gracias por brindarme la oportunidad de sentir lo que es amor de hermanos. Alex, gracias por tomar responsabilidades que no te tocaban, y por todas esas noches haciendo tareas y asignaciones juntos hasta altas horas de la madrugada… quizás rindieron su fruto. Axel, gracias por sembrar la semilla de la curiosidad en las ciencias en mí, y por todos los consejos a través de los años. Los Amo mucho. A Nova, gracias por traerme alegría en los momentos que más lo necesito.

Finalmente, quiero agradecerle a mi amada esposa, Paola Del Mar Rodríguez Ortiz, por casi 20 años de aventuras e increíbles experiencias juntos. Paola, gracias por todos los sacrificios que has hecho y que sigues haciendo por mí, por tu amor incondicional, y por ser el mejor ser humano que he conocido. Soy muy afortunado de tenerte a mi lado, y si hay alguien con quien quiero compartir cada experiencia y logro, es contigo. Dios no pudo regalarme mejor persona para compartir mi vida. Espero con esto pueda compensar un poco todo lo que has hecho por mí a través de los años, y que este pequeño logro te traiga orgullo. Te Amo Mucho…

Alex, como concluíamos casi todas esas tareas en el pasado: "Espero que este escrito le sea de mucho agrado y placer al lector, gracias."

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Introduction

Part I: Antimicrobials

Part 1.1: The threat of antimicrobial resistance

Antimicrobials are essential compounds affecting numerous, if not all, aspects of our daily lives. Unfortunately, this is often taken for granted. They are found in hygiene products and cosmetics, used as preservatives in different food items, and are employed in facilities where food and produce are collected and processed. Importantly, antimicrobials are essential for modern medical practices where they are employed to eliminate or reduce the bioburden of potentially harmful microbial organisms, in addition to their critical role as prophylactic or therapeutic agents (e.g., antivirals and antibiotics). Much of our modern way of life is enabled by the successful use of these antimicrobial compounds.

Nevertheless, these essential aspects of our lives are threatened by the rising resistance or tolerance to antimicrobials.[1, 2] This ever-present threat was so evident that, during an interview that occurred the same year that he won the Nobel prize for his discovery of penicillin, Sir Alexander Fleming stated: “In such a case the thoughtless person playing with penicillin treatment is morally responsible for the death of the man who finally succumbs to infection with the penicillin-resistant organism. I hope this evil can be averted.”.[3] Fleming further predicted that the misuse of penicillin during self-medication could lead to the selection of resistant populations, which could be transmitted between individuals, ultimately leading to the establishment of resistant bacterial populations and treatment failure. Since then, our effective antimicrobial options have been dangerously reduced and are threatened[4], probably more than ever, by resistance development, with a projected death toll that could reach 10 million deaths per year by 2050.[5]

Antimicrobial resistance poses a significant economic and human health burden. It was estimated that in 2019, nearly 5 million deaths were related to bacterial antimicrobial resistance globally, with more than a million directly attributable to resistance.[6] The main pathogens behind these infections were *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, while lower respiratory tract infections (e.g., pneumonia) were significant contributors to these estimates. In terms of the economic impact of antimicrobial resistance, several reports have estimated the costs associated with these antimicrobial-resistant infections to be over 2 billion US dollars, only in the United States.[7, 8] The multifaceted nature of antimicrobial resistance makes it an extremely difficult problem to address, with poor antibiotic stewardship in healthcare and communities, excessive use in food industries (e.g., use of antibiotics as growth promoters or pest control), and the prevalence of antimicrobial resistance mechanism in nature.[9] Antimicrobial resistance mechanisms can range from reducing the effective antimicrobial concentration by reducing permeability or increasing efflux, target modification or bypass, or antimicrobial inactivation through enzymatic activity.[10]

To mitigate these risks, both the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have listed pathogens of heightened concern for public health, including those that display high levels of antibiotic resistance (e.g., carbapenem-resistant *A. baumannii* and multi-drug resistant *P. aeruginosa*), pathogens with alarming levels of resistance to other antimicrobials (e.g., drug-resistant *Candida auris*), and those that become a threat as a consequence of antimicrobial usage (for example, *Clostridioides difficile*).[11, 12] This has bolstered research efforts into the mechanisms behind resistance to antimicrobials, and novel ways to manage or prevent resistance development.

To address a problem of this magnitude, a multi-faceted strategy must be adopted, and resistance to different classes of antimicrobials should not be overlooked. Special attention should be paid to biocides, which provide the first barrier to prevent transmission of microbial pathogens. Biocides are frequently used as preservatives, disinfectants, and antiseptics, serving to decrease the potential for transmission of pathogens, eliminate pathogen reservoirs, and protect the host from infectious agents. Yet, despite their frequent and prevalent used in multiple settings, the potential effects that exposure to these biocides can have on pathogens is often overlooked. As discussed in the following sections, resistance to biocides not only poses a threat to disinfection protocols but can also result in antibiotic cross-resistance, directly contributing to the antimicrobial resistance crisis. A better understanding of the consequences of biocide exposure can help guide the use of these important antimicrobials.

In this introductory chapter, I discuss several classes of biocides, their importance in clinical settings, and their mechanisms of action against bacteria. I then touch on the mechanisms of resistance to cationic biocides, a class of biocides that I have focused on during my dissertation research. Then, I discuss some of the relevant aspects of *P. aeruginosa*, an opportunistic pathogen that displays high intrinsic resistance to biocides and which I used throughout my dissertation research. Finally, I summarize the objectives of my dissertation research, its limitations, and its significance within the context of antimicrobial resistance, and provide a summary of the topics discussed in each chapter of my dissertation.

Part 1.2: Different classes of biocides and their importance in healthcare

Biocides are a critical component in the success of medical procedures. A classic example of this was provided by Dr. Ignaz Phillip Semmelweis, who is known as the “father of infection

control".[13] Dr. Semmelweis noticed a higher number of deaths caused by childbed fever in a child delivery ward handled by doctors dealing with cadavers and autopsies compared to a ward where midwives who had no contact with cadavers handled the deliveries. In 1847, Dr. Semmelweis put in placed the practice of chlorine washings (hand scrubs with chlorinated lime solution) for anyone handling cadavers and observed a sharp decrease in childbed fever mortality.[14] Nowadays, there are numerous classes of biocides routinely used in different aspects in healthcare, dependent upon the object or material been disinfected or sterilized following the framework for infection prevention and control devised by Dr. Earle H. Spaulding.[15, 16] For critical items (e.g., surgical instruments and catheters) that will be in contact with sterile tissues, sterilization methods are required, such as using an autoclave for heat-insensitive materials, strong oxidizing agents such as ethylene oxide or hydrogen peroxide for heat-sensitive materials, or using chemical sterilants (e.g., 0.2 % peracetic acid), only when proper cleansing to remove organic and inorganic materials precedes treatment.[17] Highly reactive biocides such as alkylating aldehydes (glutaraldehyde, formaldehyde, ortho-phthalaldehyde) and oxidizing agents (peracetic acid, hydrogen peroxide) are routinely used for high-level disinfection of semi-critical materials that will come in contact with broken skin or mucous membranes. Because of their high potency, these biocides eliminate almost all microorganisms, with the exception of a low number of bacterial spores, however, they can display high toxicity.[18] Low-level disinfection is used for non-critical objects (e.g., objects that come in contact with intact skin). This group includes, but is not limited to, biocides such as alcohols, phenols, and cationic biocides.

Aldehydes are used for high-level disinfection in healthcare, including medical equipment and materials.[19] For example, glutaraldehyde is used to disinfect equipment or materials at low temperatures, as it displays broad antimicrobial activity and high effectiveness,

even against bacterial spores. However, concerns about the use of glutaraldehyde have been raised due to the observed irritation caused by exposure to this biocide and its general toxicity.[20, 21] Ortho-phthalaldehyde is a newer aldehyde-based biocide for high-level disinfection and is used as an alternative to glutaraldehyde for disinfection of equipment that is sensitive to heat, such as endoscopic materials.[22] In addition to aldehydes, oxidizing agents are also employed for high-level disinfection. This disinfectant group of oxidizers includes highly-reactive antimicrobials such as hydrogen peroxide and peracetic acid.[18]

Milder biocides with lower reactivity are used for intermediate- or low-level disinfection in healthcare. Some oxidizing agents (hypochlorite and ozone treatment) are used to disinfect water and make it drinkable.[23, 24] Povidone-iodine, a strong antiseptic is used for intermediate to low-level disinfection during would healing, and is used in every stage of surgical procedures to prevent surgical site infections.[25, 26] Ethyl alcohol and isopropyl alcohol disinfectant properties have been recognized since the end of the 19th century.[27] Alcohols are used to decontaminate non-living surfaces and skin.[18] Triclosan is a phenolic biocide commonly found in commercial products, such as toothpaste and certain hands-sanitizers.[28] Despite concerns about its potential negative health effects, triclosan is still used as a low-level disinfection biocide to reduce surgical site infections.[29] Sodium hypochlorite is another widely used biocide that is ubiquitously present in households and healthcare facilities.[30] Cationic biocides are a class of biocide widely used as low-level disinfectants and include the bisbiguanides (e.g., chlorhexidine and alexidine), and quaternary ammonium compounds (QACs, e.g., benzalkonium chloride).[18]

Thanks to their broad antimicrobial activity, cationic biocides are widely employed in clinics to reduce the bioburden of pathogens.[31] This class of biocides include compounds with a wide range of chemical structures, modes of action, and usage. Nevertheless, it is believed that at

the in-use concentration (the concentration at which the product is used in clinical or commercial settings), they possess multiple cellular targets, the main one being cellular membranes. Modern cationic biocides were introduced in the 20th century, and since then, their use has increased in both clinical and commercial products.[32] They can be found in cosmetic and personal care products, in a range of household cleaning and disinfection products, as preservatives, eye drops, among others.[19, 33] More recently, due the COVID-19 pandemic, there was a global surge on biocide usage and demand estimated at 600%[18], particularly those containing QACs as active ingredients.[34, 35] Concerns of potential health issues caused by increased exposure to biocides, environmental damage, as well as concerns involving antimicrobial resistance have been raised.[36, 37]

Part 1.3: Biocides and their bactericidal mechanism of action

As mentioned above, biocides can be used for high-, intermediate-, or low-level disinfection based on their reactivity and overall potency dictated by their chemistries and mechanism of action.[18] Chlorine- and iodine-releasing agents, and hydrogen peroxide are oxidizing disinfectants that can be used for low- or high-level disinfection, dependent upon their reactivity and toxicity profiles. These antimicrobials act by oxidizing the amino, sulfhydryl, or thiol groups of macromolecules. Aldehydes (e.g., glutaraldehyde and glyoxal) and ethylene oxide are examples of alkylating agents that act on hydroxyl, amino, carboxyl, or sulfhydryl groups by replacing the hydrogen atom with an alkyl group. These strong antimicrobials are used for sterilization or high-level disinfection owing to their high potency and broad antimicrobial activity against bacteria, fungi, bacterial spores, and viruses. This mechanism of action can also be observed in formaldehyde-releasing agents.[19]

The antimicrobials used for low-level disinfection exhibit a diversity of mechanisms of action. For example, phenolic antimicrobial compounds (e.g., phenol and triclosan) are membrane-active disinfectants that disrupt the integrity of the cytoplasmic membrane and affect the membrane potential. Phenols antimicrobial activity has been recognized for more than a century, used as preservatives in the food industry, and as antiseptics.[38] Their antimicrobial effect lies in the progressive disruption of membranes. The bis-phenol triclosan is another widely used antimicrobial, particularly in hand sanitizers, toothpaste, and mouthwash products, despite its ban 2016 by the FDA in hand soaps due to risks associated with disruption of endocrine functions.[28] Triclosan inhibits the enoyl reductase FabI at low concentrations, ultimately leading to blockage of lipid biosynthesis. Disruption of the membrane altogether is observed at higher concentrations.[39]

On the other hand, the mechanism of action of alcohols as disinfectants is less clear, despite their abundant usage. Alcohols are frequently used in low-level disinfection of abiotic and biotic surfaces, and as preservatives at lower concentrations. Their usage as sanitizers has increased and is more frequently observed nowadays, both healthcare and community usage due to their effectiveness and broad antimicrobial spectrum, relative ease of use, and increased availability.[40] It is believed that the antimicrobial effectiveness of alcohols can be attributed to multiple mechanisms of action, including membrane disruption, protein denaturation, and metabolism interference.[19] Is important to note that their antimicrobial effectiveness is dependent on the availability of water, with an optimal alcohol concentration between 60% and 90 %. This is believed to be true for all alcohols, including those that are most frequently used: ethyl alcohol and isopropyl alcohol.

Sodium hypochlorite is another ubiquitously used compound for abiotic surfaces disinfection, in both healthcare and household settings. This chlorine-releasing agent can also be used for sterilization and antisepsis, depending on its concentration and the contact time. Although its antimicrobial activity is not fully clear, it is believed to rely on its oxidizing effects on cellular proteins. Other oxidizing agents that can also be used for low-level disinfection include improved hydrogen peroxide, a mixture of hydrogen peroxide with peracetic acid, and iodophors.[19, 41] As with other biocides that can be used for high-, intermediate-, or low-level disinfection, their antimicrobial effectiveness and potency will depend on the material being disinfected, the concentration of the biocide, and the time of contact. These biocides can oxidize and disrupt macromolecules including proteins, DNA, RNA, and membranes.

Bisbiguanides are cationic biocides commonly found in antiseptics and mouthwashes, with chlorhexidine representing one of the most recognizable and frequently used bisbiguanides. As mentioned above, chlorhexidine is an important biocide used during the delivery of babies and in post-partum care, outperforming the clinically important antiseptic povidone-iodine in these scenarios [42, 43] The use of chlorhexidine can be beneficial to both the mother and the newborn by reducing the number of wound infections in the mother and neonatal infections through the reduction of bacterial loads during delivery (both during natural or cesarean delivery). Its bactericidal activity relies on the disruption of the cell envelope, particularly the cytoplasmic membrane.[19] Even though chlorhexidine causes damage to the outer membrane and cell wall, this damage is not enough to cause cell death. It is rather the damage caused to the cytoplasmic membrane that leads to lysis. Other members of this cationic biocide group (i.e., alexidine and polymeric biguanides) generate lipid phase separation during membrane perturbation.

QACs are another group of cationic biocides commonly used for low-level disinfection. These cationic biocides are used in both healthcare and household settings as disinfectants of abiotic surfaces, antiseptics, preservatives, and also cleansing/deodorizing agents.[19] This class of cationic biocides includes a range of widely used biocides such as benzalkonium chloride (BAC), didecyldimethylammonium chloride (DDAC), cetrimide, and cetylpyridinium chloride (CPC), among others. The mechanism of action of these QACs lies on the disruption of cellular membranes. In some cases, this disruption has been shown to also affect the proton motive force (PMF) essential for bacterial viability.[44] The outer membrane of gram-negative bacteria can also be disrupted by these compounds.[19]

The breadth of mechanisms of action displayed by biocides shows the complexity of how biocides can exert their antimicrobial effects. In addition, both intrinsic and extrinsic factors can determine which biocide is appropriate for use in distinct scenarios, and how it should be used to maximize their effectiveness as antimicrobials. These considerations are of great importance because omitting them would impact the outcome of disinfection and threaten infection prevention and control protocols and could promote the spread of dangerous pathogens in both healthcare and household settings.

Part 1.4: Bacterial resistance to cationic biocides and associated concerns

Cationic biocides provide one of the first barriers to transmission and spread of harmful and pathogenic bacteria. However, resistance to cationic biocides represent a serious, but often overlooked, public health risk. Extreme cases have been observed when bacterial reservoirs in biocide solutions serve as the source of outbreaks. In 1982, 10 patients were treated for shoulder and knee joint infections. The patients were found to have been treated by orthopedic surgeons

who shared the same office. Further investigation revealed that they were infected with an epidemic strain of *Serratia marcescens.* The source of these infections was a contaminated solution of the cationic biocide benzalkonium chloride (BAC).[45]

A similar situation was reported in Taiwan, where several patients were admitted to the National Taiwan University Hospital in 1990, all with *Bacillis cereus* infections. It was later determined that these infections originated from ethyl alcohol bottles contaminated with this bacterium, with additional *B.* cereus-positive cultures isolated directly from ethyl alcohol bottles and traced back to the supplier.[46] *Burkholderia cepacia* is a gram-negative bacterium known to colonize antiseptic solutions, as exemplified by cases of infections caused by contaminated solutions of chlorhexidine (CHX) and BAC.[47, 48] A 2015 report of an increase in bloodstream infections caused by *B. cepacia complex* from October 2013 to December 2013 in South Korea was also found to be caused by contaminated commercial solutions of chlorhexidine.[49] These cases provide extreme examples of the potential consequences of resistance to different classes of cationic biocides used in healthcare, not taking into account the additional potential negative effects biocide resistance can have on different industries if disinfection protocols fail.

Bacterial resistance to biocides is dependent upon numerous factors, including intrinsic factors related to the microorganisms and their physiology, and extrinsic factors related to the environment within which the biocide exerts its action[18]. The inherent characteristics (intrinsic resistance) of the bacterium in question can establish its susceptibility to biocides. Spore-forming bacteria, such as *B. cereus* and *C. difficile*, can survive disinfection chemical treatments because of the production of endospores that are highly resistant to biocides. *Mycobacterium* spp. possess a cell envelope characterized by a mycolic acid-rich outer membrane, making them intrinsically resistant to biocides.[50] In addition, gram-negative bacteria also show high levels of intrinsic

resistance to biocides due to the presence of an outer membrane that serves as an additional permeability barrier to the action of cationic biocides, as observed with antibiotics.[51] Finally, the propensity of bacteria to live in community-like structures called biofilms provides them with additional barrier to resist biocides. These structural features provide bacteria with the ability to resist, survive, and sometimes thrive despite the antimicrobial effects of biocides, complicating infection prevention and control measures.

In addition to the variety of chemical structures and composition of biocides and the formulation in which they are prepared, additional external factors also dictate their effectiveness. Both the concentration at which a biocide is used, and the contact time allowed for biocide action are key to their effectiveness. Biocide are used as disinfectants and antiseptics at in-use concentrations (i.e., the concentration at which the biocide is applied or used in real-world conditions). However, this does not take into account that the biocide effectiveness can be reduced once applied due to environmental factors during real world applications (e.g., dilution of the biocide when applied, presence of organic materials), and hence it has been proposed the term “during use” to account for this.[52]

Other physical factors, such as the method used to deliver the biocide, the surface to which the biocide is applied to, and the temperature, can affect the antimicrobial activity of biocides. Water hardness is one of environmental factors of particular importance for cationic biocides used as disinfectants, such as QACs.[53-55] The divalent cations present in water are believed to interact with the biocides, inducing precipitation and reducing their antimicrobial activity. This highlights important considerations that must be taken to properly use biocides to ensure high antimicrobial efficacy and prevent resistance development.

Acquired mechanisms of resistance to biocides are also common.[18] These biocide resistance determinants can be particularly troublesome when cross-resistance with other antibiotics is observed. Examples have been observed in several clinically relevant pathogens. Exposure of *Acinetobacter baumannii*, an important nosocomial pathogen, to triclosan resulted in trimethoprim cross-resistance.[56] This cross-resistance phenotype was associated with increased expression of the AdeIJK efflux pump. Triclosan-induced cross-resistance to ampicillin and chloramphenicol was observed in *Escherichia coli* and cross-resistance to isoniazid has been observed in *Mycolicibacterium smegmatis*. These phenotypes were associated with changes to RNA polymerase and lipid metabolism, respectively. Exposure of *Mycobacteroides chelonae* to glutaraldehyde and of *E. coli* to povidone-iodine and chlorophene causes changes in expression of porins and modulation of permeability, resulting cross-resistance to multiple antibiotics of different classes.[18]

In *P. aeruginosa*, continuous exposure to sub-inhibitory concentrations of BAC resulted in a 12-fold increase in MIC compared to the parental strain. Moreover, this strain also displayed resistance to ciprofloxacin (256-fold increase in MIC), a fluoroquinolone antibiotic that targets DNA gyrase and DNA topoisomerase IV.[57] Interestingly, this was associated with a canonical mutation in gyrase, and the reverse (exposure to ciprofloxacin and cross-resistance to BAC) was also observed, suggesting that exposure to BAC can also lead to cross-resistance to a DNA replication inhibitor antibiotic. In addition, Mc Cay and coworkers also isolated strains with increased efflux activity associated with overexpression of the resistance–nodulation–cell division (RND) superfamily tripartite efflux systems MexAB-OprM and MexCD-OprJ. This phenotype was associated with the down regulation of *mexR*, the main repressor of the *mexAB-oprM*, and a missense mutation in *nfxB*, a negative regulator of the *mexCD-oprJ* efflux pump. To

simulate the impact of exposure to BAC in the environment, Kim *et al.* exposed a microbial community from a sediment sample to BAC for four years in a bioreactor. After metagenomic analysis, several bacterial species were isolated that displayed a higher MIC for BAC, including a *P. aeruginosa* environmental isolate. Surprisingly, exposure of this isolate to increasing concentrations of BAC resulted in mutations in *pmrB*, showing higher levels of resistance to polymyxin B. Increased MIC to other antibiotics of this isolate were associated with increased *mexCD-oprJ* overexpression. These results highlight the potential dangers of environmental exposure to disinfectants and the possibility of promoting antibiotic cross-resistance through biocide exposure.

Part II. *Pseudomonas aeruginosa*: a hard-to-kill pathogen

2.1: *Pseudomonas aeruginosa* - aspects of public health and pathogenicity

P. aeruginosa is a non-fermenting gram-negative rod-shaped opportunistic pathogen of high clinical relevance. Strains commonly possess a characteristic blue/green hue or, in some cases, a red/brown color due to the production of different pigments. Both the CDC and the WHO have communicated high levels of concern regarding this pathogen owing to its propensity for multidrug resistance.[12, 58] This threat has become evident since the COVD-19 pandemic, where the number of infections caused by *P. aeruginosa* has continued to increase. This opportunistic pathogen is of particular concern for immunocompromised patients, such as those patients suffering from hematological malignancies or cystic fibrosis.[59, 60] *P. aeruginosa* is a common causative agent of nosocomial infections in the clinic and is also commonly isolated

from wounds during armed conflicts.[61] Due to its high antimicrobial resistance (both to antibiotics and biocides), *P. aeruginosa* continues to persist as a nosocomial pathogen.

P. aeruginosa is a common cause of ventilator-associated pneumonia, and a troubling pathogen in intensive care units.[62] This pathogen is also frequently the causative agent of urinary tract infections, especially those associated with catheters. The ability of *P. aeruginosa* to form intractable biofilms on medical instruments such as catheters makes this pathogen a formidable foe within healthcare settings.[63] This pathogen is also an important cause of bloodstream infections, burn and wound infections, surgical site infections, as well as a key member in polymicrobial infections in cystic fibrosis patients.[64] The high prevalence of *P. aeruginosa* in infections associated with medical instruments shows the recalcitrant nature of this pathogen and its ability to resist biocides, highlighting the need for effective biocides to improve the effectiveness of infection prevention and control protocols and reduce the incidence of these infections.

Part 2.2: *P. aeruginosa* virulence factors

P. aeruginosa pathogenesis is characterized by its multifactorial nature, including its propensity for multidrug resistance, secreted factors, toxins produced, among other virulence factors. Secreted factors produced by *P. aeruginosa* include a range of proteases to its disposal that can be secreted as needed.[65] Elastases (elastase A and elastase B or LasA and LasB) are metalloproteases regulated through quorum-sensing systems (LasR and RhlR) that *P. aeruginosa* secretes and are implicated in its survival during infection.[66] Elastase B is autoproteolytically activated and has been shown to inactivate certain cytokines, degrade immunoglobin G (IgG), degrade collagen (type III and IV), and important for survival during corneal infection.[67, 68] On

the other hand, elastase A requires other protease (e.g., LasB, PrpL, LysC) for activation through cleavage of the LasA propeptide. LasA also has multiple roles in host-pathogen interaction including invasion of host epithelial cells, modulation of its own toxins (ExoS and ExoT) that, as cytotoxic toxins, can behave as invasion inhibitors, in addition to its role in bacterial competition through lysis of *Staphylococci*, a major member of polymicrobial infections.[66, 69] In addition, *P. aeruginosa* produced an alkaline protease (AprA) also important for immune evasion and can cleave numerous substrates relevant to infections and can inactivate human cytokines like γ-interferon and tumor necrosis factor-α.[70, 71] PrpL (also known as protease IV) is a serine protease produced by *P. aeruginosa* commonly found in isolates from patients and shown to be important to facilitate immune evasion by degrading surfactant proteins alongside elastase B to prevent cellular aggregation and uptake by macrophages, in addition to being able to degrade proteins involve in immune responses.[72, 73]

In addition to proteases, *P. aeruginosa* secretes important toxins during infection. *P. aeruginosa* type III secretion system (T3SS) is a complex machinery that includes the needle complex, the translocation apparatus, proteins involved in regulation of the T3SS, in addition to effector proteins and chaperones.[74] These are encoded by 36 genes organized in 5 different operons. Interestingly, only four proteins are secreted by *P. aeruginosa* T3SS: ExoS, ExoT, ExoU, and ExoY. These exotoxins possess different enzymatic activities, including GTPase activating protein activity and adenosine diphosphate ribosyl transferase activity (ExoS and ExoT), phospholipase activity (ExoU), and adenylate cyclase activity (ExoY).[74] These toxins are important virulence factors that endow *P. aeruginosa* with the ability to disrupt the actin cytoskeleton of host cells, prevent phagocytosis, inhibit DNA synthesis, induce cell death, among other functions. However, these effectors have different levels of importance during infection,

with ExoU having the highest impact on *P. aeruginosa* virulence, followed by ExoS.[75] Other toxins secreted by *P. aeruginosa* include the AB-type toxin Exotoxin A (also known as Pseudomonas Exotoxin or PE). This toxin is secreted through type II secretion system and induces cell death through apoptosis.[76]

Secretion of pigments are a defining characteristic of *P. aeruginosa* and an important aspect its pathogenesis. These include pyocyanin, pyoverdine, pyomelanin, and pyorubin.[77] These pigments have different role in *P. aeruginosa* physiology and virulence. Pyocyanin is a known virulence factor capable of modulating the immune response by decreasing inflammation, causing cell death, and affecting the cardiovascular, respiratory, urological, and nervous systems.[78] In addition, pyocyanin has been shown to be capable of aiding *P. aeruginosa* in antimicrobial resistance.[79] Pyoverdine is a siderophore that allows *P. aeruginosa* to scavenge iron during infection, while causing mitochondrial damage in the process.[80] Pyomelanin serve as a protective mechanism during *P. aeruginosa* infection in mice, yet it likely has additional functions as observed in other bacteria.[81] Less is known about the role of pyorubin, but production of this pigment has been observed in both environmental and clinical isolates of *P. aeruginosa*.[82, 83]

Dissertation Summary

Throughout my dissertation research, I have touched on several aspects of QACs and quaternary phosphonium compounds (QPCs). Although these studies only focus on a selection of QACs and QPCs, they further our understanding of the underlaying differences between these biocides, and how a clinically relevant pathogen adapts and responds to them. In no way do these studies encapsulate the extent of available biocide structures and their respective antimicrobial

activities, nor do they provide a complete representation of the potential microbial responses to them, as they are limited to a selected number of antimicrobial compounds and focus mostly on one pathogen. Nonetheless, through these investigations, we have uncovered important aspects about the mechanism of action of these biocides, the different resistance determinants associated with them, and how *P. aeruginosa* strains adapt when exposed to these compounds. These research efforts are presented in the following chapters.

In **Chapter II**, we explore how we can use a physicochemical property of QACs and correlate it to their bactericidal activity. In **Chapter III**, we investigate the mechanisms of action and resistance to a commonly used commercial QAC and compare this to a novel next-generation QPC that displays high potency against *P. aeruginosa*. Then, we further explore how adaptations to these biocides affect virulence and pathogenesis in *P. aeruginosa* in **Chapter IV**. Finally, in **Chapter V**, I provide an overall discussion of our findings. Additional published articles are included in the **Appendix**.

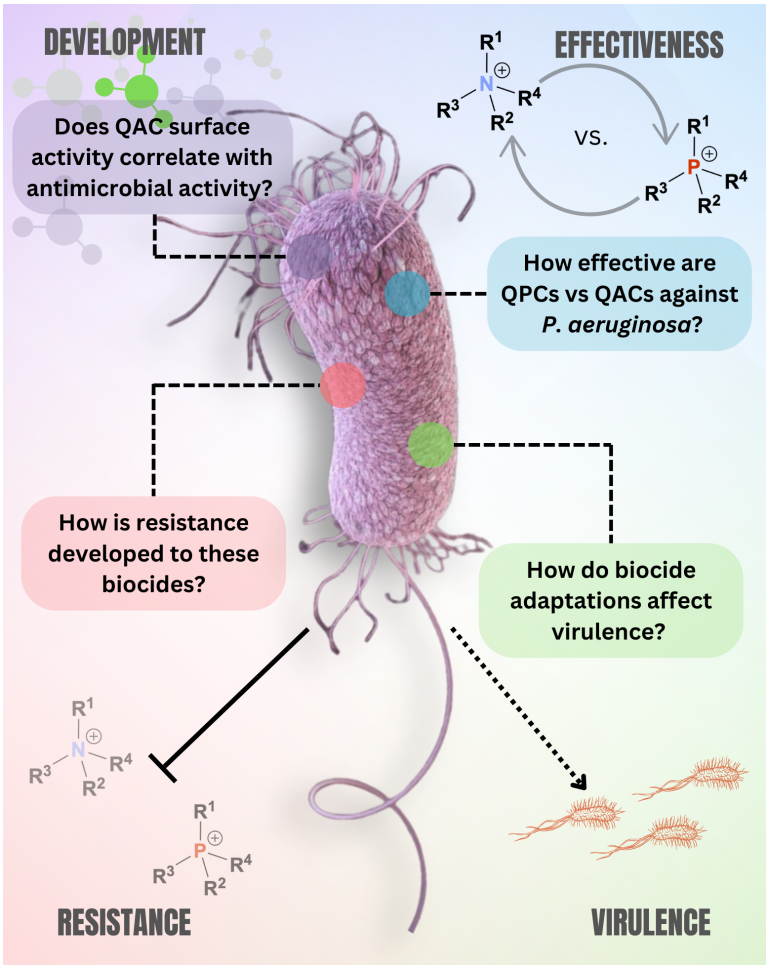

Figure 1. Dissertation research questions summary. My dissertation research indagates QACs and QPCs at different stages, from development of QACs, the effectiveness of QACs and QPCs against *P. aeruginosa*, resistance determinants associated with these cationic biocides, and how genetic adaptations associated with these compounds affect virulence factor production and pathogenesis.

Chapter II

Exploring the correlation of dynamic surface tension with antimicrobial activities of quaternary ammonium-based disinfectants

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Adapted from publication in ChemMedChem 2024 Aug 19;19(16):e202400262. doi: 10.1002/cmdc.202400262 under C.C.C. license number: 5913760331210.

Author contributions: Experiment were conceived and designed by G.G.V.C., C.A.S., A.N., R.M., K.P.C.M, W.M.W., and conducted by G.G.V.C., S.R.B.. Data interpretation and analysis was performed by G.G.V.C., C.A.S., A.N., R.M., K.P.C.M, W.M.W. The manuscript was written and edited by G.G.V.C., C.A.S., A.N., R.M., K.P.C.M., W.M.W..

Abstract

Quaternary ammonium compound (QAC) disinfectants represent one of our first lines of defense against pathogens. Their inhibitory and bactericidal activities are usually tested through minimum inhibitory concentration (MIC) and time-kill assays, but these assays can become cumbersome when screening many compounds. We investigated how the dynamic surface tension (DST) measurements of QACs correlate with these antimicrobial activities by testing a panel of potent and structurally varied QACs against the gram-positive *Staphylococcus aureus* and the gram-negative *Pseudomonas aeruginosa.* We found that DST values correlated well with bactericidal activity in real-world disinfection conditions but not with MIC values. Moreover, no correlation between these two antimicrobial activities of QACs (bactericidal and inhibition) was observed. In addition, we observed that the bactericidal activity of our QAC panel against the gram-negative *P. aeruginosa* was severely affected in the presence of hard water. Interestingly, we found that the counterion of the QAC affects the killing of bacteria in these conditions, a phenomenon not observed in most MIC assessments. Moreover, some of our best-in-class QACs show enhanced bactericidal activity when combined with a commercially available QAC. In conclusion, we determined that an intrinsic physical property of QACs (DST) can be used as a technique to screen for bactericidal activity of QACs in conditions that mimic real-world disinfection conditions.

Introduction

Biocides are often described as the first line of defense against pathogens and are a critical component for effective infection control and prevention. Quaternary ammonium compounds (QACs) are a common class of cationic biocides widely used in healthcare, agriculture, and household disinfection.[84] Their amphiphilic nature allows them to interact with, and disrupt, membranes resulting in antimicrobial activity against a diverse range of pathogens including bacteria, viruses, fungi, and parasites.[85] However, their effectiveness can be hampered by the effects of hard water. The Centers for Disease Control and Prevention (CDC) lists hard water as one of the physical factors affecting the potency of disinfectants, and it is believed that this is caused by ionic interactions and the formation of insoluble precipitates.[16] This effect has been noted since the late 1940s and represents a key metric for commercialization of disinfectants.[86] In addition, development of resistance to QACs has been increasingly reported, emphasizing the need for new QAC development.[87] The concomitant increase in resistance to biocides and other antimicrobials agents limit the available options for effective control and treatment of pathogens, creating a dire public health scenario.[88, 89]

To expand the repertoire of available biocide agents, our group and others have developed additional classes of QACs with diverse structures, as well as their phosphorous analogs.[90-95] In identifying and developing novel antimicrobial compounds efficiently, reliable methods to test important antimicrobial activities are required. The minimum inhibitory concentration (MIC) assay is widely used and defines the lowest concentration of an antimicrobial capable of preventing visible growth of an organism under defined conditions.[96] On the other hand, suspension time-kill assays provide a test that directly measures bactericidal (or killing) activity. Nonetheless, these assays are considered time-consuming, resource-intensive and tedious.[97, 98]

MIC assays are usually performed with a low inoculum (5 x 10^5CFU/mL) and long incubation time (≥ 18 hours), while suspension time-kill assays are typically performed in a timescale of minutes, oftentimes in hard water, using higher concentrations of compound and a high cell density to mimic the conditions of disinfectant usage. Ideally, a method that predicts effectiveness of QACs based on their chemical properties would facilitate the screening process of compounds before performing biological assays.

There has been a longstanding campaign to correlate antimicrobial activity with physical properties of surfactants, such as critical micelle concentration (CMC).[99, 100] Laatiris and coworkers synthetized alkanediyl α,ω-bis(dimethylammonium bromide) gemini surfactants and determined their antimicrobial activity through MIC assays, and their CMC values through conductivity and surface tension measurements.[99] They found that CMC correlated with growth inhibition of *S. aureus*, but this correlation was absent when tested against *E. coli* and *P. aeruginosa,* two gram-negative pathogens. The authors reasoned that this limited correlation was due to the MIC values of their compounds being higher than their CMC values for these bacteria. Similarly, Viscardi and coworkers investigated the correlation of several physical parameters of surfactants to their antimicrobial activity, and found correlation of the hydrophobicity of the compound with antimicrobial activity.[101] To do this, they used the software MacLog P to calculate the C log P values and saw correlation with antimicrobial activity, as determined by suspension bactericidal assay with a 5 minute contact time. However, the lack of correlation to antimicrobial activity against gram-negative pathogens of CMC, the limited temporal resolution of experiments performed in suspension bactericidal assays and the use of specialized software for calculations, reduces the accessibility of generalizable guidelines for surfactant development.

Dynamic surface tension (DST) is another quickly measurable key physical property of amphiphiles that measures the effect of compounds on surface tension in time and can be correlated with the speed of dispersion in a solution.[102] As surface-active compounds, QACs can decrease the surface tension of solutions. In 1926, Frobisher explored how addition of ST reducer or increasing the ST-reducing capacity affected the bactericidal activity of the antiseptic 4-hexylresorcinol.[103] He found that lowering ST could increase bactericidal activity. Building upon this, we decided to explore how DST correlates with both antimicrobial activities: inhibition (measured by MIC assay) and killing (measured by time-kill assay).

The goal of this study was to investigate how the surface activity of a panel of QACs correlates with these inhibitory and bactericidal antimicrobial activities. Specifically, we evaluated how DST values correlate with MIC or log-reduction values (LRV) from time-kill assays performed in conditions mimicking real-world scenarios. In addition, we evaluated how these two antimicrobial activities of QACs correlate between each other. Through our investigations, we found that DST values correlate with LRV but not with MIC values of QACs. In addition, after analyzing the efficacy of commercially available QACs, we found evidence that the counterion of QACs affect their activity in hard water. We leverage this information to increase the effectiveness of our QACs in hard water by combining them with a rapidly diffusible QAC.

Results and Discussion

Selection of QACs and further chemical characterization and bioactivity.

We selected 10 QACs based on structural diversity bioactivity (**Figure 1**).[104-107] These best-in-class QACs showed promising inhibitory activity against both the gram-positive *S.*

aureus and the gram-negative *P. aeruginosa* relative to the benchmark and commercially available mono-QACs benzyldimethyldodecylammonium chloride (BAC) and didecyldimethylammonium chloride (DDAC). This QAC panel has a median MIC value of 0.63 µg/mL against *S. aureus* and 2.5 µg/mL against *P. aeruginosa*. In contrast, the MIC values of BAC and DDAC were 0.36 µg/mL and 1.4 µg/mL against *S. aureus*, and 12 µg/mL and 43 µg/mL for *P. aeruginosa*, respectively. This QAC panel is composed of a selection of bis-cationic QACs with symmetrical alkyl chains of 10 or 12 carbons in length, plus an analogous selection of multi-QACs bearing linear or branched poly-cationic cores.

QAC antibacterial activity against the gram-positive *S. aureus* is not consistently affected by hard water.

Since the QACs display significant inhibitory activity against both gram-positive and gram-negative pathogens by MIC assays, we wanted to assess the bactericidal activity of the QACs through suspension time-kill assays.[90-95] We performed time-kill assays following the American Society for Testing and Materials (ASTM) E2315 standard protocol and report the CFU/mL log reduction values (LRV) at 1, 2, and 5 minutes relative to CFU/mL values at time 0 of untreated controls.[108] This time-assays were performed in both distilled and AOAC 400 µg/mL hard water (400 µg/mL of calcium carbonate), a level of water hardness commonly used when testing disinfectants for commercialization. Our QAC panel was tested using a final concentration of 200 µg/mL. This concentration was selected based on QAC usage during disinfection in real-world scenarios.[87, 109] Importantly, we did not observe precipitate formation of our compounds at this level of water hardness at the QAC concentration tested. To accurately measure the killing efficacy of this panel of QACs over short periods of time, their antimicrobial

activity must be neutralized after the desired contact time. This neutralizing agent must itself be nontoxic, and it must be able to neutralize the compounds almost instantly. To achieve this, we used Dey-Engley (D/E) neutralization broth and performed neutralization tests following the ASTM E1054 protocol.[110] D/E neutralization broth was developed to improve the reproducibility in testing antimicrobial agents, including QACs.[111]

We were able to effectively neutralize the bactericidal activity of the QACs using a preparation of 2X D/E broth. We did not see significant effects of the neutralizer on the viability of *S. aureus* SH1000 and *P. aeruginosa* PAO1 strains after neutralizing the QACs (see **Supplemental Figure 1**). To increase the throughput of these time-kill assays, the ASTM E2315 standard protocol was adapted to a 96-well plate format with spot plating allowing quick assessment of which compounds were most active by visual inspection (see **Supplemental Figures 6 and 7**).

Uniform MIC values were observed against the gram-positive *S. aureus* across the QAC panel, all between 0.34 μg/mL and 0.89 μg/mL (**Table 1**). However, time-kill assays revealed clear differences in bactericidal effectiveness. While QAC **3** displayed both strong inhibitory and killing activity against *S. aureus*, some of the QACs that displayed the strongest inhibitory activity by MIC assay (**7**, **9, 10**) were relatively less effective at killing in time-kill assays in early time points. Conversely, compounds **1**, **2** and **5** showed average MIC values but good bactericidal activity in deionized water. In addition, we observed that hard water strongly reduced the bactericidal activity of compounds **2**, **4** and **7** against *S. aureus*. This was particularly evident after 5 minutes of contact time for QAC **4** and **7**, with a 3-log loss in killing activity compared to that obtained in deionized water. This reduction in killing effectiveness was observed in BAC within 1 minute of contact time but was never observed in DDAC. QACs **1**, **3**,

5 and **8** behave similarly to BAC and DDAC in hard water against *S. aureus*, reducing the bacterial populations below the limit of detection after 5 minutes of contact time. Interestingly, QACs **5** and **10** performed better in hard water versus deionized water against *S. aureus*. Multi-QACs with branched cationic heads (**8**, **9**, and **10)** also showed enhanced killing in hard water against *S. aureus* (**Table 1**). Compound **4** showed the weakest potency in hard water, displaying almost complete loss of bactericidal activity even after 5 minutes of contact time, but the analogous compound **5** with two additional carbons in the alkyl chains, was markedly different. These results show the differences in inhibitory (MIC) and bactericidal (time-kill) bioactivities of these QACs against *S. aureus*.

QACs antibacterial activity against the gram-negative *P. aeruginosa* is significantly reduced by hard water.

P. aeruginosa is a gram-negative opportunistic pathogen of clinical importance due to high antibiotic resistance profiles and its prevalence as a hospital-acquired pathogen, especially in those suffering from cystic fibrosis and other immunocompromised individuals.[60] As a gram-negative bacterium, *P. aeruginosa* possesses an envelope composed of an asymmetric outer membrane made of an inner phospholipid leaflet and an outer lipopolysaccharide (LPS) leaflet, a phospholipid bilayer inner cytoplasmic membrane, and a thin peptidoglycan cell wall between these membranes.[51] This envelope acts as a barrier to multiple antimicrobials in gram-negative bacteria.[51]

To test how these QACs perform against a gram-negative pathogen in practical applications, we compared the bactericidal effectiveness of our panel against *P. aeruginosa* in deionized and hard water. In terms of growth inhibition, MIC values showed a wide range of

effectiveness in the inhibition of *P. aeruginosa*, with QAC **4** and **7** showing the weakest inhibition with 41 μg/mL and 6.5 μg/mL, respectively (**Table 2**). The other QACs in our panel showed comparable MIC values that ranged from 1.34 μg/mL to 2.9 μg/mL. Similarly, bactericidal activity measured by time-kill assays showed a range of bioactivity. Compounds **1** and **2** showed modest inhibitory activities by MIC and good bactericidal activity compared to other QACs. In contrast, compound **7** showed low inhibitory activity relative to other QACs within the panel, but its bactericidal activity was comparable to the other compounds. Similarly, BAC and DDAC showed relatively low inhibitory activity, but potent bactericidal activity as determined in time-kill assays.

The most striking difference we observed in our data was the pronounced and detrimental effects of hard water in the bactericidal activity of all QACs in our panel. We observed that only compounds **1**, **2** and **3** retained some of their bactericidal activity against *P. aeruginosa* in hard water, while the rest of the QACs within the panel were almost completely inactivated in hard water. It is tempting to speculate that the simpler structural characteristics of these QACs (**1**, **2** and **3**) makes them less susceptible to inactivation by hard water compared to the bulkier multi-QACs. It is important to note that DDAC retained its strong bactericidal activity, even in hard water. The observed bactericidal activity patterns in our time-kill data in deionized and hard water show that the inactivating effects of the latter on bactericidal activity against the gram-negative *P. aeruginosa* is general. Divalent cations are known to stabilize the outer membrane charge of gram-negative bacteria.[112] It is possible that the divalent cations present in hard water have a similar protective effect rendering the QACs ineffective. Our results highlight the need to further explore the nuances of the effects of hard water on cationic antimicrobials, especially against gram-negative pathogens.

Dynamic surface tension reduction might be a better proxy for bactericidal activity than MIC values.

With the inhibitory and bactericidal activities of this QAC panel at hand, we explored how the innate ability of these QACs to reduce surface tension correlated with these antimicrobial activities. The dynamic surface tension (DST) of our compounds was measured using a Kruss Bubble Pressure Tensiometer. We found that our QAC panel (and commercial QACs) displayed a range of DST values (**Supplemental Information**), with the commercially available mono-QACs (chloride salts) displaying the lowest values. Using this information, Pearson correlation coefficients were calculated using the DST values and the log reduction values obtained with the 12 QACs in our panel against *S. aureus* and *P. aeruginosa* at 1, 2 and 5 minutes. The statistical significance of the correlation of DST values LRV values against each bacterial strain was determined, as presented in **Table 3**. Statistically significant positive correlation of DST reduction with bactericidal activity against both *S. aureus* and *P. aeruginosa* was observed. This correlation was conserved with bactericidal activity in deionized and hard water at most timepoints with both bacterial strains. Importantly, the DST 500 msec time point showed stronger correlation with bactericidal activity than the 9,800 msec time point, particularly with bactericidal activity within shorter contact times in time-kill assays (1 and 2 minutes). However, is important to note that the correlation of DST values with bactericidal activity is not useful when other co-surfactants are present in the formula since the surface activity of the co-surfactants can mask the contribution of QACs in the DST measurements in addition to the limitations in power of this statistical analysis due to the sample size.

To our surprise, we found no significant positive or negative correlation of DST with MIC values (Pearson' r coefficients $< \pm 0.21$ for *S. aureus* and $< \pm 0.13$ for *P. aeruginosa*) or statistically significant correlation between MIC and log reduction values against *S. aureus* and *P. aeruginosa* (Pearson' r coefficients $< \pm 0.23$ for *S. aureus* and $< \pm 0.41$ for *P. aeruginosa*) in deionized or hard water at any time point tested (**Supplemental Information**). These data support the idea that the dispersion of QACs measured by DST values can be correlated with bactericidal activity but not MIC values. This could offer a cost-effective method to screen QACs for high bactericidal activity.

Effect of counterion in bactericidal activity in hard water.

Since we noted that the commercially available BAC and DDAC are the chloride salts, we explored if the counterion (bromide versus chloride salts) had a role in the effectiveness of one of our QAC. To our knowledge, the effects of counterion in the antimicrobial activity of diverse QACs has been explored, but not in relation to the activity in hard water.[113] We decided to investigate whether the inclusion of chloride or bromide counterions had an impact on the activity of QAC **3**, as this QAC showed reduced activity in hard water against *P. aeruginosa*. Interestingly, we found that the chloride salt of QAC **3** was much more active in hard water compared to the bromide salt, both tested at a final concentration of 200 μg/mL (**Figure 2**). A higher antimicrobial activity has also been associated with the chloride salt of gemini quaternary ammonium salts against fungal pathogens.[114] This counterion-dependent activity increase of QACs has been mainly attributed to effects of the counterion in solubility.[84] It is believed that the counterion does not directly affect the activity of QACs.[101] These data provide additional

evidence in support of the importance of the counterion selection during synthesis of QACs, and the potential effect on bactericidal activity.

Combination of QACs with DDAC show enhanced bactericidal activity.

Leveraging the information obtained of the effect of the chloride counterion in bactericidal activity, we reasoned that combinations of our QACs with DDAC could overcome the negative effects of hard water on bioactivity because of the effects of the chloride counterions provided by DDAC. We tested these combinations against both *S. aureus* and *P. aeruginosa* to ensure that the mixtures of QACs were still as active against both bacterial strains. No major difference was observed in bactericidal activity against *S. aureus*, with most combinations showing the same potent bactericidal efficacy (**Table 4**). Importantly, combinations of DDAC with some of the compounds in our QAC panel outperformed the BAC and DDAC combination commonly found in commercial products in shorter timeframes. In contrast, combination of QACs with DDAC against *P. aeruginosa* showed more pronounced differences in activity. Interestingly, only the combination of QAC **8** with DDAC outperformed the BAC and DDAC combination against *P. aeruginosa*, with over 1 log reduction higher after 2 minutes of contact time compared to the commercially used QAC combination. Compound **4** also showed the lowest bactericidal activity against *P. aeruginosa*, which correlates with the results obtained in time-kill assays when testing this QAC alone. Whether the difference in bactericidal activity observed with QAC combinations are due to the interaction of the QACs with the bacterial membranes or interactions between the QACs themselves is not known. These data suggest that combinations of QACs could be used to improve the bactericidal activity against difficult to eradicate gram-negative pathogens, such as *P. aeruginosa*.

Conclusion

Biocides play an essential role in the control of microbial pathogens in a wide range of industries. Nevertheless, increased usage of biocides such as QACs is leading to higher rates of antimicrobial resistance development.[115] MIC and time-kill assays have been a cornerstone in antimicrobial research, allowing easy determination of inhibitory and bactericidal activity of different antimicrobial agents. However, these assays can become cumbersome when screening numerous antimicrobials. Our data indicates that DST measurements have good correlation with bactericidal activity under real-world disinfection conditions. However, DST values do not correlate with inhibitory activity of QACs determined by MIC assays. Interestingly, we found no statistical correlation between inhibitory capacity and bactericidal activity of in our QAC panel. In addition, we observed that the bactericidal effectiveness of our QAC panel was significantly reduced against gram-negative *P. aeruginosa* in hard water, but less so against the gram-positive *S. aureus* (**Table 1** and **Table 2**). To investigate additional factors that affect the potency of QACs in hard water, we evaluated the effect of counterions in QAC activity in hard water and the effectiveness of QAC combinations with DDAC. We found that the chloride salt of a tested QAC outperformed the corresponding bromide salt. Of note, the chloride salts of both BAC and DDAC were used in this study, and they also retained partial and full bactericidal activity in hard water against *P. aeruginosa*, respectively. Thus, the effect of counterion in QAC bactericidal activity in hard water should be further explored. In addition, we found that rational combinations of QACs could help mitigate the effects of hard water in bactericidal activity. This study provides evidence that DST can be used as a quick and effective technique to broadly screen QACs for bactericidal activity in real-world disinfection conditions. Finally, this study

underlines the need to further explore strategies to overcome the detrimental effects of hard water to QAC bactericidal activity as novel and potent biocides are developed.

Acknowledgements

Funding was provided by the NIH (GM119426 to W.M.W.; DK126467 to G.G.V.C.; T32 GM008602 to C.A.S.) and the Stepan Corporation.

General Biological Information

P. aeruginosa PAO1 strain was streaked onto lysogeny broth (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) at 37 ºC overnight. Single colonies were used to inoculate LB liquid cultures (5 mL) and incubated for 18-24 hours at 37 ºC with shaking. Similarly, *S. aureus* SH1000 strain was streaked onto Tryptic Soy Broth (TSB) agar plates and liquid cultures were prepared using TSB. All optical density measurements were taken at 600 nm wavelength (OD_{600}) using a Synergy H1 Plate Reader from Agilent. For time-kill assays, Microbial Nutrient Test Agar (MCA) plates (BD, 255320) were used to generate a lawn of bacteria by spread plating 250 µL/plate and incubated at 37 ºC overnight. Bacteria were scraped from lawn and resuspended in 1 mL of sterile water to generate a dense bacterial suspension for assays. Of note, this bacterial collection method from plates is known to increase tolerance to biocides compared to liquid suspensions.[108]

Minimal Inhibitory Concentration (MIC) Assays

MIC microdilution assays were performed in a 96-well plate using Mueller-Hilton broth (MHB) following CLSI protocols.[116] 100 µL of 1 mM stocks of QACs prepared in 10 % DMSO and deionized water were added to the plates and serially diluted by 2-fold to give a wide range of actives concentrations in a 100 µL volume. Some QACs stocks were diluted using AOAC 400 µg/mL hard water instead to test if MIC values changed when using hard water. No difference in MIC values was observed in the tested QACs. Bacteria were grown overnight in MHB at 37 ºC, and then subcultured in fresh MHB until exponential growth phase was reached (OD_{600} ~ 0.5). These actively growing bacteria were diluted to ca. 10^6 CFU/mL and 100 µL of this bacterial

suspension was added to each well for a final concentration of 5 x 10^5 CFU/mL in a 200 µL final volume. After inoculation, plates were incubated statically for 24 hours at 37 ºC. MIC values were determined as the lowest concentration of compound that prevented visible growth of bacteria. A minimum of three biological replicates were performed per assay.

Neutralization Assays

Dey/Engley (D/E) neutralizing broth (BD, 281910) was prepared following the manufacturer's protocol at a 2X concentration and sterilized by autoclaving. Neutralization assays were performed following ASTM E1054 protocol.[110] Overnight cultures of bacteria were diluted to ~5,000-50,000 CFU/mL. 0.1 mL of this suspension was used to prepare an inoculation control in 9.9 mL of sterile deionized water. 9.9 mL of 2X D/E neutralization broth were inoculated in the same manner and used as the neutralizer control. QACs neutralization was tested by adding 1 mL of QAC at 2000 µg/mL (final concentration 200 µg/mL) to 9 mL of 2X D/E broth already inoculated with 0.1 mL of the bacterial suspension. These suspensions were vortex to mix and left untouched for ~ 2 minutes before plating on MCA plates for recovery. Plates were incubated overnight at 37 ºC statically. Recovery rate was calculated by comparing the recovered CFU/mL of treated samples to the inoculum (untreated) control.

AOAC 400 µg/mL Hard Water

Association of Official Analytical Collaboration International (AOAC) 400 µg/mL hard water was prepared following EPA SOP: MB-30-02. Briefly, solution 1 was prepared adding 16.94 g of $MgCl_2$:6 H_2O and 18.50 g of CaCl2 to deionized water and brought to a final volume of 250 mL volumetrically. This solution was sterilized by filtration using a 0.22 µM membrane filter.

Solution 2 was prepared in a similar fashion by adding 14.01 g of NaHCO3 to DI-H2O and brought to a final volume to of 250 mL volumetrically and sterilized by membrane filtration. Per 1 L of AOAC 400 µg/mL hard water, 1 mL of solution 1 and 4 mL of solution 2 were added and sterilized by membrane filtration. This final 400 µg/mL sterile AOAC hard water solution was freshly prepared before each experiment and used within 1 day of preparation. Stocks of Solution 1 and 2 were stored at 4 ºC for maximum of 1 week.

Time-kill assays

Suspension time-kill assays were performed in a 96-well plate to facilitate assay and reduce materials used following ASTM E2315 standard protocol.[108] Briefly, dense bacterial suspensions were used to inoculate solutions of active in either deionized or hard water at a final volume of 1 mL using sterile 1.5 mL microcentrifuge tubes, with a final active compound concentration of 200 µg/mL. These suspensions were constantly mixed by inverting the tubes to maintain a homogenous solution throughout the experiment. An inoculation control without active was always included. After specific contact times (0, 0.5, 1, 2, and 5 minutes), 10 µL were transferred to 90 µL of 2X D/E broth for neutralization in a 96-well plate and mixed by pipetting. 10-fold serial dilutions were made in 2X D/E broth in the 96-well plate and 5 µL of these dilutions were spot plated onto pre-dried MCA plates. MCA plates were incubated at 37 ºC overnight, and CFU/mL values were determined after incubation accounting for the dilutions used. CFU/mL bacterial log reduction was calculated using the untreated control also diluted in 2X D/E broth and plated alongside the treated samples.

Statistical Analyses

All statistical analyses were performed using the GraphPad Prism 9.0 software (San Diego, CA). When the value of $P < 0.05$, it was considered statistically significant. Pearson correlation coefficients and simple linear regression models were used to determine statistical significance of correlations.

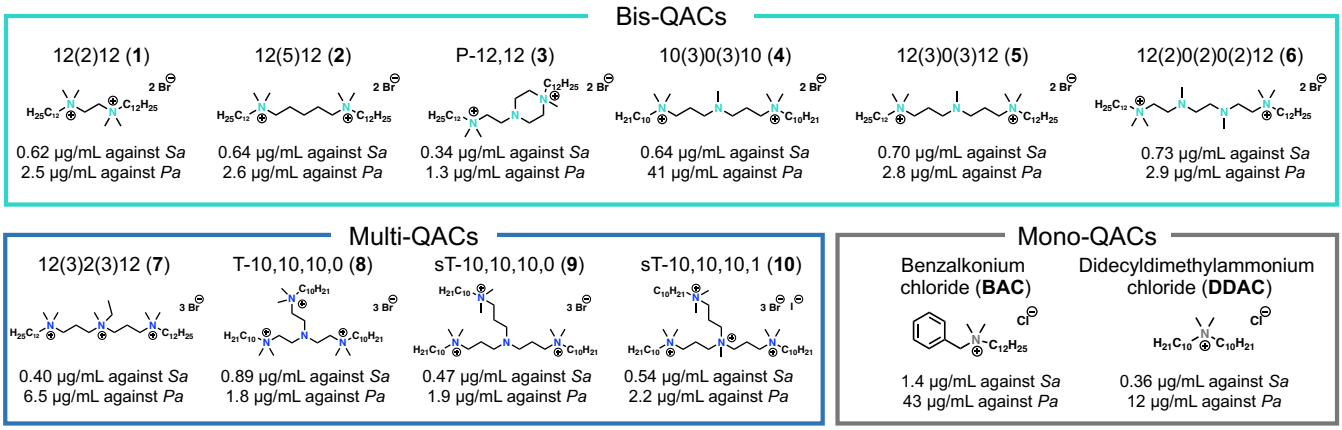


Figure 1. Best-in-class quaternary ammonium compounds (QACs) structure and minimal inhibitory concentrations (MIC). Best-in-class QACs were selected based on their biological activity measured using conventional MIC assays for *S. aureus* (*Sa*) and *P. aeruginosa* (*Pa*) as well as diversity of structures. The commercially available mono-QACs benzalkonium chloride (benzyldimethyldodecylammonium chloride) and didecyldimethylammonium chloride were also included as part of the QAC panel for this study.

Table 1. CFU/mL log reduction of *S. aureus* by QAC panel (n = 2).

		Log reduction values (LRV); CFU/mL					
		1 minute		2 minutes		5 minutes	
QAC[a]	MIC (µg/mL)	Deionized Water	Hard Water[b]	Deionized Water	Hard Water[b]	Deionized Water	Hard Water[b]
QAC 1	0.62	1.9	1.3	2.6	3.4	5.7	6.0
QAC 2	0.64	3.8	2.4	6.2	3.7	6.2	5.0
QAC 3	0.34	4.4	3.5	5.0	5.0	5.9	6.2
QAC 4	0.64	0.4	0.0	0.7	0.8	3.3	0.3
QAC 5	0.70	1.1	2.6	2.1	6.1	2.8	6.1
QAC 6	0.73	1.1	2.4	3.7	2.9	2.3	3.7
QAC 7	0.40	0.3	0.8	0.9	2.4	6.0	3.0
QAC 8	0.89	0.4	1.3	1.6	3.4	3.8	6.0
QAC 9	0.47	0.5	0.9	0.7	2.1	2.1	5.4
QAC 10	0.54	0.5	1.0	0.8	2.5	1.1	4.7
BAC	0.91	4.0	2.9	4.6	5.2	5.3	6.3
DDAC	0.36	5.5	5.1	5.8	6.1	6.1	6.1

[a] *QACs final concentration used was 200 µg/mL.*
[b] *AOAC 400 µg/mL hard water.*

Table 2. CFU/mL log reduction of *P. aeruginosa* by QAC panel (n = 2).

QAC[a]	MIC (µg/mL)	Log reduction values (LRV); CFU/mL					
		1 minute		2 minutes		5 minutes	
		Deionized Water	Hard Water[b]	Deionized Water	Hard Water[b]	Deionized Water	Hard Water[b]
QAC 1	2.5	2.0	0.5	2.9	1.3	4.6	2.5
QAC 2	2.6	4.3	0.6	5.7	1.4	5.7	3.4
QAC 3	1.3	2.7	0.4	2.6	0.9	2.9	1.2
QAC 4	41	2.5	0.1	2.7	0.4	3.1	0.1
QAC 5	2.8	2.0	0.6	1.7	0.0	3.3	0.0
QAC 6	2.9	2.5	0.0	3.0	0.0	3.2	0.6
QAC 7	6.5	2.3	0.2	1.9	0.9	3.1	0.1
QAC 8	1.8	2.8	0.0	3.4	0.0	4.8	0.0
QAC 9	1.9	2.4	0.2	2.5	0.2	3.0	0.0
QAC 10	2.2	1.9	0.2	1.9	0.2	3.1	0.1
BAC	43	4.5	1.8	5.3	2.2	5.9	2.4
DDAC	12	3.9	4.1	5.8	5.1	5.8	5.8

[a] *QACs final concentration used was 200 µg/mL.*
[b] *AOAC 400 µg/mL hard water.*

Table 3. Pearson correlation coefficients calculated using the dynamic surface tension (DST) values and log reduction values (LRV) of the 12 QACs within our panel.

	S. aureus (LRV)			*P. aeruginosa* (LRV)		
DI water	1 minute	2 minutes	5 minutes	1 minute	2 minutes	5 minutes
Kruss DST at 500-msec[a]	0.81*	0.79*	0.78*	0.64*	0.72*	0.65*
Kruss DST at 9800-msec[a]	0.62*	0.62*	0.71*	0.43	0.58*	0.64*
Hard water						
Kruss DST at 500-msec[a]	0.76*	0.55	0.42	0.71*	0.80*	0.82*
Kruss DST at 9800-msec[a]	0.60*	0.48	0.51	0.56	0.66*	0.72*

[a] DST values were measured using a QAC concentration of 1 mg/mL.

* Statistically significant correlation P-value < 0.05 (critical value of r = 0.576).

Highlighted values in white: Power (1-β = 0.8) by *post hoc* analysis using G*Power.

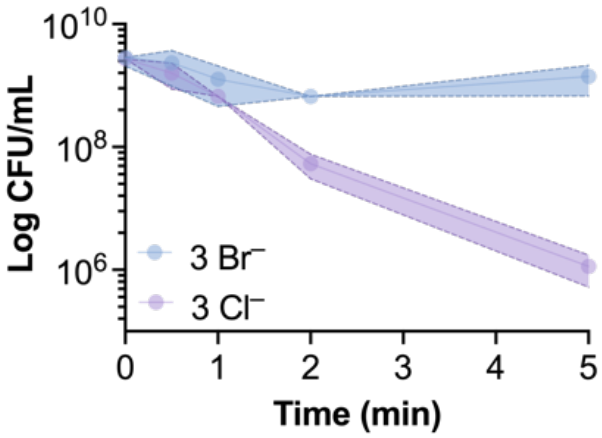
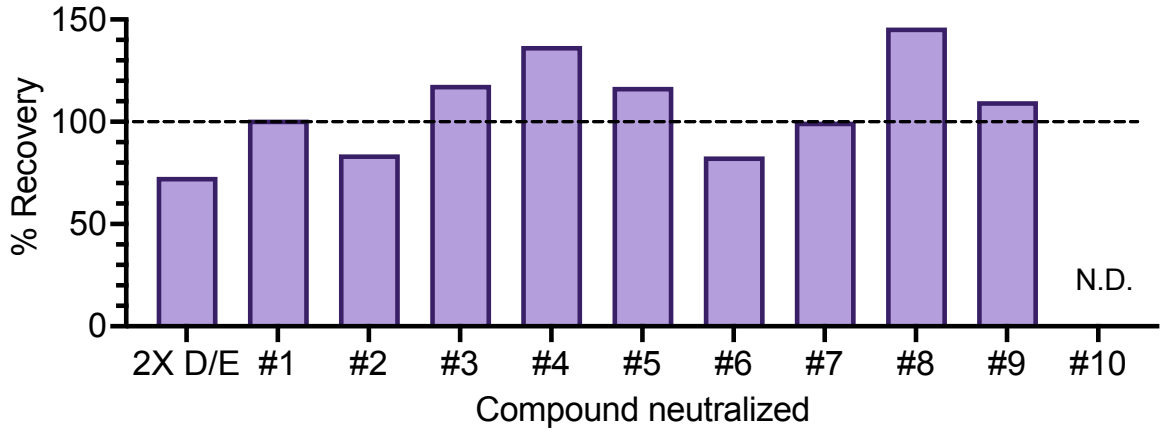


Figure 2. Chloride counterion QACs are more tolerant to hard water effectiveness reduction. P-12,12 Br^- (**3**) and P-12,12 Cl^- (**3** Cl^-) were tested by time-kill assay against *P. aeruginosa* and the reduction in CFU/mL over time was measured in triplicates.

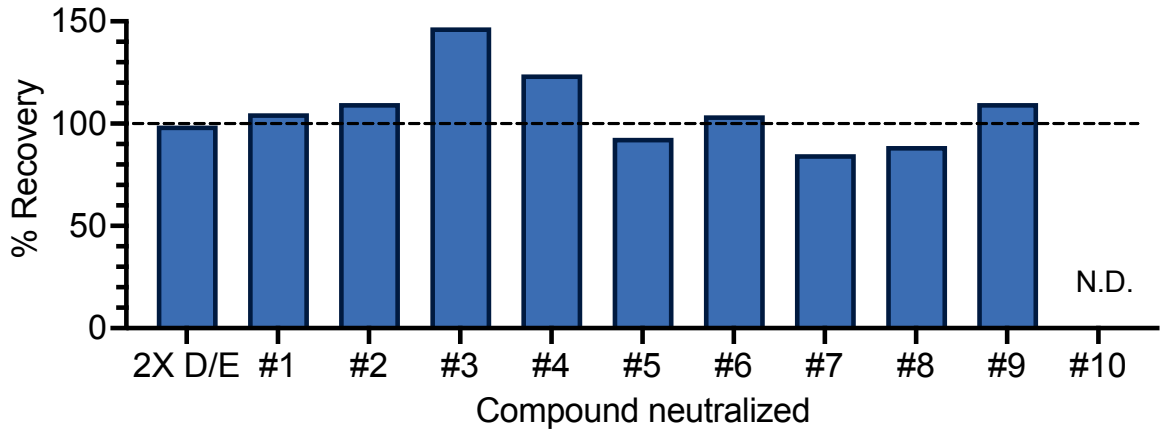
Table 4. CFU/mL log reduction values (LRVs) of *S. aureus* and *P. aeruginosa* by combined QACs (QAC combination concentration = 200 µg/mL) in AOAC 400 µg/mL hard water (n = 2).

	***S. aureus* (LRV)**			***P. aeruginosa* (LRV)**		
Combination	1 minute	2 minutes	5 minutes	1 minute	2 minutes	5 minutes
1 + DDAC	4.3	5.2	5.8	2.3	2.6	3.9
2 + DDAC	1.9	2.2	6.1	2.0	2.3	3.9
3 + DDAC	5.9	5.9	5.9	1.9	2.6	2.5
4 + DDAC	3.5	5.8	5.8	1.7	1.8	2.2
5 + DDAC	5.8	5.8	5.8	1.9	2.7	3.3
6 + DDAC	5.7	5.8	5.8	2.9	3.9	4.9
7 + DDAC	2.5	5.8	5.8	1.7	2.7	3.1
8 + DDAC	5.7	5.7	5.7	3.0	4.6	4.1
9 + DDAC	5.6	4.7	6.1	2.3	2.6	3.5
10 + DDAC	4.9	5.8	5.8	1.6	1.7	3.8
BAC + DDAC	2.8	4.2	5.7	2.8	3.1	4.7

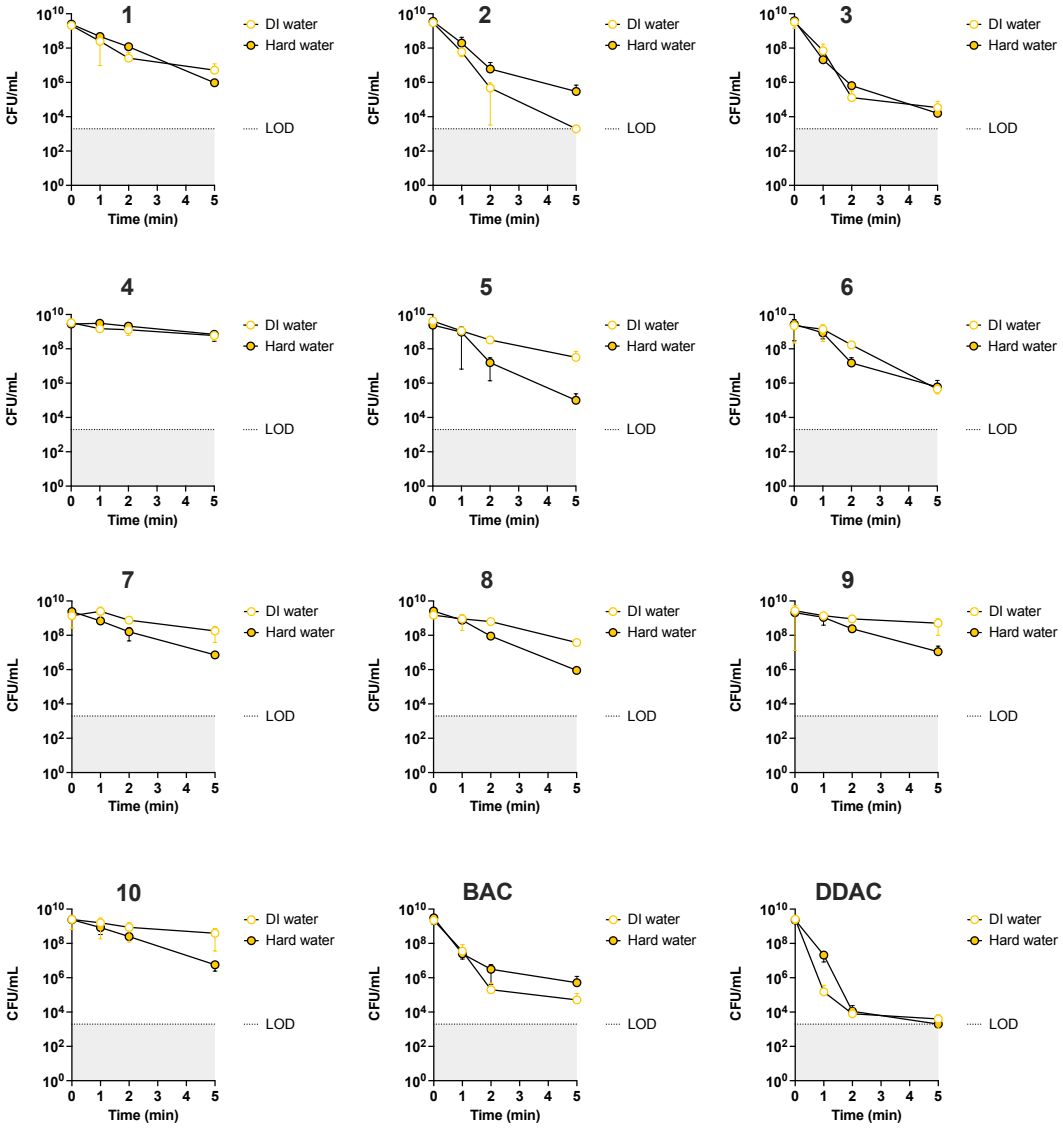
S. aureus SH1000



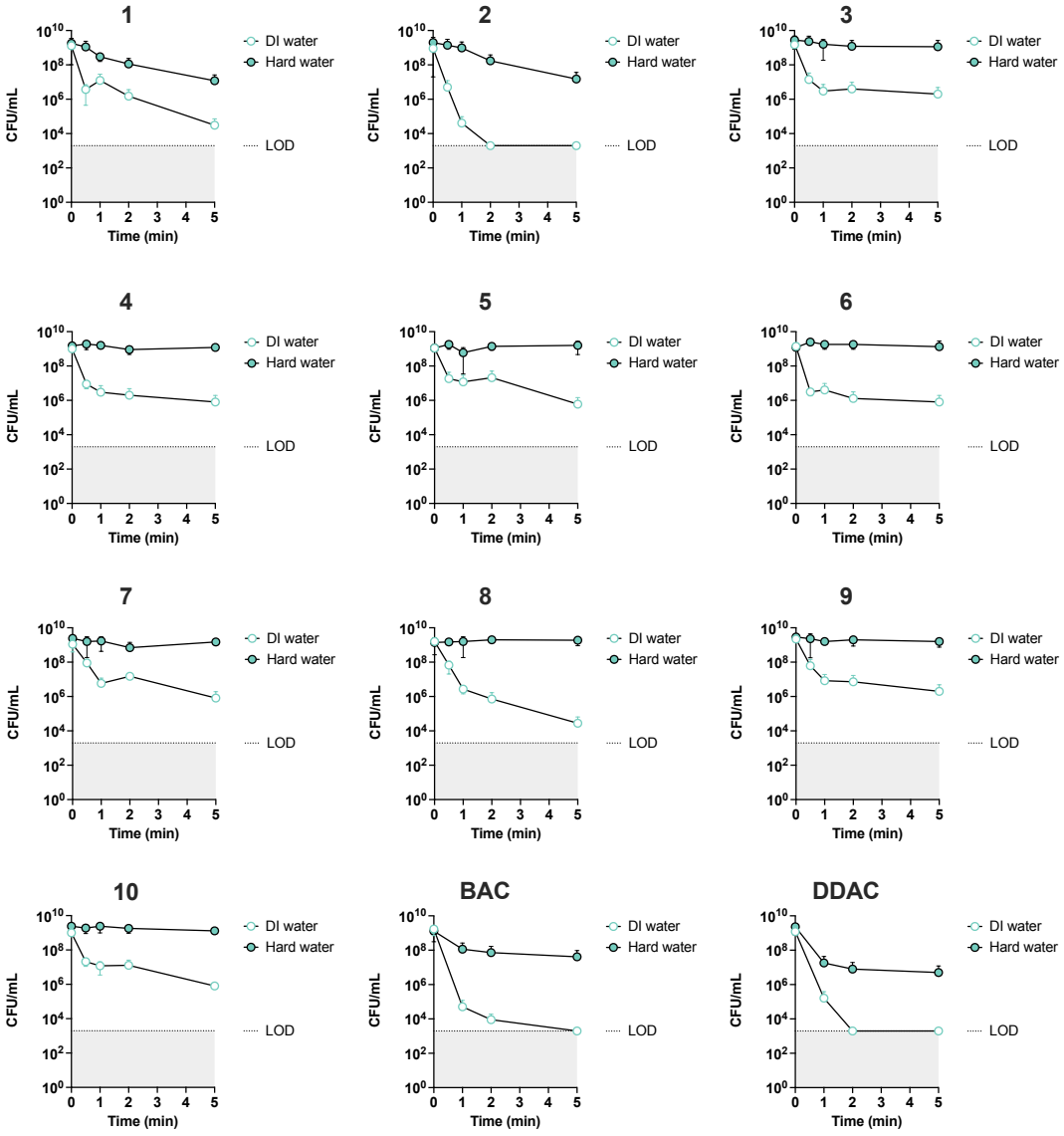
P. aeruginosa PAO1



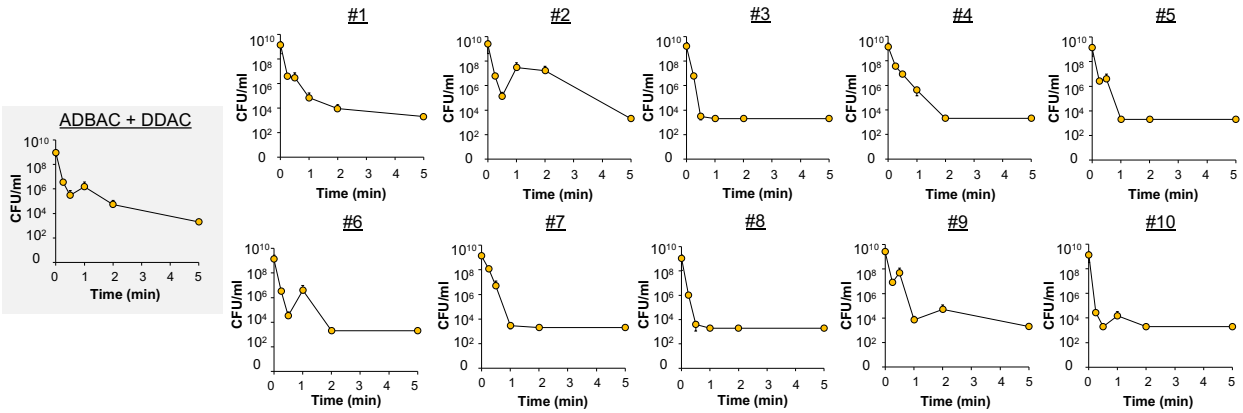
Supplementary Figure 1: Neutralization assays with *S. aureus* SH1000 (top) and *P. aeruginosa* PAO1 (bottom) using 2X Dey-Engley (D/E) neutralization broth. 2X Dey-Engley neutralization broth toxicity was also measured (2X D/E). Average of two biological replicates is plotted for each strain. Inoculum control was normalized to 100 % (dashed line). Final concentration of QACs tested was 200 µg/mL and contact time for QACs was 2 minutes. Compound #10 neutralization was not determined (N.D.) since previous experiment showed effective neutralization of very similar compounds within the panel.



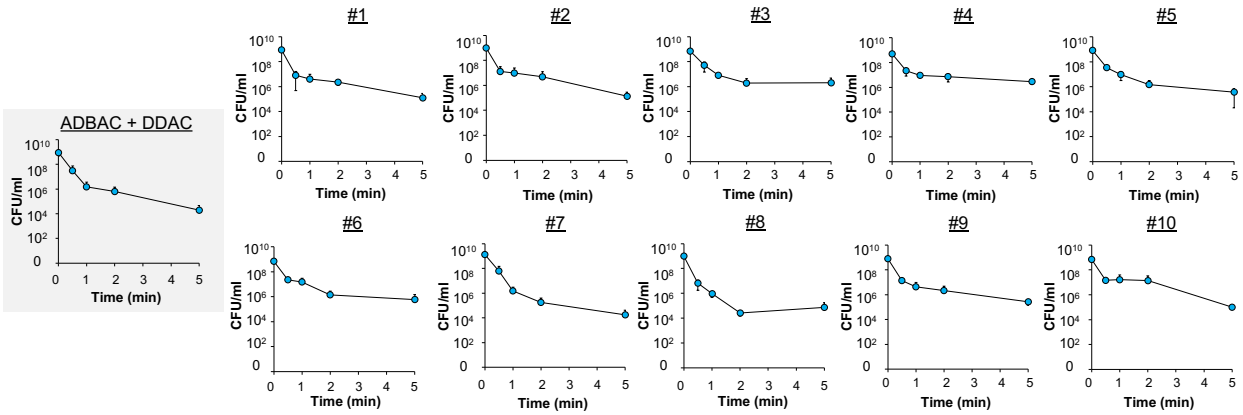
Supplementary figure 2. QACs time-dependent killing effectiveness of *S. aureus* is infrequently affected by hard water. *S. aureus* was exposed to QACs at a concentration of 200 µg/mL for 0.5, 1, 2 and 5 minutes in distilled water (empty symbol) and AOAC 400 µg/mL hard water (filled symbol). After QAC neutralization at specific times, bacterial cells were spot plated. CFU/mL values were calculated for treated samples and untreated samples (0 minutes). Limit of detection (LOD) shaded in gray.



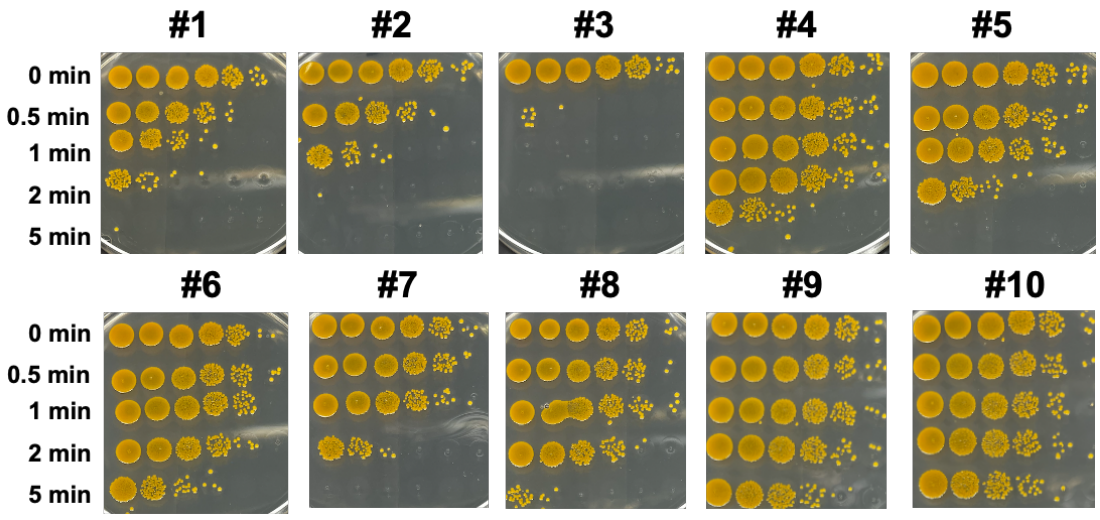
Supplementary figure 3. QACs time-dependent killing effectiveness of *P. aeruginosa* is significantly affected by hard water. *P. aeruginosa* was exposed to QACs at a concentration of 200 µg/mL for 0.5, 1, 2 and 5 minutes in distilled water (empty symbol and 400 µg/mL hard water (filled symbol). After QAC neutralization at specific times, bacterial cells were spot plated (left panel). CFU/mL values were calculated for treated samples (right panel) and untreated sample (0 minutes). Limit of detection (LOD) shaded in gray.



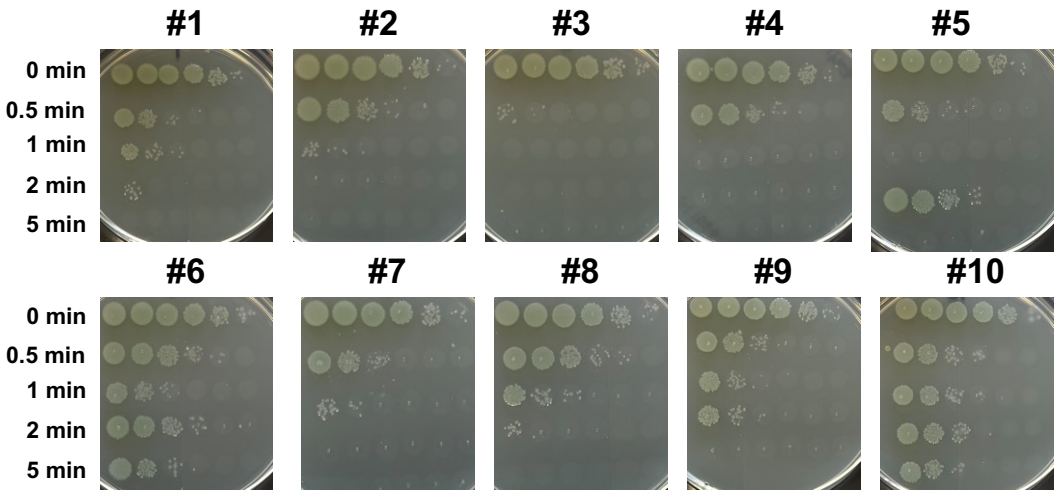
Supplementary figure 4. Combination of DDAC and QAC tested in hard water against *S. aureus.* *S. aureus* was exposed to mixes of QACs or QPCs and DDAC (100 µg/mL each) and the reduction in CFU/mL was measured over time in AOAC 400 µg/mL hard water.



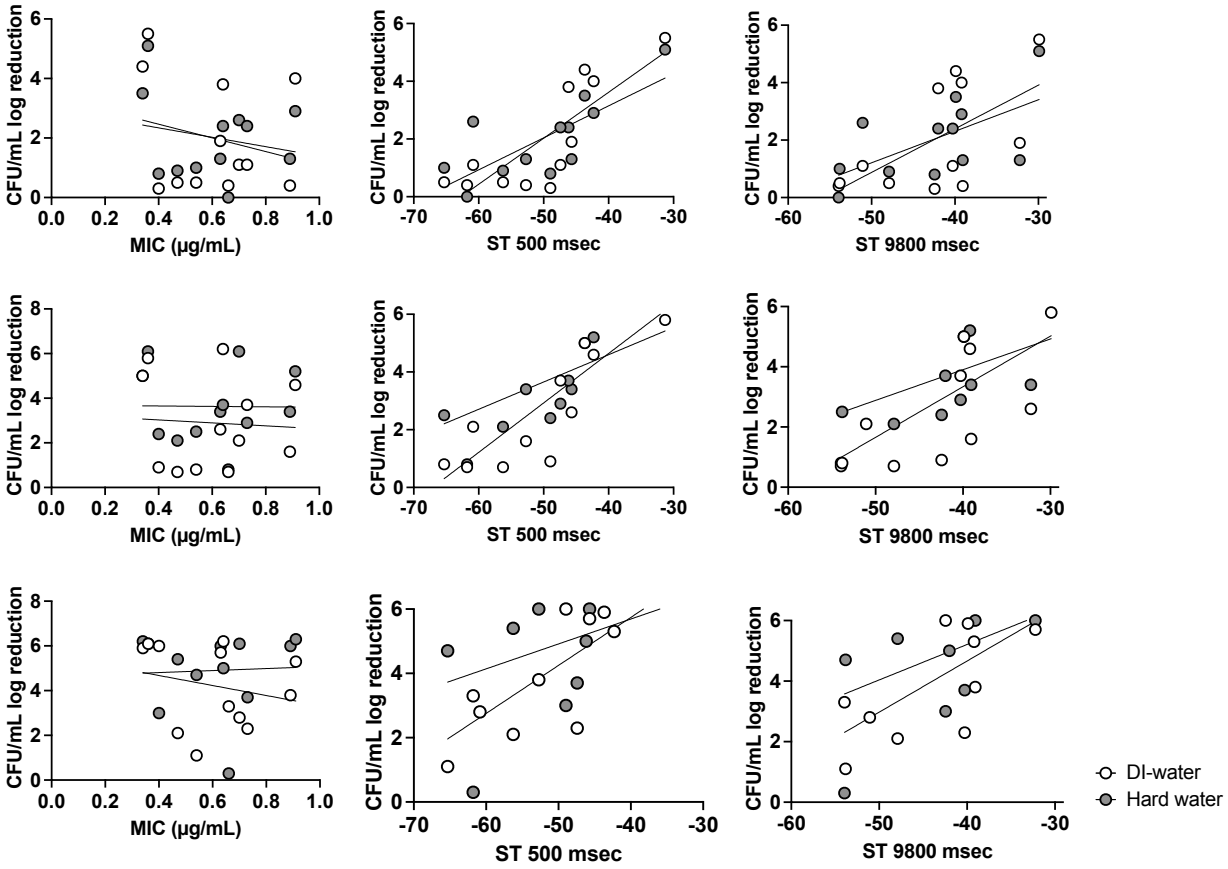
Supplementary figure 5. Combination of DDAC and QAC tested in hard water against *P. aeruginosa.* *P. aeruginosa* was exposed to mixes of QACs or QPCs and DDAC (100 µg/mL each) and the reduction in CFU/mL was measured over time in AOAC 400 µg/mL hard water.



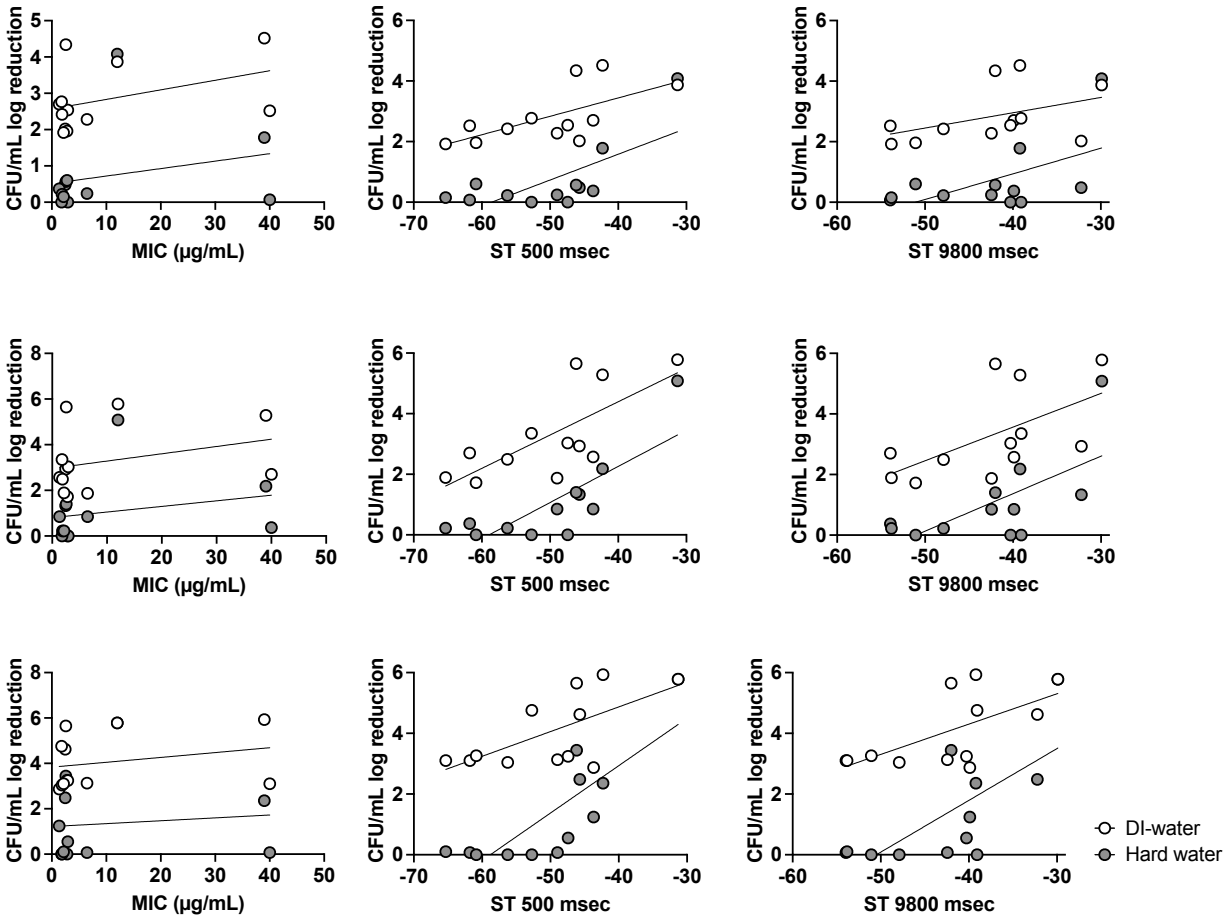
Supplementary figure 6. Example of spot plating with time-kill assay perform in deionized water with *S. aureus* SH1000. *S. aureus* was exposed to QACs at a concentration of 200 µg/mL for 0.5, 1, 2 and 5 minutes in distilled water and pictures were taken after 24 hours of recovery in Microbial Content Test Agar (MCA) plates. Increasing dilutions are plated (10^{-1}-10^{-6}) on each plate from left to right.



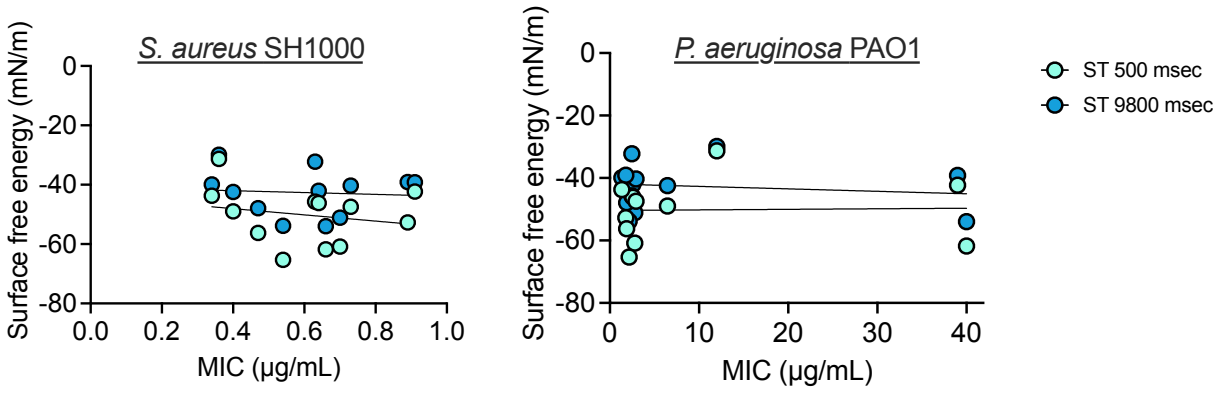
Supplementary figure 7. Example of spot plating with time-kill assay perform in deionized water with *P. aeruginosa* PAO1. *P. aeruginosa* was exposed to QACs at a concentration of 200 µg/mL for 0.5, 1, 2 and 5 minutes in distilled water and pictures were taken after 24 hours of recovery in Microbial Content Test Agar (MCA) plates. Increasing dilutions are plated (10^{-1}-10^{-6}) on each plate from left to right.



Supplementary figure 8. Correlation plots between QACs MIC and surface tension (ST) values and CFU/mL log reduction against *S. aureus* SH1000. Correlation plots and simple linear regressions between QACs MIC values (left), and Kruss ST at 500 msec (center) and 9800 msec (right) values and CFU/mL log reduction values against *S. aureus* SH1000. CFU/mL log reduction were plotted for time-kill assays performed in deionized (DI) water (empty circles) and AOAC 400 µg/mL hard water (filled circles). Pearson's r correlation coefficients are presented in **Table 4**.



Supplementary figure 9. Correlation plots between QACs MIC and surface tension (ST) values and CFU/mL log reduction against *P. aeruginosa* PAO1. Correlation plots and simple linear regressions between QACs MIC values (left), and Kruss ST at 500 msec (center) and 9800 msec (right) values and CFU/mL log reduction values against *P. aeruginosa* PAO1. CFU/mL log reduction were plotted for time-kill assays performed in deionized (DI) water (empty circles) and AOAC 400 µg/mL hard water (filled circles). Pearson's r correlation coefficients are presented in **Table 4**.



Supplementary figure 10. Correlation plots between QACs MIC values and surface tension (ST) values against *S. aureus* SH1000 and *P. aeruginosa* PAO1. Correlation plots and simple linear regressions between QACs MIC values (x-axis), and Kruss ST at 500 msec (light blue) and 9800 msec (dark blue) values against *S. aureus* SH1000 (left panel) and *P. aeruginosa* PAO1 (right panel).

Supplementary Table 1: Dynamic surface tension (DST) values of QAC panel.

QAC	Kruss BPT Mobile: DST at 500-msec (mN/m)	Kruss BPT Mobile: DST at 9800-msec (mN/m)
12(2)12	45.71	32.23
12(5)12	46.16	42.01
P-12,12	43.68	39.9
10(3)0(3)10	61.8	53.95
12(3)0(3)12	60.86	51.07
12(2)0(2)0(2)12	48.96	42.44
12(3)2(3)12	47.42	40.27
T-10,10,10,0	52.73	39.07
sT-10,10,10,0	56.26	47.91
sT-10,10,10,1	65.33	53.82
BAC	42.3	39.2
DDAC	31.3	29.9

**QACs formulated at 1000 μg/mL in DI water

Supplementary Table 2: Pearson correlation coefficient and P values calculated using MIC values and CFU/mL log-reduction against *S. aureus*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water
Pearson r	-0.2313	-0.2126	-0.06062	-0.01103	-0.2297	0.04762
P value	0.4695	0.5071	0.8516	0.9729	0.4726	0.8832
Significance (alpha = 0.05)	No	No	No	No	No	No

Supplementary Table 3: Pearson correlation coefficient and P values calculated using DST values at 500 msec and CFU/mL log-reduction against *S. aureus*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water
Pearson r	0.8139	0.7581	0.7948	0.5503	0.7789	0.4171
P value	0.0013	0.0043	0.002	0.0638	0.0028	0.1773
Significance (alpha = 0.05)	Yes	Yes	Yes	No	Yes	No

Supplementary Table 4: Pearson correlation coefficient and P values calculated using DST values at 9800 msec and CFU/mL log-reduction against *S. aureus*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water
Pearson r	0.6219	0.601	0.6253	0.4759	0.707	0.5057
P value	0.0308	0.0387	0.0297	0.1178	0.0101	0.0935
Significance (alpha = 0.05)	Yes	Yes	Yes	No	Yes	No

Supplementary Table 5: Pearson correlation coefficient and P values calculated using MIC values and CFU/mL log-reduction against *P. aeruginosa*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water	MIC (µg/mL) vs. CFU/mL log-reduction in DI water	MIC (µg/mL) vs. CFU/mL log-reduction in hard water
Pearson r	0.4125	0.2518	0.3091	0.2426	0.2514	0.09823
P value	0.1827	0.4299	0.3283	0.4475	0.4306	0.7613
Significance (alpha = 0.05)	No	No	No	No	No	No

Supplementary Table 6: Pearson correlation coefficient and P values calculated using DST values at 500 msec and CFU/mL log-reduction against *P. aeruginosa*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water	DST 500 msec vs. CFU/mL log-reduction in DI water	DST 500 msec vs. CFU/mL log-reduction in hard water
Pearson r	0.6442	0.708	0.7211	0.7983	0.6468	0.8199
P value	0.0238	0.01	0.0081	0.0019	0.023	0.0011
Significance (alpha = 0.05)	Yes	Yes	Yes	Yes	Yes	Yes

Supplementary Table 7: Pearson correlation coefficient and P values calculated using DST values at 9800 msec and CFU/mL log-reduction against *P. aeruginosa*. A $P < 0.05$ is reported as significant.

Contact time	1 minute		2 minutes		5 minutes	
	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water	DST 9800 msec vs. CFU/mL log-reduction in DI water	DST 9800 msec vs. CFU/mL log-reduction in hard water
Pearson r	0.43	0.5635	0.5847	0.6639	0.6368	0.7183
P value	0.163	0.0564	0.0459	0.0186	0.026	0.0085
Significance (alpha = 0.05)	No	No	Yes	Yes	Yes	Yes

Supplementary Table 8: Pearson correlation coefficient calculated using MIC values (µg/mL) and CFU/mL log-reduction against *S. aureus* and *P. aeruginosa*. A $P < 0.05$ is reported as significant. No statistical significance achieved.

	S. aureus			*P. aeruginosa*		
DI water	1 minute	2 minutes	5 minutes	1 minute	2 minutes	5 minutes
MIC[a]	-0.23	-0.06	-0.23	0.41	0.31	0.25
Hard water						
MIC[a]	-0.21	-0.01	0.05	0.25	0.24	0.10

[a] MIC values in µg/mL were used.

Supplementary Table 9: Raw CFU/mL values obtained in suspension time-kill assays with *P. aeruginosa* and P-12,12 (**3**) bromide and chloride salts.

P-12,12 Br-

Time (min)	P-12,12 Br A	P-12,12 Br B	P-12,12 Br C
0	3000000000	3600000000	2000000000
0.5	3400000000	800000000	2800000000
1	2200000000	800000000	800000000
2	600000000	600000000	800000000
5	2200000000	800000000	1200000000

P-12,12 Cl-

Time (min)	P-12,12 Cl A	P-12,12 Cl B	P-12,12 Cl C
0	3200000000	2200000000	2800000000
0.5	2400000000	1400000000	1000000000
1	800000000	600000000	600000000
2	40000000	80000000	40000000
5	1000000	600000	1800000

Chapter III

Highly effective biocides against *Pseudomonas aeruginosa* reveal new mechanistic insights across gram-negative bacteria

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Author contributions: Experiment were conceived and designed by G.G.V.C., C.A.S., M.E.M., K.P.C.M, W.M.W., and conducted by G.G.V.C., C.A.S., M.E.M., R.A.A., K.R.M.L., S.S.. Data interpretation and analysis was performed by G.G.V.C., C.A.S., M.E.M., K.P.C.M, W.M.W.. The manuscript was written and edited by G.G.V.C., C.A.S., K.P.C.M., W.M.W..

Abstract

Pseudomonas aeruginosa is a major nosocomial pathogen that persists in healthcare settings despite rigorous disinfection protocols due to intrinsic mechanisms conferring resistance. We sought to systematically assess cationic biocide efficacy against this pathogen using a panel of multidrug-resistant *P. aeruginosa* clinical isolates. Our studies revealed widespread resistance to commercial cationic disinfectants that are the current standard of care, raising concerns about their efficacy. To address this shortcoming, we highlight a new class of quaternary phosphonium compounds that are highly effective against all members of the panel. To understand the difference in efficacy, mechanism of action studies were carried out which identified a discrete inner-membrane selective target. Resistance selection studies implicated the SmvRA efflux system (a transcriptionally regulated, inner membrane-associated efflux system) as a major determinant of resistance. This system is also implicated in resistance to two commercial bolaamphiphile antiseptics, octenidine and chlorhexidine, which was further validated herein. In sum, this work highlights, for the first time, a discrete inner-membrane specific mechanism for the bolaamphiphile class of disinfectants that contrasts with the prevailing model of indiscriminate membrane interactions of commercial amphiphiles paving the way for future innovations in disinfectant research.

Introduction

Cationic biocides are commonly found in antiseptic and disinfectant products that provide the first line of defense against microbial pathogens[89, 117]. Their utility extends from household and agricultural disinfection to biotic and abiotic surface decontamination in healthcare settings[118]. Due to their widespread use, tens of millions of pounds of cationic biocides are produced in the United States annually for disinfection[37]. Benzalkonium chloride and didecyldimethylammonium chloride are examples of highly effective cationic biocides used in healthcare settings due to their low toxicity and wide-spectrum efficacy. However, over decades of use, resistance to these biocides has increased at an alarming rate, threatening their utility[119]. Biocide resistance has more recently been exacerbated by the COVID-19 pandemic, as stringent disinfection protocols became widespread and usage increased[115]. Moreover, resistance to biocides has been shown to promote antibiotic resistance[120, 121].

Resistance to cationic biocides in gram-negative bacteria is especially troubling due to the ever-shortening list of effective treatments against such pathogens. A major goal of this study was to evaluate the efficacy of both commercially available and our best-in-class cationic biocides against a panel of *Pseudomonas aeruginosa* clinical isolates. *P. aeruginosa* is an opportunistic gram-negative pathogen that is responsible for over 500,000 deaths annually and is currently ranked as a Serious Threat by the CDC, and a pathogen of Critical Priority by the WHO[60]. Recently, Stribling *et al.* reported the decade-long persistence of *P. aeruginosa* strains in a hospital and how proper infection control was essential to suppress spread, highlighting the importance of effective disinfection protocols[122].

Cationic biocides generally act upon bacterial cells by binding to and subsequently disrupting the phospholipid cell membrane[31]. With two cellular membranes, gram-negative bacteria possess an added barrier to the uptake of disinfectants and other antimicrobials[123]. Resistance to well-studied quaternary ammonium compound (QAC) disinfectants in gram-negative bacteria includes the expression of efflux pumps, upregulation of polyamines like spermidine, membrane lipid changes, and even biodegradation[124]. These mechanisms typically confer cross-resistance against other QACs leading to widespread resistance for these biocides. Illustrating this point, we recently reported the identification of a clinical isolate of *Acinetobacter baumannii* that is resistant to most, if not all, commercially available classes of QACs[125]. These findings suggest the presence of underlying mechanistic subtleties and underscore the importance of understanding how gram-negative bacteria can develop resistance to these biocides. There is a paucity of innovation in this field, and this lack of mechanistic nuance in the biocide mode of action has led to our current dire situation.

As detailed below, we conducted a screen against a panel of multi-drug resistant *P. aeruginosa* hospital-acquired clinical isolates and observed broad resistance to QAC biocides but, excitingly, superior efficacy of our novel quaternary phosphonium compounds (QPCs). While exploring the bactericidal and resistance mechanisms of QPC P6P-10,10, we uncovered distinct, structurally predictable mechanistic determinants of cationic biocides in gram-negative bacteria that have profound implications for disinfectant resistance mechanisms. Through this work, we have elucidated how the chemical properties of cationic biocides influence the specificity of membrane targets in a panel of high-priority gram-negative pathogens and, in turn, how this is reflected in their antimicrobial activity. These findings provide insight for rational design of cationic biocides against gram-negative bacteria.

Results

Pseudomonas aeruginosa clinical isolates are broadly cationic biocide resistant.

We sought to interrogate the efficacy of cationic biocides against a panel of *P. aeruginosa* clinical isolates from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN)[126]. This panel was originally designed to maximize genetic diversity of *P. aeruginosa* strains, but it also provides a diverse range of antibiotic resistance phenotypes. Thus, the panel provides an excellent avenue to study antimicrobial resistance in this bacterial species. We selected a subset of twenty genetically diverse multiple drug-resistant, extensively drug-resistant, and pan-drug-resistant members, and collected IC_{90} values for four commercial disinfectants, twelve of our best-in-class QACs, and two of our best QPCs. Initially, we determined MICs for each cationic biocide listed and observed superior antimicrobial activity of our QPCs (**Fig S1**). However, we were met with trailing growth for certain QACs in different isolate strains, corresponding with a heteroresistance phenotype like our previous observations in *A. baumannii*[127]. Due to the presence of resistant subpopulations above the MIC, we used IC_{90} values as a proxy for disinfectant efficacy to standardize our results (**Fig. 1**). Across the panel, we observed a high degree of cationic biocide resistance in both commercially available QACs and our next-generation QACs, compared to previously reported results with laboratory strain PAO1 and our screen with *A. baumannii*[127]. We observed no apparent correlation between antibiotic resistance and disinfectant resistance, but we found each of the PA7-related species displayed a high degree of disinfectant resistance. The PA7 clade is a taxonomic outlier that possess an extended resistance spectrum and typically possesses an increased biofilm forming

character[128]. This represents the first work to our knowledge on a potential connection between disinfectant resistance and the PA7 clade.

Next generation QPCs are effective against highly antibiotic resistant *P. aeruginosa* strains.

In addition, we tested the effectiveness of two QPCs against this panel of *P. aeruginosa* strains. We observed the superior inhibitory efficacy of QPCs P6P-10,10 and P6P-12A,12A (**13** and **14** in **Fig. 1**, respectively) where traditional nitrogen-centered disinfectants fell short. Whereas many QACs displayed trailing growth obscuring the MIC, QPCs P6P-10,10 and P6P-12A,12A had distinct MICs averaging in the single- digit micromolar range across the panel. To understand the ability of QPCs to overcome disinfectant resistance mechanisms, we sought to further investigate any mechanistic differences that might be present.

The outer membrane of *P. aeruginosa* is not appreciably influenced by the presence of P6P-10,10.

We used various membrane disruption assays to study the effects of P6P-10,10 on the outer membrane of *P. aeruginosa* lab strain PAO1[129-132]. Starting with the N-phenyl-1-naphthylamine (NPN) uptake assay, we were surprised to see that there was minimal uptake of NPN induced by P6P-10,10 treatment when compared to commercial QACs benzalkonium chloride (BAC) and dodecyl dimethyl ammonium chloride (DDAC) at sub-MIC concentrations (**Fig. 2A**). Upon dosing P6P-10,10 at sequentially higher concentrations above the MIC, we observed a dose-dependent increase in NPN uptake, but it still was substantially less than the other known membrane disrupters (**Fig. S2A**). Testing at a lower cell density (OD_{600} = 0.05), we observed similar results (**Fig. S2B**). Additionally, BAC was tested at increasingly higher

concentrations and no significant changes were observed, suggesting that BAC saturates the outer membrane well below the MIC (**Fig. S2C**). To further investigate the effect of P6P-10,10 on the outer membrane, we used a lysozyme permeability assay to test the effects of biocide at the MIC. Again, we observed that P6P-10,10 exerts no appreciable permeabilizing effect on the outer membrane of *P. aeruginosa*. However, QACs BAC and DDAC displayed potent membrane permeabilizing effects (**Fig. 2B**), suggesting distinct mechanisms of action of these cationic biocides. Additionally, a nitrocefin hydrolysis assay supported the previous results and indicated that P6P-10,10 exerts a minimal effect on the outer membrane of *P. aeruginosa* at the MIC (**Fig. 2C**).

P6P-10,10 selectively targets the inner membrane of *P. aeruginosa.*

To understand the effects of the QPC on the inner membrane, we used a 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3$-(5)] membrane depolarization procedure to measure inner membrane disruption at a low cell density (OD_{600} = 0.05)[133]. P6P-10,10 displayed appreciable depolarization at 0.5x and 1x MIC comparable to BAC (**Fig. 2D**). Furthermore, by using a Laurdan generalized polarization (GP) assay, we observed similar dose-dependent responses[134]. A dose-dependent decrease in GP upon P6P-10,10 treatment was observed, which correlates with an increase in membrane fluidity consistent with an inner membrane disruption mechanism (**Fig. 2E**). Additionally, P6P-10,10 dose-dependently induced membrane disruption, as determined by increase in fluorescence of the dye propidium iodide (**Fig. 2F**)[135]. The higher concentration of QPC required to induce these membrane perturbations compared to the DiSC3-(5) assay is due to a sizeable inoculum effect of P6P-10,10 on PAO1, where a significant increase in MIC was observed when a larger inoculum was used (**Fig. S3**). These inner membrane

disruption assays suggest that, while P6P-10,10 has no appreciable effect on the outer membrane, it possesses an inner membrane-specific mechanism of action, distinct from QACs BAC and DDAC. To the best of our knowledge, this is the first specific chemotype that is selective for the inner membrane of gram-negative bacteria and may shed light on a new mechanism of action for the future design of disinfectants.

Antagonism assays support an inner membrane-specific mechanism of action.

To further explore the inner membrane specificity of P6P-10,10, we performed two different antagonism assays designed to probe this putative mechanism of action. Spermidine (Spd) is a cationic polyamine of gram-negative bacteria. In *P. aeruginosa*, Spd has been shown to localize to and protect the outer membrane from antibiotic treatment[136, 137]. In addition, Kwon *et al.* demonstrated that addition of exogenous Spd antagonizes activity of the cationic membrane disrupter polymyxin B[138]. Furthermore, an increase in Spd production is a known transcriptional response to treatment with BAC, presumably because it masks the negative potential on the exterior of the cell[124]. To probe the mechanism of our lead QPC, *P. aeruginosa* was treated with BAC, DDAC, and P6P-10,10 in the presence of 5 mM Spd. We hypothesized that if BAC and DDAC target the outer membrane but P6P-10,10 does not, then we would observe Spd antagonism with BAC and DDAC but not against P6P-10,10. As hypothesized, we observed that exogenous Spd antagonized BAC and DDAC, as evidenced by the increase in MIC (**Fig. S4**). However, the MIC of P6P-10,10 remained unchanged, further supporting our hypothesis that the outer membrane is not the target of P6P-10,10. The second antagonism assay involved the use of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP). CCCP decouples the electrical potential of the cytoplasmic membrane, and dosing at subinhibitory concentrations has

shown to be advantageous to probe systems related to the inner membrane of bacteria[139, 140]. Previous results while exploring P6P-10,10 in *P. aeruginosa* have illustrated that CCCP is antagonistic to QPC treatment[92]. We replicated this previous experiment and demonstrated that CCCP is antagonistic to P6P-10,10, while having no profound effect on BAC treatment under the experimental conditions tested (**Fig. S4**). Taken together, these results further support the inner membrane specificity of P6P-10,10 activity.

Distinct mechanisms of resistance to cationic biocides.

We hypothesized that since these cationic biocides display distinct modes of action, resistance to these compounds would be achieved through distinct mechanisms. In a recent study, distinct intrinsic mechanisms of resistance were identified in *A. baumannii* using transposon-directed insertion-site sequencing (TraDIS) and a panel of 10 biocides[141]. To explore acquired resistance mechanisms to cationic biocides, we performed a resistance selection assay by exposing the reference *P. aeruginosa* PAO1 strain and four additional clinical isolates with different MDR profiles to sub-inhibitory concentrations of BAC and P6P-10,10 over a period of 15 days, and isolated mutants with stable increases in MIC for both cationic biocides (**Fig. 3A**). One resistant mutant from each genetic background was selected and subjected to whole-genome sequencing to identify resistance determinants associated with decreased susceptibility to these biocides. We observed no overlap in resistance mutations to these cationic biocides, consistent with these biocides having a distinct mode of action (**Fig. 3B**). In addition, while adaptation to P6P-10,10 can be pinpointed to a few genetic loci, BAC adaptation is associated with mutations in multiple genetic loci across the genome (**Fig. 3C**). Loss of function mutations in *htrB1* were frequently identified in the BAC-resistant mutants (3/5 strains) in addition to mutations in genes

associated with several cellular functions including DNA replication and repair, biofilm production, and virulence, among others (**Fig. 3C**). In contrast, loss of function mutations in *smvR,* the negative transcriptional regulator of SmvA, were identified in all P6P-10,10 resistant mutants, indicating this efflux system is involved in resistance to this QPC. Some BAC-resistant mutants show a small increase in P6P-10,10 MIC, but the converse was not observed. (**Table S4**). These data suggest that SmvA is a major bolaamphiphile resistance determinant in *P. aeruginosa*. Adaptations to cationic biocides with distinct mechanisms of action result in completely different resistance profiles in *P. aeruginosa*.

SmvA is a major P6P-10,10 resistance determinant.

SmvR is a Tet-like repressor of SmvA, a Major Facilitator Superfamily (MFS) efflux pump that has been shown to provide resistance to other cationic biocides. Derepression of SmvA can occur by binding of the cationic compound to SmvR, causing a conformational change that prevents it from effectively binding to DNA, or by loss of function mutations in *smvR* (**Fig. 4A**). With 14 transmembrane domains, SmvA is classified within the drug:proton antiporter (DHA) DHA2 member alongside QAC efflux pump (QacA) and LfrA, likely sharing an "asymmetric rocker-switch" motion coordinated by extracellular loops[142, 143]. Interestingly, a loss of function mutation in *smvR* was identified in strains resistant to P6P-10,10 in all genetic backgrounds (**Fig. 4B**). To assess the role of the SmvRA system in P6P-10,10 resistance, we performed genetic complementation test by introducing a wildtype SmvR copy with its predicted native promoter in pUCP30T vector to restore repression of SmvA, and measured susceptibility to P6P (**Fig. 4C**)[144]. Restoration of SmvR function partially restored susceptibility to P6P-10,10 as measured by MIC assays (**Fig. 4D**). In addition, SmvR complementation restores expression

of SmvA close to wildtype levels, as measured by RT-qPCR (**Fig. 4E**). This also correlated with an increased accumulation rate of Hoechst 33342, used as a proxy for efflux activity (**Fig. 4F**). To further corroborate the involvement of SmvRA in P6P-10,10 resistance, we exposed the wildtype and P6P-10,10-resistant strains harboring either pUCP30T with a functional copy of SmvR or the empty vector as control to increasing concentrations of P6P-10,10 and observed that introduction of a functional copy of SmvR led to a similar cationic biocide susceptibility profile as wildtype *P. aeruginosa* PAO1 (**Fig. 4G**). In sum, these data unveil SmvA as a main resistance determinant to the novel P6P-10,10, and highlight the importance of this MFS efflux system in cationic biocide resistance. Further, these data exhibit different mutations associated with distinct modes of action for cationic biocides.

Cationic biocides possess distinct mechanisms in gram-negative bacteria that are structurally predictable.

Due to the shared resistance determinant among OCT, CHX, and P6P-10,10 in gram-negative species, we hypothesized that they share an inner membrane-specific mechanism of action in *P. aeruginosa* [134, 145, 146]. Using the NPN uptake assay for outer membrane permeabilization, we observed no appreciable NPN uptake upon treatment with OCT or CHX when compared to BAC and DDAC, like P6P-10,10. From the presence of *smvR* mutations in other gram-negative species, we hypothesized that this inner membrane-specific mechanism of action was not limited to *P. aeruginosa*. To test our hypothesis, we assessed the mechanism of action of P6P-10,10, OCT, and CHX in additional gram-negative species *Escherichia coli* MC4100 and *Acinetobacter baumannii* ATCC 19606. NPN uptake assays revealed that P6P-10,10, OCT, and CHX do not appreciably perturb the outer membrane in any of the species

tested (**Fig. S5**). Importantly, this demonstrates that cationic biocides act via different mechanisms in gram-negative bacteria and uncovers a mechanistic difference that was previously unknown. This was additionally validated by antagonism experiments with antibiotics with reduced uptake in gram-negative species in combination with inner membrane specific biocides (**Fig. S7**). With this new knowledge in hand, we revisited our initial *P. aeruginosa* panel and tested CHX and OCT against the twenty clinical isolates. For these multicationic disinfectants, we observed potency like that of the QPCs tested (**Fig. 5, S4**).

Through examining the molecular structures between the P6P-10,10, OCT, and CHX compared to QACs BAC and DDAC, we observed the presence of spatially separated, charged moieties in the former three. These compounds possess cationic warheads all separated by 6-8 methylene moieties. We posit that by possessing these cationic moieties separated by a linker, these biocides selectively disrupt the inner membrane of gram-negative bacteria without producing a strong effect on the outer membrane. Future studies will further probe this phenomenon to better understand how the chemical composition of each membrane leads to its chemo-selectivity.

Discussion

A longstanding problem in medicinal chemistry is the challenge associated with targeting specific biological targets and cellular components over others. Here we report that cationic antimicrobials P6P-10,10, OCT, and CHX, display the ability to passively diffuse through the outer membrane of gram-negative bacteria and target the cytoplasmic membrane. Certain bolaamphiphiles are known to penetrate membranes without disruption[147-149] while others have been optimized for membrane-disrupting activity[150-152]. Here, we observe bolaamphiphiles that

non-disruptively penetrate the bacterial outer membrane and selectively perturb the cytoplasmic membrane, providing a chemical basis for cytoplasmic membrane targeting in gram-negative bacteria. It has been noted that the segmented amphiphilicity of the bolaamphiphiles endow this class of molecules with complex self-assembly behavior, which may lead to this novel mechanism of action[153]. The opposing polar heads separated by a sufficiently long alkyl chain have been proposed to adopt a U-shaped (in the outer leaflet) and transmembrane conformations in the lipid bilayer of the bacterial cytoplasmic membrane[154]. Molecular dynamics studies of mono- and multicationic QACs have suggested an alternative mechanism of membrane integration and disruption in a one-two fashion of electrostatic attraction and subsequent membrane penetration and disruption[155]. This work uncovers that the mechanistic differences of cationic disinfectants expand beyond lipid bilayer interactions, providing a framework for cationic biocide selectivity determination.

Despite having a primarily external target in bacteria, certain cationic biocides—almost counterintuitively—possess an internally regulated resistance mechanism: transcriptional flux of efflux pump expression. In gram-positive species, QacA is a classic example of an efflux pump that confers resistance against cationic species[156, 157]. Interestingly, QacA is regulated by the negative transcriptional regulator QacR, which requires the binding of substrate before it is released from DNA to allow transcription of the downstream gene *qacA*[158]. In the current work, we observe an analogous system with SmvRA conferring resistance against bolaamphiphilic disinfectants in *P. aeruginosa*. Since SmvA is in the cytoplasmic membrane, these cationic species are only exported from the cytoplasm to the periplasm. Efflux from the cytoplasm may be sufficient to mitigate the harmful effects of the biocides or additional export mechanisms such as passive diffusion out of the cell or some other unknown export process may exist. Requiring

intracellular accumulation of amphiphile (though presumably at very low concentrations) to induce resistance mechanisms suggests that internal effects of disinfectant are not negligible. This highlights how the linear mechanism of action that is presented is simply the sum average of all the interactions occurring between the lipid membranes and the cationic amphiphiles.

From this perspective, the inner membrane selectivity observed is likely the result of a high degree of localization at the inner membrane and unfavorable on-off binding kinetics between the outer membrane and the bolaamphiphiles. Furthermore, the diffuse charge of the phosphorous cation or the delocalized, lipophilic ammonium cations could contribute to this effect. In contrast, non-bolaamphiphile disinfectants interact strongly with the outer membrane, leading to disruption. This effect can be amplified by increasing the affinity between the membrane and the amphiphile by adding cationic centers, as evidenced by the potency of multicationic QACs. While multicationic QACs are effective against a range of antimicrobial-resistant pathogens, results from initial screen display the superiority of the bolaamphiphiles against highly resistant biocide resistant clinical isolates. Importantly, we demonstrate how the distinct mechanism of action of spaced-cationic biocides can overcome resistance to traditional QACs in gram-negative bacterial species *P. aeruginosa*, which is notoriously difficult to eradicate through biocide treatment[141].

P. aeruginosa is an opportunistic pathogen that possesses a range of complicated clinical manifestations, especially in patients with cystic fibrosis[60, 159]. This pathogen has received the highest risk level by both the World Health Organization and the CDC due to its propensity to develop antimicrobial resistance with almost a third of all isolates of Europe demonstrating resistance to at least one antimicrobial treatment group [11, 160, 161]. Recently, McGann *et al*. reported a case of a wounded soldier co-infected with six bacterial strains, three of which were

extremely drug-resistant *P. aeruginosa* strains, highlighting the danger this pathogen poses during conflicts and the need for effective biocides and disinfection protocols against this pathogen[61].

Cationic biocides, such as QACs, are an important part of disinfection protocols in the healthcare and food industries as a barrier to prevent the spread of this pathogen and others. This widespread use of biocides across industrial settings has led to increased resistance as a consequence [115]. As alternative compounds to the overused QACs, our group has developed a series of QPCs that show improved activity against a diverse group of pathogenic bacteria[91, 92, 162-167]. The effectiveness of one such compound, P6P-10,10, against *A. baumannii* strains displaying high levels of resistance to diverse cationic biocides suggested that P6P-10,10 might possess a distinct mode of action compared to common QACs[12].

In this work, we investigated the mechanistic nuances of cationic biocide antimicrobial activity by comparing the QPC P6P-10,10 to commercially available QACs. We tested these disinfectant compounds against a panel of highly antibiotic-resistant *P. aeruginosa* clinical isolates, and, as anticipated, observed high levels of resistance to a wide range of biocides (**Fig. 1**). Strikingly, the QPCs maintained strong antimicrobial activity against all the isolates, suggesting that these compounds evade preexisting mechanisms of antibiotic and biocide resistance. Biocides are believed to have multiple cellular targets, with membrane disruption being the primary mechanism of action [168]. While BAC and DDAC showed potent outer membrane disruption, P6P-10,10 minimally affected the outer membrane. In contrast, when the integrity of the inner membrane was assessed, P6P-10,10 exhibited strong and dose-dependent disruption, suggesting that P6P-10,10 preferentially targets the inner membrane in *P. aeruginosa*.

We have also shown that increased expression of the MFS efflux pump SmvA is the main resistance mechanism to the QPC, P6P-10,10, with all P6P-10,10-resistant strains having a loss of function mutation in its negative regulator SmvR (**Fig. 4**). Genetic complementation studies indicated that, while SmvA is the predominant resistance determinant for P6P-10,10, complete repression of SmvA expression or mutations in other genes (e.g., *pssA*) are likely required for full complementation (**Fig. 4**). Importantly, SmvA is also associated with resistance to octenidine and chlorhexidine, two bolaamphiphiles that share a preference to disrupt the inner membrane[134, 145, 146]. These results provide a link between chemical nature of these cationic biocides, their cellular target, and the determinants associated with resistance.

In summary, this study uncovers several key mechanistic nuances of how different cationic biocides exert their antimicrobial effects on gram-negative bacteria, how their structural properties can provide insights into their mechanism of action, and how this information can be used to predict resistance determinants that decrease susceptibility to these compounds.

Materials and Methods

Bacterial strains and growth conditions.

All strains and plasmids are listed in **Table S1**. Bacterial strains were streaked onto lysogeny broth (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) at 37 ºC overnight. Liquid cultures were inoculated with single colonies from plates and incubated for 18-24 hours at 37 ºC with shaking. Media was supplemented with gentamicin (30-60 μg/mL) for vector maintenance in *P. aeruginosa* strains, as needed. *P. aeruginosa* clinical isolates were obtained from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN).

Growth curves were performed in 96-well flat-bottom plates (Falcon®, 351172) with shaking. Optical density was measured at a wavelength of 600 nm (OD_{600}) every 10 minutes and growth was monitored over 24 hours. OD_{600} measurements were obtained using a SpectraMax iD3 plate reader (Molecular Devices, United States). Growth curve experiments were performed on different days with independent biological replicates with at least 3 technical replicates per strain/condition.

Minimum Inhibitory Concentration (MIC) Assays

To determine the MIC values, compounds were serially diluted two-fold from stock solutions (1.0 mM) to yield twelve 100 μL test concentrations, wherein the starting concentration of DMSO was 2.5%. Overnight cultures of each strain were diluted to ca. 10^6 CFU/mL in MHB and regrown to mid-exponential phase, as determined by OD_{600}. All cultures were then diluted again to ca. 10^6 CFU/mL and 100 μL were inoculated into each well of a U-bottom 96-well plate (Falcon, 351177) containing 100 μL of compound solution. Plates were incubated statically at 37 ºC for 72 hours upon which wells were evaluated visually for bacterial growth. The MIC was determined as the lowest concentration of compound resulting in no bacterial growth visible to the naked eye, based on the highest value in three independent experiments. Aqueous DMSO controls were conducted for each strain. Strains of *S. aureus* MSSA (SH1000), *E. coli* (MC4100), *P. aeruginosa* (PAO1), *A. baumannii* (ATCC 17978), CA-MRSA (USA300-0114), and HA-MRSA (ATCC 33591) were grown with shaking at 37 °C overnight from freezer stocks in 5 mL of the indicated media: SH1000, MC4100, USA300-0114, and PAO1 were grown in BD

Mueller–Hinton broth (MHB), whereas ATCC 33591 was grown in BD tryptic soy broth (TSB). MRSN isolates were grown in MHB in the same fashion as previously described.

Calculation of IC_{90} Values

To determine the IC_{90} values for each compound against the clinical isolates, the OD_{600} for each compound concentration against each strain was recorded. Using Prism 9 (GraphPad software, v. 9.3.1), the IC_{90} values for each disinfectant compound against each strain were calculated. The OD600 measurements were used as inputs, then normalized to fit 0% (equal to media blank) to 100% (maximum OD_{600} for each strain). The analysis was then performed on the normalized data using the dose-response model with a least squares regression fit, wherein outliers (Q=1%) were excluded and no weighting method was applied.

NPN Uptake Assay

P. aeruginosa PAO1 were grown overnight in LB, then regrown from a 1:100 dilution in fresh media for 5 hours to an OD_{600} of 0.5. Cells were harvested by centrifugation (4000 rpm, 25ºC, 10 min), washed twice with assay buffer (5 mM HEPES, 5 mM glucose, pH 7.2), and resuspended in assay buffer to a final OD_{600} of 1. Then, 100 µL of washed cells and 100 µL of assay buffer containing 20 µM NPN were together and incubated for 10-30 min. 198 µl of cells and NPN added to a 96-well optical-bottom black plate. Either 2 µL of a chemical compound or the corresponding solvent was added to each well, and fluorescence was immediately monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm for 7 minutes at 30 second intervals.

F_{obs} = NPN + cells + compound

$F_{control}$ = NPN + cells

F_b = NPN

NPN uptake = $(F_{obs} – F_B)$—$(F_{control}-F_B)$

20 μM NPN in assay buffer was made from a 5 mM stock of NPN in acetone.

Lysozyme Permeability Assay

P. aeruginosa PAO1 were grown overnight in LB, then regrown from a 1:100 dilution in fresh media. Midlog phase bacteria (OD_{600} = 0.4 – 0.6) were harvested, washed once, and resuspended in HEPES buffer (5 mM HEPES at pH 7.2 and 5 mM sodium azide) to an optical absorbance of OD_{600} = 1. Then, 98 μL of bacterial suspension was added to a 96-well plate containing 100 μL of lysozyme solution in PBS. OD_{600} was then measured. The final concentration of lysozyme was 50 μg/mL, and the final OD_{600} was 0.5. Either 2 μL of a chemical compound, or the corresponding solvent, was added to each well. The turbidity of the sample was measured after the lysis process reached equilibrium (as seen by a stabilization in the OD_{600} after mixing) and every 10 s after stabilization for 30 s. Relative values were normalized to PBS as 0% and 99% isopropanol as 100%.

Nitrocefin Hydrolysis Assay

P. aeruginosa PAO1 cells were grown overnight in LB, then regrown (1:100 dilution) in fresh media to an OD_{600} of 0.4–0.5. Cells were centrifuged, washed in PBS, and resuspended to OD_{600} = 0.02 in 20 mM PBS with 1mM $MgCl_2$ at pH 7.2. A volume of 50 μL of the cell

suspension was added to a clear, flat-bottom 96-well plate containing 50 µL of PBS with a final concentration of 30 µM nitrocefin and the 2-fold dilution of compound. Plates were incubated at 37 °C in a stationary incubator and read from 0 min to 60 min at 10 min intervals at 490 nm to monitor nitrocefin hydrolysis. Reads were normalized using the corresponding no cell control wells.

$DISC_3(5)$ Depolarization Assay

P. aeruginosa PAO1 were grown overnight in LB, then regrown from a 1:100 dilution in fresh media. Midlog phase bacteria (OD_{600} = 0.4 – 0.6) were harvested, washed once, and resuspended in HEPES buffer (5 mM HEPES at pH 7.2) to an optical absorbance of OD_{600} = 0.05. Then, 100 µL of 10 mM EDTA was added to 5 mL of resuspended cells for a final concentration of 200 µM EDTA. The bacterial solution was then gently mixed and then let sit for 2 minutes. Afterwards, 5 µL of 0.75 mM $DISC_3(5)$ was added to the solution for a final concentration of 0.75 µM. Following another gentle mix, the solution was left to incubate in the dark at 37ºC. After incubation, 125 µL of 4M KCl was added to the cells for a final concentration of 100 mM KCl. Finally, 198 µL of cells and $DISC_3(5)$ added to a 96-well optical-bottom black plate. Either 2 µL of a chemical compound, or the corresponding solvent, was added to each well. The excitation wavelength was 622 nm, and the emission wavelength was 670 nm. The release of $DISC_3(5)$ was measured by the increase in fluorescence of $DISC_3(5)$ for 60 min as a measure of inner membrane depolarization.

Propidium Iodide

P. aeruginosa PAO1 were grown overnight in LB, then regrown from a 1:100 dilution in fresh media for 5 hours to an OD_{600} of 0.6. *P. aeruginosa* cells were harvested (4000 rpm, 25°C, 10 min), washed, and resuspended in PBS buffer at pH 7.2. Then, 50 µL of a 1.5 mM solution of propidium iodide (PI) was added to the resuspended cells. Following a 60-minute incubation, 198 mL of cells and PI added to a 96-well optical-bottom black plate. Either 2 µL of a chemical compound, or the corresponding solvent, was added to each well. The excitation wavelength was 535 nm, and the emission wavelength was 617 nm. The uptake of PI was measured by the increase in fluorescence of PI for 30 min as a measure of inner membrane permeabilization.

Laurdan GP

An overnight culture of *P. aeruginosa* PAO1 was grown to OD_{600} = 0.4 and diluted to 10^5 CFU/mL in HEPES buffer, followed by 60 min incubation with 2.5 µM Laurdan at 37 °C in the dark. Following incubation with Laurdan, 198 mL of cells was added to a 96-well plate. Subsequently, 2 mL of compound was added to the wells. The Laurdan fluorescence intensities were measured using a Biotek Synergy H1 spectrophotometer with emission wavelengths of 435 nm and excitation at 490 nm, and the temperature was maintained at 37 °C. Laurdan GP was calculated using the equation GP = (I435 − I490)/(I435 + I490).

Antagonism Assays

Respective QPC and QAC compounds were serially diluted two-fold from stock solutions (1.0 mM) to yield twelve test concentrations of 50 µL each, wherein the starting concentration of DMSO was 2.5%. To each well containing 50 µL of the QAC or QPC solution, 50 µL of CCCP (50 µM) and Spd (5 mM) in H_2O at the designated test concentration was added. Overnight *P.*

aeruginosa (PAO1) cultures were regrown to mid-exponential phase and diluted to ca. 10^6 CFU/mL in MHB and as determined by OD_{600}. Subsequently, 100 µL were inoculated into each well of a U-bottom 96-well plate (Corning, 351177) containing 100 µL of compound solution. Plates were incubated statically at 37ºC for 24 hours upon which wells were evaluated visually for bacterial growth. The MIC was determined as the lowest concentration of compound resulting in no bacterial growth visible to the naked eye, based on the highest value in three independent experiments.

Whole genome sequencing

Genomic DNA extraction, library preparation and Illumina sequencing were performed at the SeqCenter (Pittsburgh, Pennsylvania, USA) using 200Mbp as the minimum read count per sample. Data was analyzed using breseq (version 0.38.1) as previously described, using the contigs option (-c) when needed [169]. The following annotated reference genomes were obtained from NCBI and used for the analysis: NC_002516.2 for strain PAO1 and isogenic mutants, and for strains MRSN6220, MRSN6241, MRSN409937, MRSN5524 and isogenic mutants, sequences were obtained from Bioproject PRJNA446057. To identify mutations in isolated resistant mutants, the breseq output was compared to the one obtained from their respective parental (wildtype) strain. Genetic variation also identified in the wildtype parental strain were removed. All the genetic variants are reported in **Table S2** (P6P-10,10 resistance associated genetic variations) and **S3** (BAC resistance associated genetic variations). Genomic positions and COG functions of genes with mapped genetic variants were obtained in Pseudomonas.com. CLC Genomics Workbench and BLASTn were used to mapped genetic variants to genomic positions.

Complementation studies

For genetic complementation, the *smvR* (PA1283) coding sequence containing its predicted native promoter (predicted using SAPPHIRE) was amplified, by PCR, ligated into pUCP30T vector (BamHI and EcoRI sites) and plated onto LB agar plates supplemented with gentamicin (60 µg/mL) for selection of transformants (Emory Integrated Genomics Core) [170]. This vector containing *smvR* was confirmed by sequencing. 100 ng of plasmid DNA of empty vector (pUCP30T) and vector containing *smvR* were transformed by electroporation (settings: 25 µF; 200 Ω; 2500 V on a BTX™ Gemini X2 Electroporation System) into *P. aeruginosa* PAO1 electrocompetent cells prepared as previously described [171].

Growth curves with P6P-10,10

Two-fold dilutions of the compound P6P-10,10 were prepared in a flat-bottom 96-well plate (Falcon®, 351172). *P. aeruginosa* strains were grown overnight in MHB at 37 ºC with shaking (200 ppm) from single colonies grown on LB plates or LB gentamicin 60 µg/mL, when needed. Overnight culture media was supplemented with gentamicin 30 µg/mL for plasmid maintenance, as needed. Cultures were then diluted (1:100 dilution) in fresh MHB (no antibiotic) and grown until mid-logarithmic growth phase was reached and then normalized to ca. 10^6 CFU/mL right before the growth experiment. These fresh bacterial suspensions were used as inoculum in a 1:1 dilution (final cell density ca. 5 x 10^5 CFU/mL). Plates were incubated at 37 ºC with shaking and OD_{600} was measured every 10 minutes to monitor growth over a 20-hour period. Six replicates were performed distributed in two different dates using two different stock solutions of P6P-10,10. Plasmid maintenance was confirmed after each experiment by plating 5 µL aliquots onto LB gentamicin 60 µg/mL plates.

RT-qPCR experiments

Relative gene expression analysis was performed as previously described using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit in an Applied Biosystems StepOnePlus Real-Time PCR System. Data obtained from three independent biological replicates and was normalized to the housekeeping gene *rpoD*.[172]

Hoechst 33342 dye accumulation assay

Hoechst 33342 accumulation assays were performed as previously described [173]. Briefly, overnight cultures of *P. aeruginosa* in LB were diluted in fresh media and grown until mid-logarithmic phase and normalized to an $OD_{600} = 0.5$. Bacterial cultures were pelleted by centrifugation (10,000 g x 3 minutes) and resuspended in PBS. 180 µL of this suspension were used to inoculate a flat-bottom 96-well plate. After two readings, Hoechst 33342 dye was added to final concentration of 2.5 µM in a final volume of 200 µl per well, including a PBS control. Fluorescence was measured from the top of the wells using 360 nm and 460 nm wavelengths as excitation and emission, respectively. Readings were taken every two minutes for a total of 60 minutes. All experiments were performed with at least 3 biological replicates.

Acknowledgments

We would like to acknowledge Dr. Patrick McGann for the generous distribution of clinical isolates. Funding was provided by the NIH (GM119426 to W.M.W.; DK126467 to G.G.V.C.; T32 GM008602 to C.A.S.).

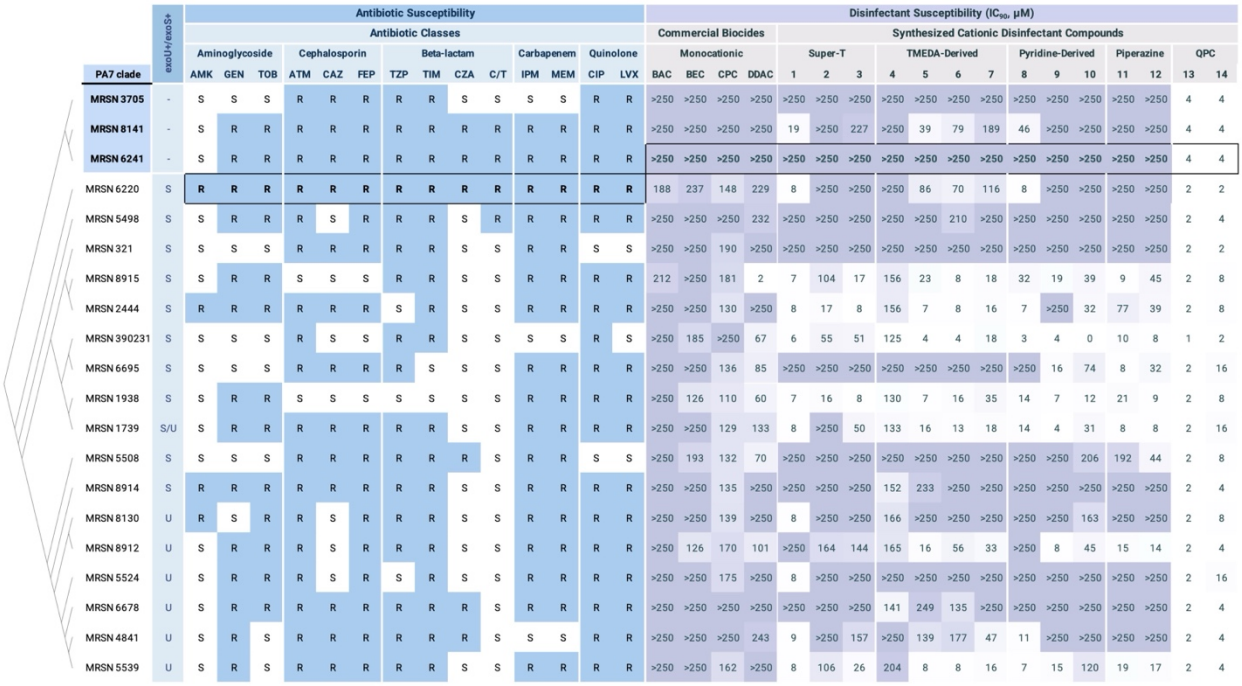


Figure 1. Susceptibility of the *P. aeruginosa* clinical isolates to a panel of 14 antibiotics, 4 commercial QACs, and 14 of our previously reported cationic disinfectant compounds. For the structures of cationic biocides 1-14, see Supporting Information. The listed antibiotic susceptibilities were previously reported by Lebreton *et al.*, wherein resistance (*R*) or susceptibility (*S*) was determined according to CLSI guidelines. Antimicrobials are grouped by drug class, and their susceptibilities are mapped against the phylogeny of the clinical isolates, generated using RAxML from alignment of the core genomes. Antibiotic abbreviations: AMK, amikacin; GEN, gentamicin; TOB, tobramycin; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; CZA, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin. Cationic biocides abbreviations: BAC, benzalkonium chloride, BEC, benzethonium chloride, CPC, cetylpyridinium chloride, DDAC, didecyldimethylammonium chloride.

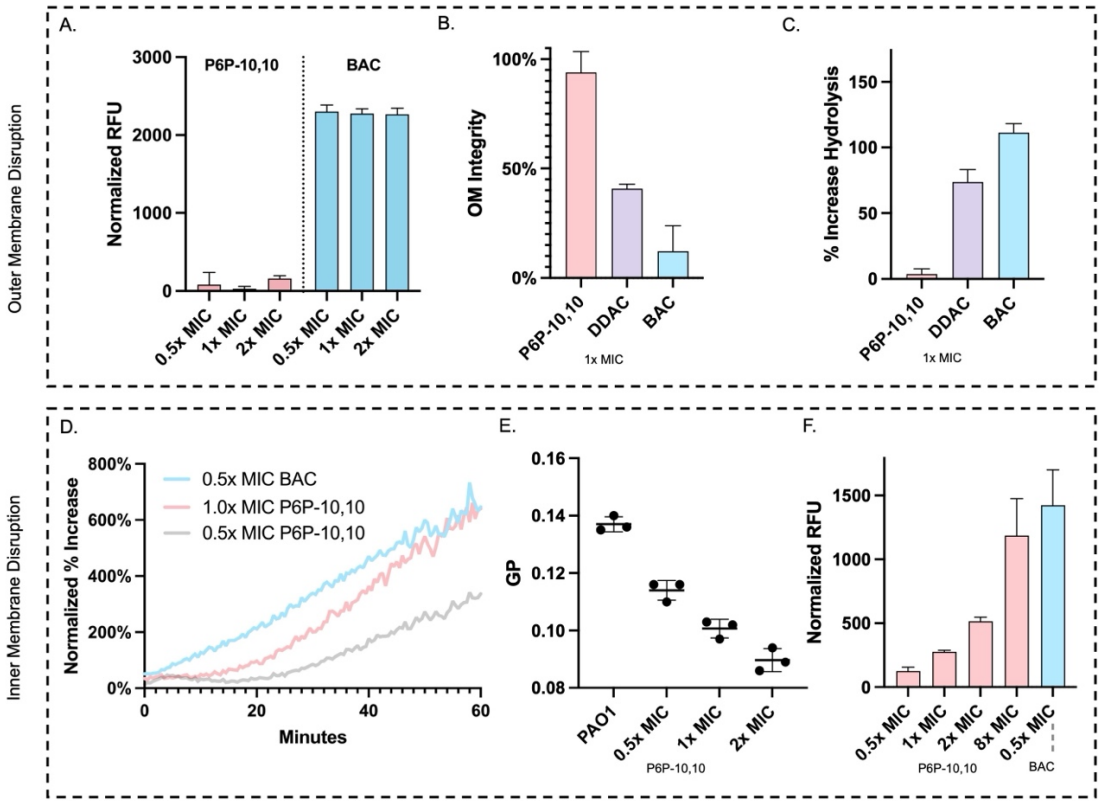


Figure 2. Evaluation of cationic biocide activity on the outer and inner membrane of *P. aeruginosa* PAO1. A) NPN uptake was assessed fluorometrically to measure outer membrane perturbation. B) Lysozyme permeability assay to assess outer membrane integrity through OD_{600} measurements upon lysozyme and disinfectant treatment. C) Effect of disinfectant treatment on nitrocefin hydrolysis mediated by outer membrane disruption. D) Inner membrane depolarization measured by $DiSC_3$-(5) upon QAC and QPC treatment. E) Laurdan generalized polarization (GP) to assess inner membrane fluidity, where lower values indicate increases in fluidity. F) Propidium iodide cytoplasmic entry as a measure of inner membrane disruption.

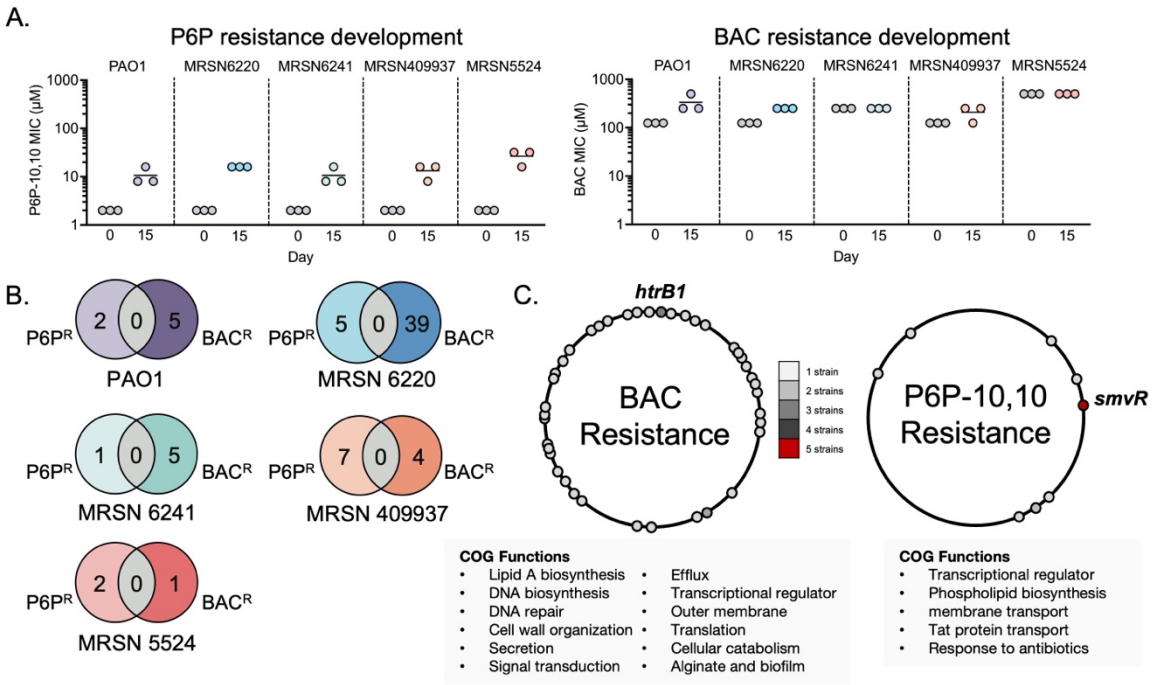


Figure 3. Distinct cationic biocide resistance profiles in response to P6P-10,10 and BAC. A) MIC values for P6P-10,10 (left) and BAC (right) before (Day 0) and after completion of resistance selection assay (Day 15). Three independent biological replicates were performed per strain. B) Venn diagrams of genetic variants identified in P6P- and BAC-resistant mutants of 5 *P. aeruginosa* strains: PAO1, MRSN6220, MRSN6241, MRSN409937 and MRSN5524. C) Schematic of genes with identified genetic variations associated with resistance to P6P-10,10 and BAC. Genes were mapped to *P. aeruginosa* PAO1 reference genome for simplicity. Cluster of orthologous genes (COG) cellular functions of genes with mutations identified in adaptation to each cationic biocide is reported.

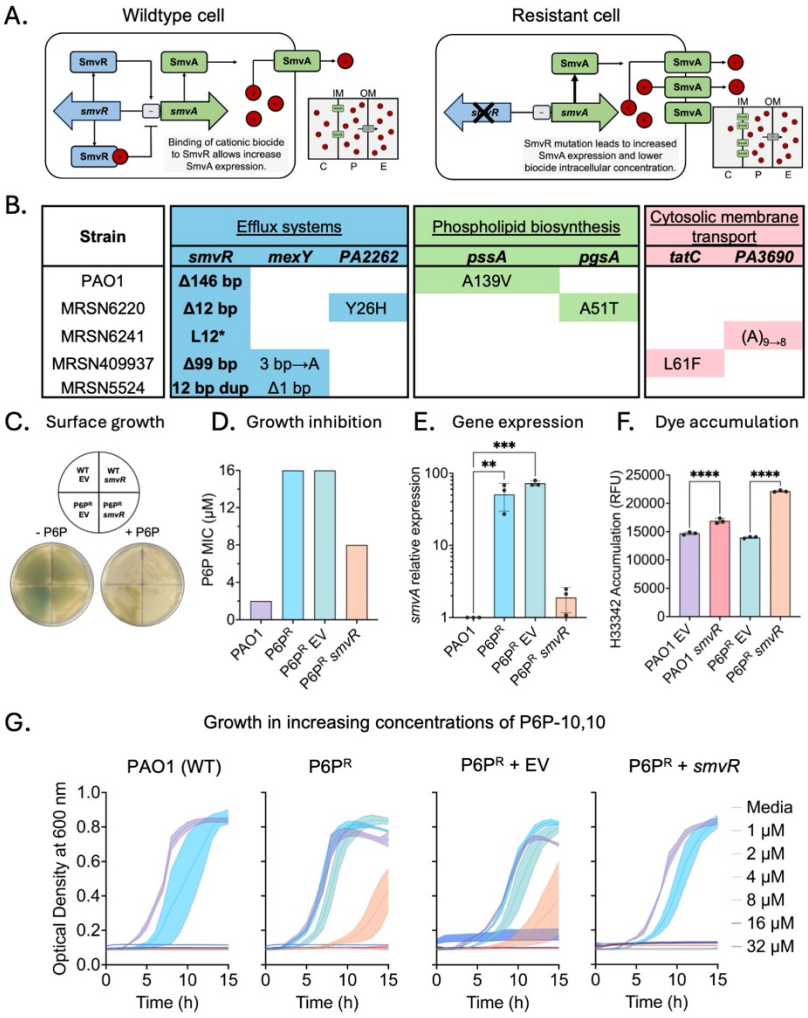


Figure 4. The efflux system SmvRA is a major resistance determinant of the QPC, P6P-10,10. A) Schematic of regulation of SmvA efflux pump expression. B) Mutations identified associated with P6P resistance in the 5 *P. aeruginosa* genetic backgrounds. C) *P. aeruginosa* PAO1 (WT) and P6P-resistant (P6P^{R}) strains with either empty vector (pUCP30T) or vector containing a functional copy of *smvR* with its native promoter (pUCP30T-*smvR*) incubated in LB plates with and without P6P-10,10 (700 μg). D) MIC values of PAO1 (WT) and P6P-resistant (P6P^{R}) strains with either empty vector (pUCP30T) or vector containing a functional copy of *smvR*. E) RT-qPCR expression analysis of smvA. F) Hoechst 33342 accumulation assay in strains with either empty vector (pUCP30T) or vector containing a functional copy of *smvR*. G) Growth curves of *P. aeruginosa* PAO1 (WT) and P6P-resistant (P6P^{R}) strains

with either empty vector (pUCP30T) or vector containing a functional copy of *smvR* in the presence of increasing concentrations of P6P-10,10. The mean and standard deviation (SD) of 6 replicates is plotted. RT-qPCR and dye accumulation was analyzed using one-way ANOVA with Tukey correction for multiple comparisons. **, p-value ≤ 0.01; ***, p-value ≤ 0.001; ****, p-value ≤ 0.0001.

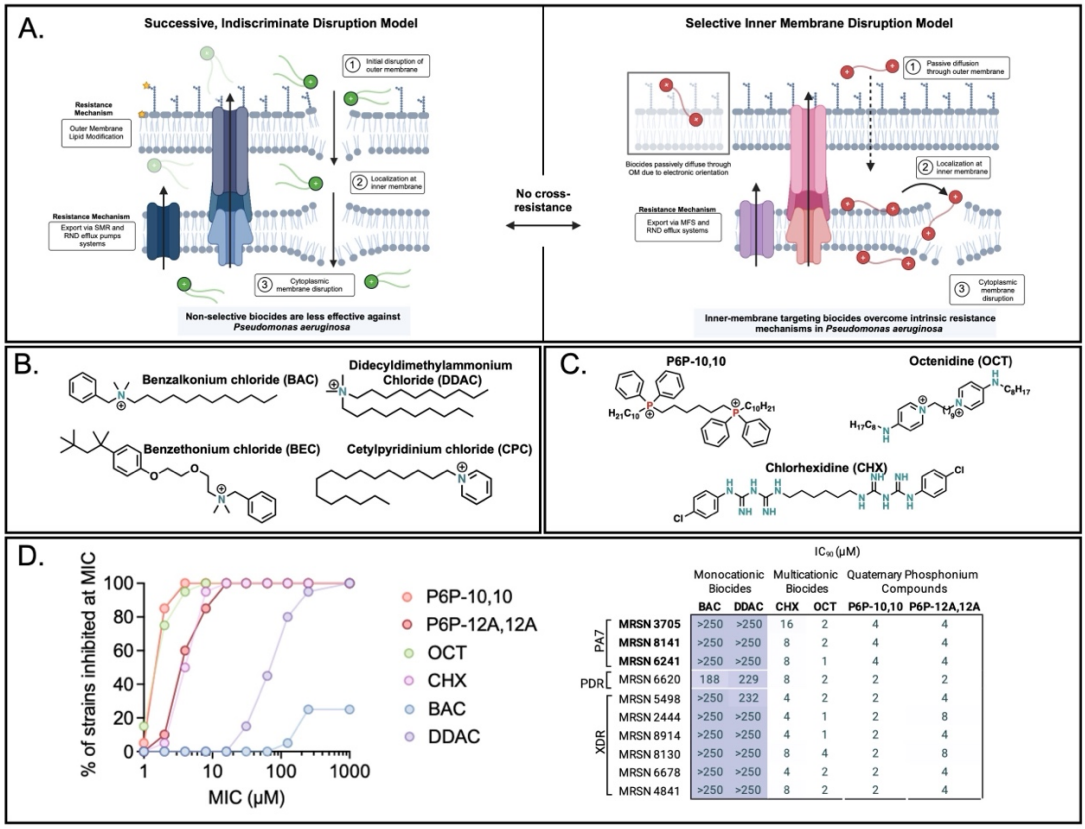


Figure 5. Mechanistic insights of cationic biocide classes from this study A. Mechanistic proposal of delocalized lipophilic cationic biocides contrasted with traditional QACs BAC and DDAC, whereby DLC biocides selectively disrupt the inner membrane while QACs show nonselective disruption of both outer and inner membranes **B.** Structures of QACs BAC, BEC, CPC, and DDAC. **C.** Structures of disinfectants with delocalized lipophilic cations in this study: P6P-10,10, octenidine, and chlorhexidine **D.** CHX and OCT possesses comparable activity to P6P-10,10 and P6P-12A,12A against a panel of 20 clinical isolates of *P. aeruginosa.*

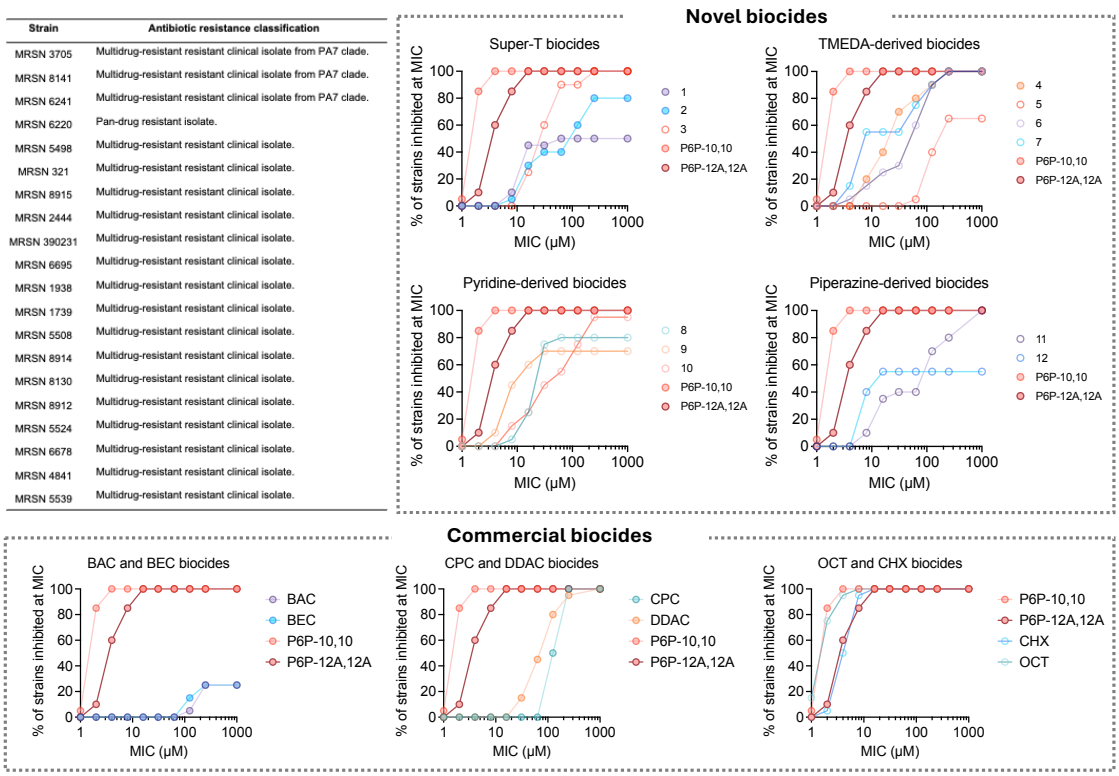


Figure S1. Cumulative inhibition of 20 selected *P. aeruginosa* strains by commercial and novel biocides determined by MIC assays. 20 *P. aeruginosa* clinical isolates with different antibiotic resistance profiles were selected and their susceptibility to novel biocides synthetized by our group and commercial biocides was tested by MIC assay. The cumulative percentage inhibition of the 20 *P. aeruginosa* strains was calculated and plotted against biocide concentration (μM). A trailing growth effect was observed with some of the biocides with specific strains, and IC_{90} values were calculated instead and presented in **Figure 1**.

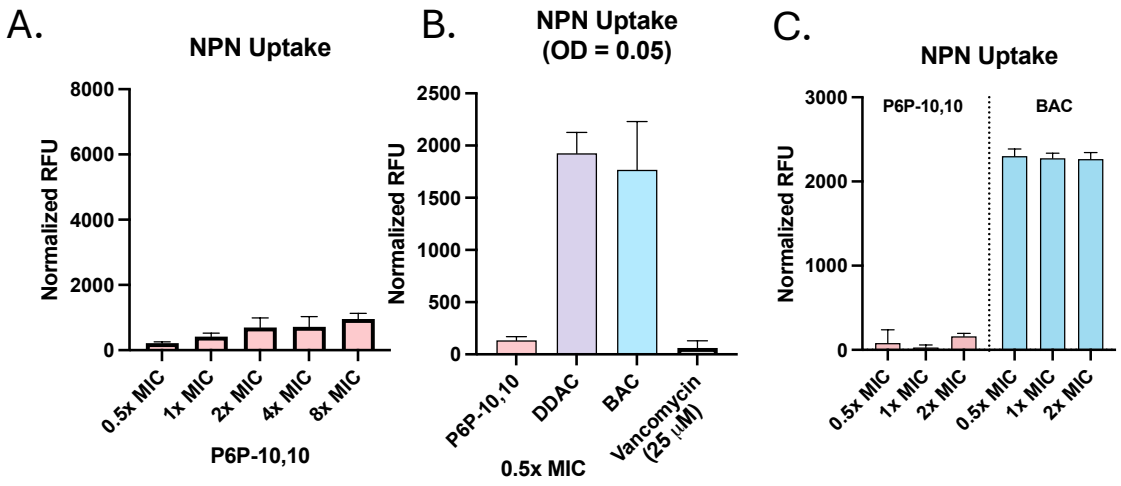


Fig S2. NPN assays varying concentration of cationic biocide and cell density. A) NPN uptake assay with increasing amount of P6P-10,10 to assess outer membrane interactions. B) NPN uptake assay at a 10-fold lower cell density to assess whether inoculum effect influences NPN uptake results with vancomycin as a negative control. C) NPN uptake with P6P-10,10 and BAC at 0.5x, 1x, and 2x relative MIC values show a saturation of outer membrane disruption due to BAC below MIC and no strong effects of higher concentrations of P6P-10,10.

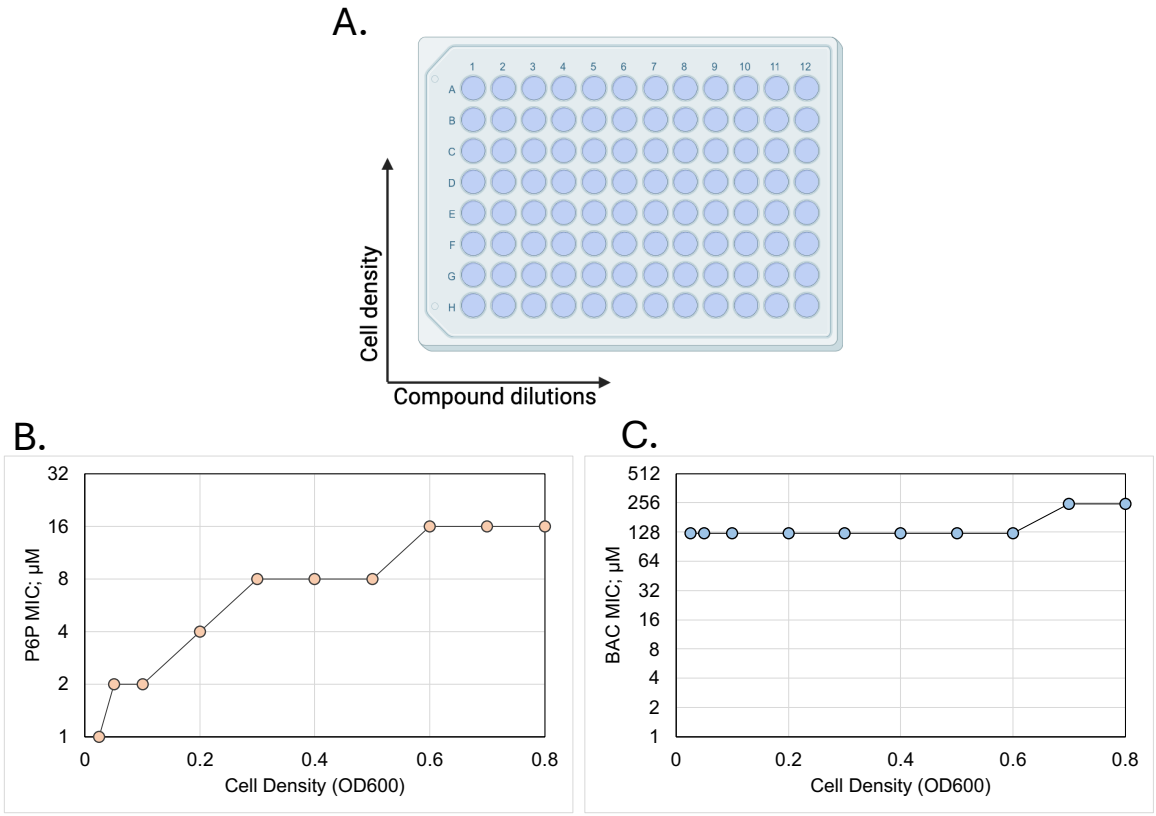
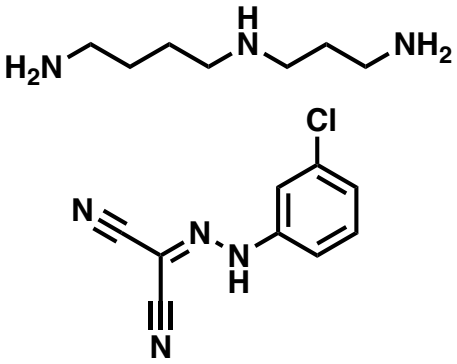


Figure S3: Inoculum effect on P6P-10,10 and BAC antimicrobial activity against *P. aeruginosa* PAO1. A) Schematic of 96-well plate setup to test inoculum effects on P6P-10,10 and BAC minimum inhibitory concentration (MIC). Increasing concentrations of PAO1 cells were exposed to two-fold serial dilutions of the compounds. MIC values were determined after 24 hours of incubation. B) Inoculum effect on P6P-10,10 antimicrobial effectiveness. C) Inoculum effect on BAC antimicrobial effectiveness.

A.



Spermidine (Spd)

Carbonyl cyanide 3-chlorophenyl hydrazone (CCCP)

B.

MIC, µM (± 5mM Spd)

BAC		DDAC		P6P-10,10	
- Spd	+ Spd	- Spd	+ Spd	- Spd	+ Spd
100	300	32	50	2	2

C.

MIC, µM (± 50 µM CCCP)

BAC		DDAC		P6P-10,10	
- CCCP	+ CCCP	- CCCP	+ CCCP	- CCCP	+ CCCP
125	125	32	63	2	40

Figure S4. Spermidine and carbonyl cyanide 3-chlorophenyl hydrazone antagonism assays in *P. aeruginosa* PAO1. A) Chemical structures of spermidine (Spd) and carbonyl cyanide 3-chlorophenyl hydrazone (CCCP). B) MIC data for BAC, DDAC, and P6P-10,10 in the presence of 5mM Spd. C) MIC data for BAC, DDAC, and P6P-10,10 in the presence of 50 mM CCCP.

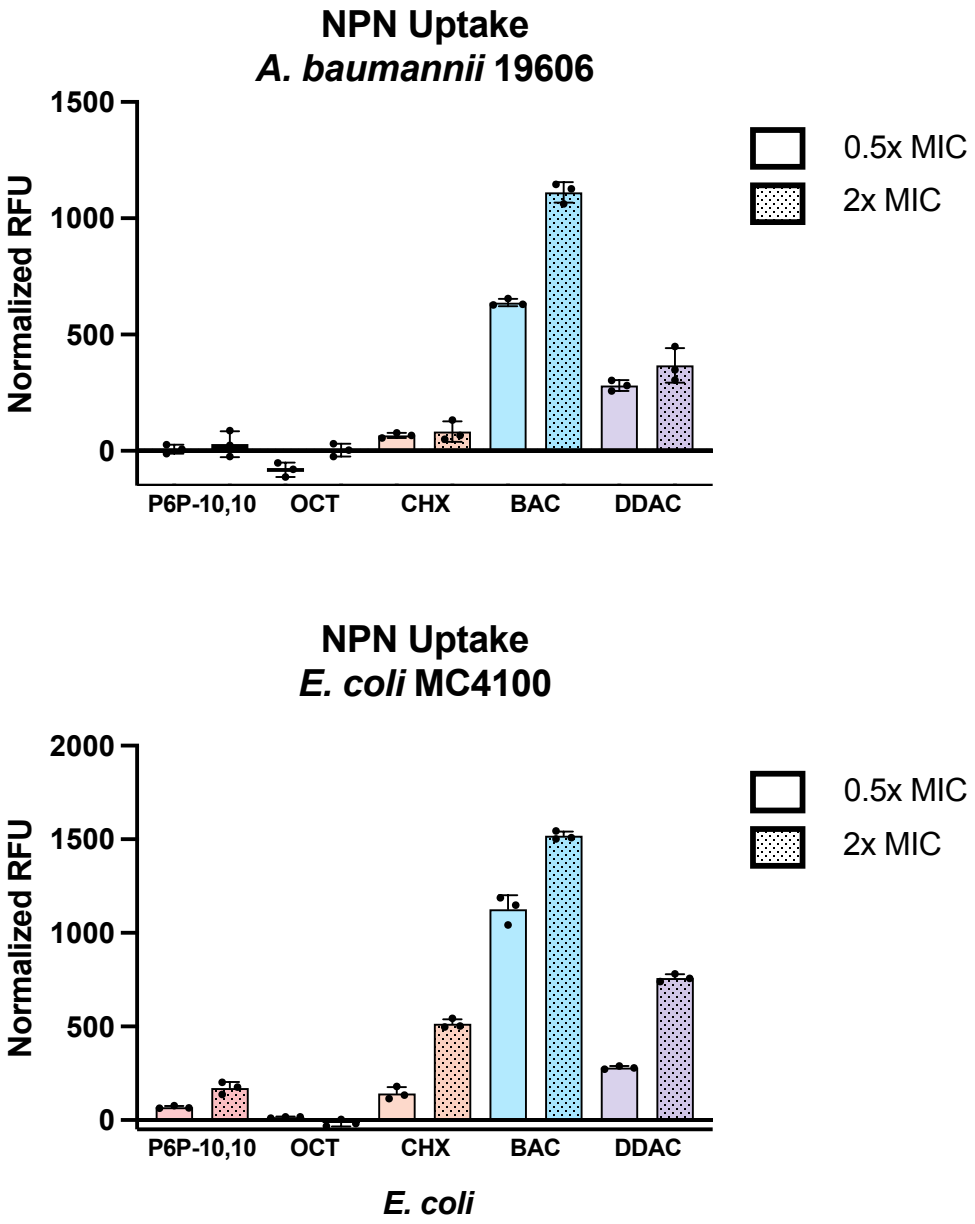


Fig S5. NPN uptake assays in additional gram-negative species. A) NPN uptake was assessed fluorometrically to measure outer membrane perturbation in *A. baumannii* ATCC 19606 at 0.5x and 2x MIC. B) NPN uptake was assessed fluorometrically to measure outer membrane perturbation in *E. coli* MC4100 at 0.5x and 2x MIC.

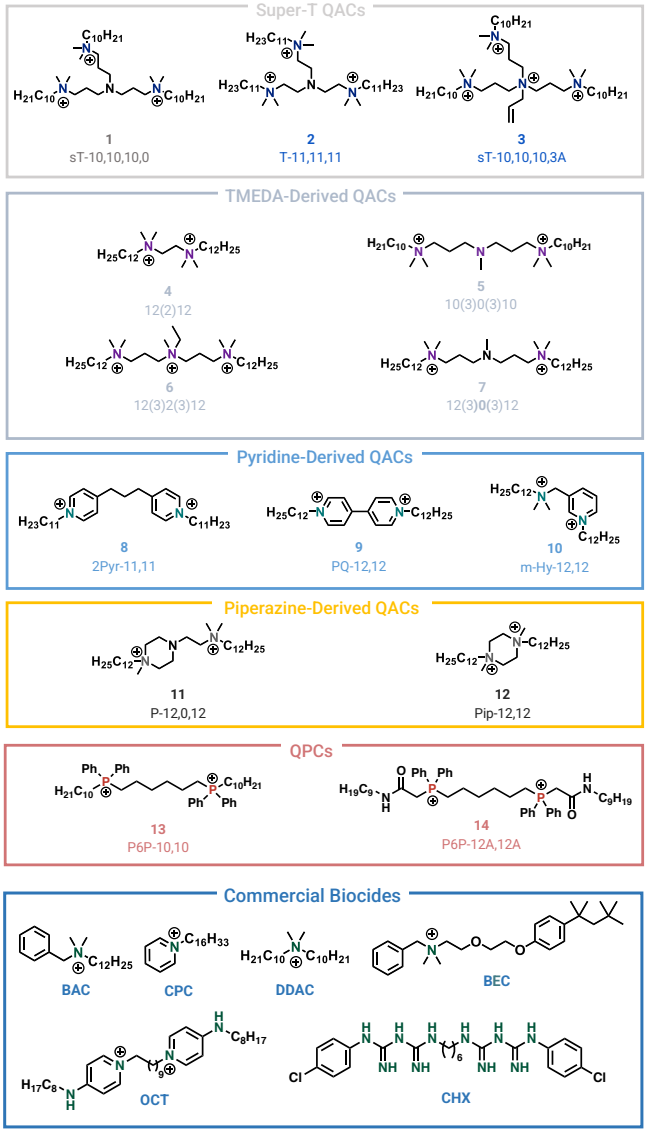


Fig S6. Cationic biocides used in the study grouped by class. Super-T QACs: sT-10,10,10,0 (**1**) , T-11,11,11 (**2**), sT-10,10,10,3A (**3**). TMEDA-Derived QACs: 12(2)12 (**4**), 10(3)0(3)10 (**5**), 12(3)2(3)12 (**6**), 12(3)0(3)12 (**7**). Pyridine-Derived QACs: 2Pyr-11,11 (**8**), PQ-12,12 (**9**), m-Hy-12,12 (**10**). Piperazine-Derived QACs: P-12,0,12 (**11**), Pip-12,12 (**12**). QPCs: P6P-10,10 (**13**), P6P-12A,12A (**14**). Monocationic commercial biocides: benzalkonium chloride (**BAC**), cetylpyridinium chloride (**CPC**), didecyldimethylammonium chloride (**DDAC**), benzethonium chloride (**BEC**). Multicationic commercial biocides: octenidine dichloride (**OCT**), chlorhexidine (**CHX**).

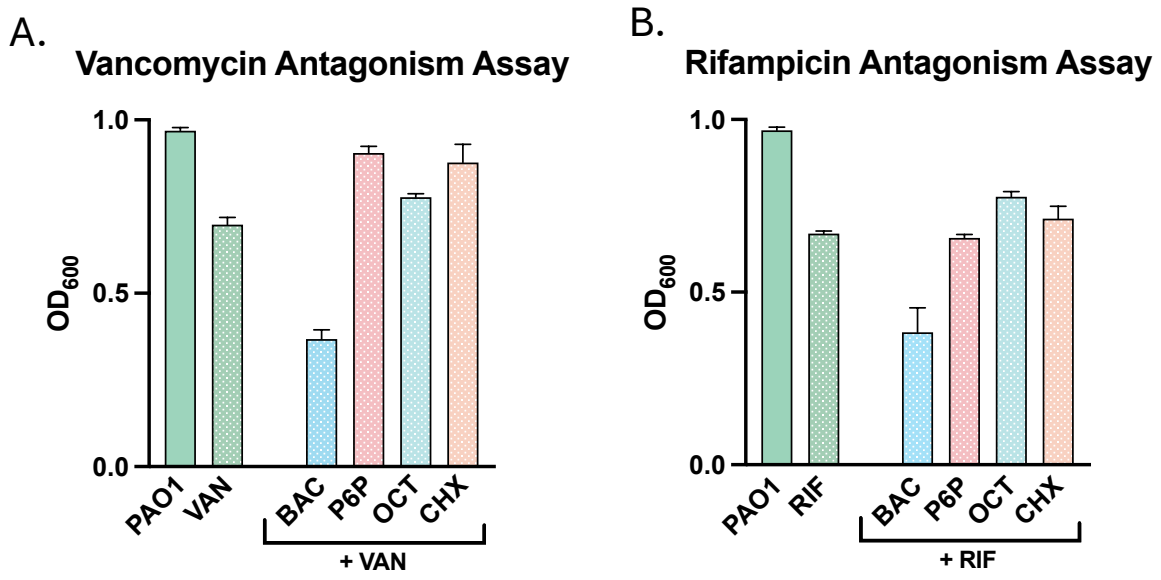


Fig S7. Antagonism assays with gram-positive antibiotics. NPN uptake assay with 0.5x MIC of cationic biocide and A) 16 μM of vancomycin or B) 8 μM of rifampicin. The combination of BAC and antibiotic results in a lower maximum OD_{600} than antibiotic alone, presumably through permeabilizing the membrane and facilitating accumulation. The inner membrane selective biocides had no additive effect on vancomycin and rifampicin.

Table S1: Strains and plasmids used in this study.

Strain name	Description	Source
Strains		
DH5α	*E. coli* cloning strain	Invitrogen
PAO1	*P. aeruginosa* laboratory strain	18
PAO1 BAC^R	Isolated BAC-resistant mutant derived from PAO1.	This study.
PAO1 P6P^R	Isolated P6P-resistant mutant derived from PAO1.	This study.
MRSN 6220	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 6220 BAC^R	Isolated BAC-resistant mutant derived from MRSN6220.	This study.
MRSN 6220 P6P^R	Isolated P6P-resistant mutant derived from MRSN6220.	This study.
MRSN 6241	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 6241 BAC^R	Isolated BAC-resistant mutant derived from MRSN6241.	This study.
MRSN 6241 P6P^R	Isolated P6P-resistant mutant derived from MRSN6241.	This study.
MRSN 409937	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 409937 BAC^R	Isolated BAC-resistant mutant derived from MRSN409937.	This study.
MRSN 409937 P6P^R	Isolated P6P-resistant mutant derived from MRSN409937.	This study.
MRSN 5524	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 5524 BAC^R	Isolated BAC-resistant mutant derived from MRSN5524.	This study.
MRSN 5524 P6P^R	Isolated P6P-resistant mutant derived from MRSN5524.	This study.
MRSN 3705	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 8141	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 5498	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 321	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 8915	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 2444	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 390231	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 6695	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 1938	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 1739	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 5508	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 8914	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 8130	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 8912	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 6678	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 4841	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
MRSN 5539	Clinical isolate from DoD *P. aeruginosa* diversity panel.	15
Plasmids		
pUCP30T	Parental plasmid used for *trans*-complementation (Tet^R)	25
pUCP30T::*smvR*	pUCP30T containing *smvR* with its predicted native promoter	This study.

Table S2: Genetic variants identified in P6P-resistant strains.

gene	position	mutation	PAO1 locus tag	description
PAO1 P6P-R				
[*smvR*]–[PA1284]	1,394,696	Δ146 bp	PA1283	[*smvR*], [PA1284]
pssA ←	5,271,885	A139V (GCG→GTG)	PA4693	phosphatidylserine synthase
MRSN6220 P6P-R				
DY941_RS06195 ←	18,779	$(ATCAGCGCCGCC)_{2\rightarrow1}$	PA1283	TetR/AcrR family transcriptional regulator
DY941_RS06850 ←	79,961	N187S (AAC→AGC)	PA1157	winged helix-turn-helix domain-containing protein
DY941_RS09310 →	223,123	E128* (GAG→TAG)	PA0663	hypothetical protein
pgsA ←	60,954	A51T (GCC→ACC)	PA2584	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
DY941_RS23730 ←	30,560	Y26H (TAC→CAC)	PA2262	MFS transporter
MRSN6241 P6P-R				
DY940_RS05995 →	317,974	L12* (TTG→TAG)	*PA1283 (smvR)*	*smvR* TetR/AcrR family transcriptional regulator
MRSN409937 P6P-R				
[DY952_RS03800]	338,867	Δ99 bp	*PA1283 (smvR)*	*smvR*
tatC ←	66,282	L61F (CTT→TTT)	*PA5070*	twin-arginine translocase subunit TatC
intergenic	15,636	$(A)_{9\rightarrow8}$	*PA3690 / PA3691*	DUF4398 domain-containing protein/heavy metal translocating P-type ATPase
mexY ←	37,600	2 bp→CA	*PA2018*	multidrug efflux RND transporter permease subunit MexY
mexY ←	37,603	3 bp→A	*PA2018*	multidrug efflux RND transporter permease subunit MexY
MRSN5524 P6P-R				
DY943_RS12890 ←	17,893	$(ATCAGCGCCGCC)_{2\rightarrow3}$	*PA1283*	smvR
mexY →	76,038	Δ1 bp	*PA2018*	multidrug efflux RND transporter permease subunit MexY

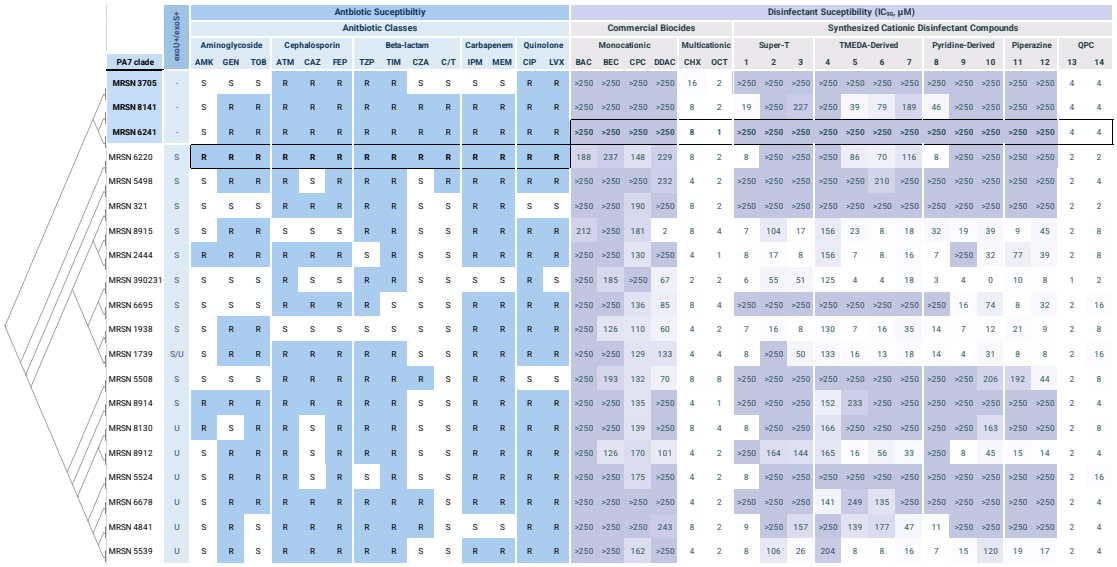
Table S3: Genetic variants identified in BAC-resistant strains.

gene	position	mutation	PAO1 locus tag	description
PAO1 BAC-R				
cupC2 →	1,074,592	$(C)_{6\rightarrow5}$	PA0993	chaperone CupC2
aprD →	1,351,186	Y147C (TAC→TGC)	PA1246	alkaline protease secretion protein AprD
PA2867 →	3,220,511	G302S (GGC→AGC)	PA2867	probable chemotaxis transducer
mutS →	4,055,923	N467D (AAC→GAC)	PA3620	DNA mismatch repair protein MutS
PA4667 →	5,235,708	coding (712/1773 nt)	PA4667	hypothetical protein
MRSN6220 BAC-R				
DY941_RS02760 ←	17,679	$(T)_{9\rightarrow8}$	PA5133	murein hydrolase activator EnvC
DY941_RS03340 ←	4,227	G340G (GGC→GGT)	PA5021	potassium/proton antiporter
dipA ←	9,293	Δ13 bp	PA5017	phosphodiesterase DipA
DY941_RS05315 ←	31,937	Y16Y (TAC→TAT)	PA1463	chemotaxis protein CheW
DY941_RS05340 ←	37,996	D39D (GAC→GAT) G164G (GGA→GGG)	PA1458	chemotaxis protein CheA
lasR ←	66,473		PA1430	transcriptional regulator LasR
DY941_RS06180 ←	15,375	V219I (GTC→ATC)	PA1286	MFS transporter
phoQ ←	56,267	L312P (CTG→CCG)	PA1180	two-component system sensor histidine kinase PhoQ
DY941_RS07355 →	30,120	R192C (CGT→TGT)	PA0291	OprD family porin
intergenic	83,144	C→T	PA0373	insulinase family protein/signal recognition particle-docking protein FtsY
DY941_RS08390 →	50,598	R103W (CGG→TGG)	PA0483	GNAT family N-acetyltransferase
DY941_RS11375 →	22,861	S10S (AGC→AGT)	PA3937	taurine ABC transporter ATP-binding protein
DY941_RS11615 →	70,312	S116S (AGC→AGT)		regulatory protein GemA
DY941_RS11900 ←	122,033	F68L (TTC→CTC)	PA3886	TIGR01459 family HAD-type hydrolase
intergenic	22,126	A→G	PA3828/29 intergenic	alpha/beta hydrolase/LPS export ABC transporter permease LptF
DY941_RS13070 →	59,888	V71A (GTT→GCT)	PA4440	alpha/beta hydrolase
murD →	14,515	V313V (GTA→GTG)	PA4414	UDP-N-acetylmuramoyl-L-alanine--D-glutamate ligase
intergenic	2,853	C→T	PA2042	APC family permease/serine/threonine transporter SstT
ligD →	120,803	D298G (GAC→GGC)	PA2138	DNA ligase D
tagH ←	9,833	$(GGCTGT)_{10\rightarrow7}$	PA0081	type VI secretion system-associated FHA domain protein TagH
DY941_RS16375 ←	56,939	S213S (AGC→AGT)	PA0780	AraC family transcriptional regulator
DY941_RS16525 →	86,312	G71S (GGC→AGC)	PA0751	AbrB family transcriptional regulator
intergenic	11,056	G→A	PA3300/01	long-chain-fatty-acid--CoA ligase FadD2/alpha/beta hydrolase
DY941_RS18410 ←	26,646	Δ2 bp	PA0011	lysophospholipid acyltransferase
DY941_RS19265 ←	208,282	$(CCG)_{2\rightarrow3}$	PA5418	sarcosine oxidase subunit alpha
DY941_RS20300 ←	3,314	V117A (GTG→GCG)		hypothetical protein
cdsA ←	19,282	P132L (CCG→CTG)	PA3651	phosphatidate cytidylyltransferase
rpsB ← / → *map*	23,774	T→C	PA3656	30S ribosomal protein S2/type I methionyl aminopeptidase
pheT →	23,793	Y658C (TAC→TGC)	PA2739	phenylalanine--tRNA ligase subunit beta
DY941_RS22510 ←	47,540	R114G (AGA→GGA)		inovirus Gp2 family protein
intergenic	26,063	$(G)_{6\rightarrow7}$	PA2379/80	hypothetical protein/(2Fe-2S)-binding protein
DY941_RS23690 ←	20,551	Δ1 bp	PA2270	TetR family transcriptional regulator
mutS →	45,820	T112P (ACC→CCC)	PA3620	DNA mismatch repair protein MutS
intergenic	7,090	$(C)_{6\rightarrow7}$	PA4028/29	hypothetical protein/DedA family protein
DY941_RS25610 →	50,523	V38I (GTC→ATC)	PA4076	hypothetical protein
DY941_RS25645 ←	1,754	N719S (AAC→AGC)	PA4082	filamentous hemagglutinin N-terminal domain-containing protein
DY941_RS27435 ←	2,249	A189T (GCC→ACC)	PA1015	IclR family transcriptional regulator
DY941_RS27520 →	19,818	M1M (ATG→GTG) †	PA1028/29	DUF2384 domain-containing protein
DY941_RS28400 ←	25,649	R54C (CGC→TGC)	PA4173	RidA family protein
MRSN6241 BAC-R				
DY940_RS01155 →	88,254	Δ15 bp	*PA0011*	lysophospholipid acyltransferase
intergenic	22,050	$(TCTCCTG)_{10\rightarrow9}$	intergenic	Hpt domain-containing protein/type 1 fimbrial protein
DY940_RS07645 ←	181,779	V389A (GTG→GCG)	*PA1561*	methyl-accepting chemotaxis protein
DY940_RS24055 ←	79,343	$(TTCTCGATG)_{3\rightarrow2}$	*PA4670*	ribose-phosphate pyrophosphokinase
DY940_RS27745 →	50,110	T225P (ACC→CCC)	*PA5332*	exodeoxyribonuclease III
MRSN409937 BAC-R				
tpbB ←	159,229	A196T (GCG→ACG)	*PA1120*	diguanylate cyclase TpbB
DY952_RS25190 ←	100,076	H229R (CAT→CGT)	*PA0011*	lysophospholipid acyltransferase
algB →	78,955	P205S (CCG→TCG)	*PA5483*	sigma-54-dependent response regulator transcription factor AlgB
DY952_RS29250 →	59,032	A21V (GCG→GTG)	*PA2270*	TetR family transcriptional regulator
MRSN5524 BAC-R				
DY943_RS04370	2,749	IS5	PA3450	glycosyltransferase family 4 protein

Table S4: BAC and P6P-10,10 MIC values in resistant mutant and parental strains.

	MIC (μM)	
Strain	**BAC**	**P6P-10,10**
PAO1	125	2
BAC^R	250-500	2-4
$P6P^R$	125-250	8-16
MRSN 6220	125	2
6220 BAC^R	250	2
6220 $P6P^R$	125	16
MRSN 6241	250	2
6241 BAC^R	250	2-4
6241 $P6P^R$	125-250	8-16
MRSN 409937	125	2
409937 BAC^R	125-250	1-4
409937 $P6P^R$	125	8-16
MRSN 5524	500	2
5524 BAC^R	500	2-4
5524 $P6P^R$	500	8-16

Table S5. Susceptibility of the *P. aeruginosa* clinical isolates to a panel of 14 antibiotics, 4 commercial QACs and 2 multicationic biocides, and 14 of our previously reported cationic disinfectant compounds.



		Antibiotic Susceptibility														Disinfectant Susceptibility (IC_{50}, μM)													
		Anitbiotic Classes														Commercial Biocides						Synthesized Cationic Disinfectant Compounds													
		Aminoglycoside			Cephalosporin			Beta-lactam				Carbapenem		Quinolone		Monocationic				Multicationic		Super-T			TMEDA-Derived				Pyridine-Derived			Piperazine		QPC	
PA7 clade	exoU+/-exoS+	AMK	GEN	TOB	ATM	CAZ	FEP	TZP	TIM	CZA	C/T	IPM	MEM	CIP	LVX	BAC	BEC	CPC	DDAC	CHX	OCT	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MRSN 3705	-	S	S	S	R	R	R	R	R	S	S	S	S	R	R	>250	>250	>250	>250	16	2	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	4	4
MRSN 8141	-	S	R	R	R	R	R	R	R	R	R	R	R	R	R	>250	>250	>250	>250	8	2	19	>250	227	>250	39	79	189	46	>250	>250	>250	>250	4	4
MRSN 6241	-	S	R	R	R	R	R	R	R	R	R	R	R	R	R	**>250**	**>250**	**>250**	**>250**	**8**	**1**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	4	4
MRSN 6220	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	188	237	148	229	8	2	8	>250	>250	>250	86	70	116	8	>250	>250	>250	>250	2	2
MRSN 5498	S	S	R	R	R	S	R	R	R	S	R	R	R	R	R	>250	>250	>250	232	4	2	>250	>250	>250	>250	>250	210	>250	>250	>250	>250	>250	>250	2	4
MRSN 321	S	S	S	S	R	R	R	R	R	S	S	R	R	S	S	>250	>250	190	>250	8	2	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	2	2
MRSN 8915	S	S	R	R	S	S	S	R	R	S	S	R	R	R	R	212	>250	181	2	8	4	7	104	17	156	23	8	18	32	19	39	9	45	2	8
MRSN 2444	S	R	R	R	R	R	R	S	R	S	S	R	R	R	R	>250	>250	130	>250	4	1	8	17	8	156	7	8	16	7	>250	32	77	39	2	8
MRSN 390231	S	S	S	S	R	S	S	R	R	S	S	S	S	R	S	>250	185	>250	67	2	2	6	55	51	125	4	4	18	3	4	0	10	8	1	2
MRSN 6695	S	S	S	S	R	R	R	R	S	S	S	R	R	R	R	>250	>250	136	85	8	4	>250	>250	>250	>250	>250	>250	>250	>250	16	74	8	32	2	16
MRSN 1938	S	S	R	R	S	S	S	S	S	S	S	R	R	R	R	>250	126	110	60	4	2	7	16	8	130	7	16	35	14	7	12	21	9	2	8
MRSN 1739	S/U	S	R	R	R	R	R	R	R	S	S	R	R	R	R	>250	>250	129	133	4	4	8	>250	50	133	16	13	18	14	4	31	8	8	2	16
MRSN 5508	S	S	S	S	R	R	R	R	R	R	S	R	R	S	S	>250	193	132	70	8	8	>250	>250	>250	>250	>250	>250	>250	>250	>250	206	192	44	2	8
MRSN 8914	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R	>250	>250	135	>250	4	1	>250	>250	>250	152	233	>250	>250	>250	>250	>250	>250	>250	2	4
MRSN 8130	U	R	S	R	R	S	R	R	R	S	S	R	R	R	R	>250	>250	139	>250	8	4	8	>250	>250	166	>250	>250	>250	>250	>250	163	>250	>250	2	8
MRSN 8912	U	S	R	R	R	S	R	R	R	S	S	R	R	R	R	>250	126	170	101	4	2	>250	164	144	165	16	56	33	>250	8	45	15	14	2	4
MRSN 5524	U	S	R	R	R	S	R	S	R	S	S	R	R	R	R	>250	>250	175	>250	4	2	8	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	2	16
MRSN 6678	U	S	R	R	R	R	R	R	R	R	S	R	R	R	R	>250	>250	>250	>250	4	2	>250	>250	>250	141	249	135	>250	>250	>250	>250	>250	>250	2	4
MRSN 4841	U	S	R	S	R	R	R	R	R	R	S	S	S	R	R	>250	>250	>250	243	8	2	9	>250	157	>250	139	177	47	11	>250	>250	>250	>250	2	4
MRSN 5539	U	S	R	S	R	R	R	R	R	S	S	R	R	R	R	>250	>250	162	>250	4	2	8	106	26	204	8	8	16	7	15	120	19	17	2	4

Super-T QACs: sT-10,10,10,0 (**1**) , T-11,11,11 (**2**), sT-10,10,10,3A (**3**). TMEDA-Derived QACs: 12(2)12 (**4**), 10(3)0(3)10 (**5**), 12(3)2(3)12 (**6**), 12(3)0(3)12 (**7**). Pyridine-Derived QACs: 2Pyr-11,11 (**8**), PQ-12,12 (**9**), m-Hy-12,12 (**10**). Piperazine-Derived QACs: P-12,0,12 (**11**), Pip-12,12 (**12**). QPCs: P6P-10,10 (**13**), P6P-12A,12A (**14**). Monocationic commercial biocides: benzalkonium chloride (**BAC**), cetylpyridinium chloride (**CPC**), didecyldimethyl ammonium chloride (**DDAC**), benzethonium chloride (**BEC**). Multicationic commercial biocides: octenidine dichloride (**OCT**), chlorhexidine (**CHX**)

Chapter IV

Adaptations to cationic biocide exposure differentially influences virulence factors and pathogenicity in *Pseudomonas aeruginosa*

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Author contributions: Experiment were conceived and designed by G.G.V.C., C.A.S., E.L.B., G.M.W., K.P.C.M, W.M.W., and conducted by G.G.V.C., C.A.S., E.L.B., G.M.W., S.S.. Data interpretation and analysis was performed by G.G.V.C., C.A.S., E.L.B., G.M.W., S.S.. The manuscript was written and edited by G.G.V.C., C.A.S., E.L.B., G.M.W., K.P.C.M., W.M.W..

Abstract

Cationic biocides (CBs), which include quaternary ammonium compounds (QACs), are employed to mitigate the spread of infectious bacteria, but resistance to such surface disinfectants is rising. CB exposure can have profound phenotypic implications that extend beyond allowing microorganisms to persist on surfaces. *Pseudomonas aeruginosa* is a deadly bacterial pathogen that is intrinsically tolerant to a wide variety of antimicrobials and is commonly spread in healthcare settings. In this study, we pursued resistance selection assays to the QAC benzalkonium chloride and quaternary phosphonium compound P6P-10,10 to assess the phenotypic effects of CB exposure in *P. aeruginosa* PAO1 and four genetically diverse, drug-resistant clinical isolates. In particular, we sought to examine how CB exposure affects defensive strategies and the virulence-associated "offensive" strategies in *P. aeruginosa*. We demonstrated that development of resistance to BAC is associated with increased production of virulence-associated pigments and alginate as well as pellicle formation. In an *in vivo* infection model, CB-resistant PAO1 exhibited a decreased level of virulence compared to wildtype, potentially due to an observed fitness cost in these strains. Taken together, these results illustrate the significant consequence CB resistance exerts on the virulence-associated phenotypes of *P. aeruginosa.*

Introduction

Disinfectants stand at the front lines in the fight against bacterial infections, which are associated with one in eight deaths worldwide.[174] Effective infection prevention and control rests on the ability to eliminate human pathogens from high-contact surfaces and provide sterile surfaces in healthcare settings,[19] though disinfectant use extends to a broader range of settings including agriculture, food industry, cosmetics, and domestic cleaning.[87] Chemical disinfectants fall into different classes including chlorine and chlorine-releasing compounds, peroxides, phenolics, and cationic biocides (CBs). CBs are among the most widely used disinfectants and include subclasses such as biguanides (e.g. chlorhexidine) and quaternary ammonium compounds (QACs) like benzalkonium chloride (BAC) and didecyldimethyl ammonium chloride (DDAC).[118] These cationic surfactants act upon bacteria by disturbing their cell envelopes, leading ultimately to membrane lysis and death.[19]

However, in recent years, resistance to disinfectants such as CBs has emerged, being further exacerbated following the COVID-19 pandemic, which resulted in a significant increase in disinfectant usage thereby inducing pressure toward disinfectant resistance.[115] In addition to extended surface survival, disinfectant resistance can lead to profound phenotypic implications in bacteria that are equally concerning. For example, disinfectant resistance has been associated with an increase in virulence factor production, reduced metabolism and growth rate, and increased biofilm production.[120, 175]

Pseudomonas aeruginosa is an opportunistic gram-negative bacterial human pathogen of particular concern. Notoriously difficult to treat due to its wealth of resistance mechanisms to antibiotics and disinfectants alike, *P. aeruginosa* is responsible for over half a million deaths per year worldwide.[6] Through a combination of defense strategies and virulence factors, *P.*

aeruginosa can establish deadly infections in immunocompromised hosts, especially in cystic fibrosis patients.[176] This bacterium possesses several defense strategies that protect it from the innate immune system and exogenous molecules like antibiotics. Biofilm and pellicle formation, alginate production, and intrinsic and acquired drug resistance mechanisms provide extensive protection for *P. aeruginosa* in infection settings.[60] Furthermore, the pathogenicity *P. aeruginosa* lies in what can be regarded as its "offense" strategies.[177, 178] This includes secreted factors (pyocyanin, pyoverdine, and proteases), cellular motility, and additional ways of gaining competitive advantages in the presence of other organisms; these factors are summarized in **Figure 1**. Concerningly, *P. aeruginosa* can be transmitted from one person to another through contaminated equipment or surfaces in healthcare settings.[179] Because of its high priority rating by the CDC and the WHO, it is crucial to assess the effect that exposure to cationic biocides has on the defensive and offensive strategies of *P. aeruginosa*.

Prolonged subinhibitory exposure to disinfectants has become an environmental reality due to increased CB prevalence in nature.[180] Previous reports have demonstrated that long term exposure to BAC can promote antibiotic resistance in bacteria including *P. aeruginosa.*[57, 120, 181] Kim *et al*. demonstrated that isolates of *P. aeruginosa* from river sediment that were exposed to BAC over a 3-year time span developed cross-resistance to clinically relevant antibiotics such as ciprofloxacin and chloramphenicol. Mc Cay *et al.* showed that after 33 serial passages of a *P. aeruginosa* clinical isolate in subinhibitory concentrations of BAC, the strain became less susceptible to ciprofloxacin; however, susceptibility to polymyxin B increased. Loughlin *et al*. also reported the development of serially passaged *P. aeruginosa* strains in sublethal amounts of BAC to explore antibiotic cross-resistance. In their studies, it was observed that the BAC-

resistant mutants exhibited an increase in resistance to polymyxin B with no MIC changes against imipenem, ciprofloxacin, and tobramycin.

In this study, we explored the effects of sublethal CB exposure utilizing *P. aeruginosa* strains from a recently developed panel of diverse clinical isolates from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN).[126] By using isolates from this panel, we are able to probe strains from a wide range of antibiotic susceptibility profiles and genetic backgrounds in addition to the laboratory reference strain PAO1. From the panel, we selected the pan-resistant isolate MRSN6220, extensively drug-resistant strains MRSN6241 and MRSN5524, and multidrug resistant strain MRSN409937. The disinfectants selected for this study included the most common CB BAC, used in a wide range of applications like hospital-surface disinfection, wound sterilization, pool water disinfection, and eggshell sanitization,[87] as well as the novel quaternary phosphonium compound P6P-10,10, previously reported by our research groups.[92] This compound is a promising biocide, showing significantly higher potency (~100X) compared to other commercial disinfectants. By comparing these two dramatically different cationic biocides, we hoped to assess whether structural diversity in CBs influences the adaptation phenotypes in *P. aeruginosa* after long-term exposure.

Herein, we disclose that exposure to a pair of disparate CB structures results in changes in bacterial defensive and offensive phenotypes with distinct implications in pathogenesis. Exposure to structurally distinct biocides can lead to differential production of virulence factors and overall bacterial physiology of *P. aeruginosa* strains. This underlines the importance of the potential implications of disinfection protocols on virulence and pathogenicity, and calls for the rational selection and usage of disinfectants.

Results and Discussion

CB resistance in P. aeruginosa influences antibiotic susceptibility.

We isolated CB-resistant *P. aeruginosa* strains after exposure to subinhibitory concentrations of BAC and P6P-10,10 for 15 days. To evaluate the effect of CB resistance development on antibiotic susceptibility, we tested our CB-resistant and parental strains against 12 antibiotics with differing modes of action including aminoglycosides, monobactams, cephalosporins, carbapenems, fluoroquinolones, and polymyxins. No significant trends were observed in cross-resistance between antibiotics and CB resistance, in contrast with the previous reports mentioned.[57, 120] Nevertheless, our results suggest that some resistance development to CBs may increase susceptibility to certain antibiotic classes in *P. aeruginosa* (**Table 1).** The BAC-resistant strains of MRSN6241 and MRSN409937 showed enhanced susceptibility to aminoglycosides, while the BAC-resistant PAO1 strain showed reduced sensitivity to aminoglycosides. Previously, aminoglycoside antagonism has been observed in PAO1 resulting from a reduction in membrane polarization as a tolerance mechanism.[121] In two clinical isolates, however, an increase in susceptibility is observed. For the BAC-resistant MRSN6241 strain, a drastic change in aminoglycoside susceptibility shifts the MIC for gentamicin and tobramycin back into a clinically susceptible range compared to the parental strain. In addition to changes in aminoglycoside susceptibility, PAO1 and each MRSN strain had a CB-resistant derived strain that displayed an increased susceptibility to a fluoroquinolone antibiotic. Most notably again, BAC-resistant MRSN6241 had a three-to-four-fold decrease in MIC for ciprofloxacin and levofloxacin. Additionally, we observed a two-to-three-fold decrease in MIC for the BAC-resistant mutant of MRSN409937 when tested against cephalosporins and carbapenems

(**Supplemental Figure S1**). The "P6P^{R}" strains showed little to no MIC difference in most cases, although the P6P-10,10-resistant mutant of MRSN409937 was markedly more susceptible to the three aminoglycosides tested. Overall, our results contrast with the previous studies which report cross-resistance or no change for BAC-resistant *P. aeruginosa* clinical and environmental isolates.

CB resistance increases alginate production in P. aeruginosa.

We sought to assess whether our resistance selection efforts resulted in any CB-resistant strains that increased alginate production to mitigate the effects of disinfectant treatment. The exopolysaccharide alginate encapsulates *P. aeruginosa* cells and has been shown to protect the bacteria from threats such as phagocytosis and disinfectant treatment.[182-184] *P. aeruginosa* strains infecting the lungs of cystic fibrosis (CF) patients frequently become overproducers of alginate, and this mucoid phenotype is crucial for establishing chronic infections in the lungs of CF patients.[185] To our knowledge, there have been no previous observations on whether CBs could influence alginate production in *P. aeruginosa*; though alginate has been shown to protect against QACs such as BAC and CTAB.[184]

We quantified alginate production of both the parental and CB-resistant strains and found a significant increase in alginate production in the BAC-resistant strains of PAO1, MRSN6241, and MRSN5524, and in the P6P-10,10-resistant strains of MRSN5524. Our findings suggest that the QAC BAC is more likely to induce this phenotype change in *P. aeruginosa* compared to quaternary phosphonium compound P6P-10,10 (**Fig. 2A**). Due to the rise of BAC resistance in the environment, this increased alginate phenotype could have profound implications on CF patients with CB-resistant *P. aeruginosa* infections.

Formation of biofilm in the solid-liquid interphase is not changed in the CB-resistant strains of P. aeruginosa.

Bacteria are commonly found in bacterial aggregates called biofilms. Within these biofilms bacteria engage in community-like behaviors that enhance their survival including acquisition of nutrients, cooperative and competitive interactions, and protection from antimicrobials.[186] Biofilms are composed of a matrix of extracellular polymeric substances produced by bacteria that include nucleic acids, lipids, secreted proteins, polysaccharides, and water.[187, 188] Biofilm development renders pathogens less susceptible to antimicrobials such as antibiotics and disinfectants which can lead to recalcitrant infections. For example, Henly *et al.* reported that uropathogenic strains of *E. coli* adapted to BAC showed increased biofilm formation.[189]

In *P. aeruginosa*, the main components of biofilms are the polysaccharides Pel (positively charged), Psl (charge-neutral), and alginate (negatively charged).[190] Production of Pel and Psl is observed during infection of cystic fibrosis patients and reduces the effectiveness of antimicrobial treatments, while alginate is the main polysaccharide produced by mucoidal strains and is associated with chronic infections.[191, 192] Since we observed increased alginate production associated with CB resistance, we sought to first quantify biofilm production at the solid-liquid interface in the parent and CB-resistant strains using crystal violet staining in minimal medium.

Surprisingly, no differences were observed in biofilm formation at the liquid-surface interface in the CB-resistant strains derived from the laboratory reference strain PAO1 or the strains derived from the clinical isolates panel (**Fig. 2B**). When compared to the parental PAO1 strain, the BAC-resistant strain produced on average less biofilm compared to the other isogenic strains, however this reduction in biofilm production was not significant. A similar result was

also observed in the BAC-resistant strain derived from MRSN409937 clinical isolate. Interestingly, the CB-resistant strains derived from MRSN5524 showed lower overall biofilm production, but not in a significant manner. Biofilm formation measured in lysogeny broth (LB) rich media also showed no significant difference between the CB-resistant strains and the parental strains (data not shown). Our data indicate CB resistance does not lead to increased biofilm formation at the solid-liquid interface in *P. aeruginosa*.

Pellicle formation is increased in the BAC-resistant but not in the P6P-resistant strain derived from PAO1.

Bacteria can also form floating biofilms at the air-liquid interface known as pellicles. Pellicles are mainly composed of exopolysaccharides, usually cellulose. In *P. aeruginosa,* the pellicle matrix is composed of the Pel and Psl polysaccharides which are rich in glucose and in mannose, respectively.[193] Regulation of pellicle formation is complex and species-specific, and its matrix composition varies between bacterial species. Pellicle formation in the air-liquid interface allows bacteria to readily acquire oxygen from the air and nutrients from the media.[193, 194] In *Bacillus subtilis*, exposure to sub-lethal concentrations of the biocide chlorine dioxide activates the membrane-bound histidine kinase KinC promoting biofilm formation in the form of pellicle.[195] Pellicle formation in *P. aeruginosa* requires proteins encoded in the *pel* and *psl* operons responsible for the synthesis of the required polysaccharides to build the matrix.[196]

When the CB-resistant strains derived from PAO1 were grown in LB, we observed an evident increase in pellicle formation in the BAC-resistant compared to the other isogenic strains (**Fig. 2C**). This perceived increase in biofilm biomass at the air-liquid interface suggested that pellicle formation was enhanced in the BAC-resistant strain. To quantify this difference in

pellicle formation, we used biofilm peg plates and performed the assay as previously described.[197] We found a significant increase in pellicle formation in the BAC-resistant strain when compared to the parental isogenic strain (**Fig. 2D**). No significant difference in pellicle formation was observed in the P6P-resistant strain. These data indicate CB resistance differentially influences biofilm formation at the air-liquid interface with only BAC resistance promoting pellicle formation in *P. aeruginosa*.

BAC adaptation reduces swimming behavior in clinical isolates.

Motility processes such as swimming and swarming play a vital role in pathogenesis of *P. aeruginosa* infections. Swimming—single cellular movement with flagella—aids *P. aeruginosa* in locating an infection site whereas the swarming motion, which is coordinated multicellular movement with flagella, is useful for the development of biofilms.[198] Together, these motility processes are multifaceted and assist in the pathogen's defense to the host immune response. Previous studies have shown that exposure to a range of biocides can negatively affect motility in gram-negative species. Nordholt *et al.* observed a reduction in motility for *E. coli* BAC-resistant mutants.[199] This same result was observed in a long-term *E. coli* BAC exposure study where the authors hypothesized that downregulation of motility could be a survival mechanism as the energy to produce flagella is high, thus this energy could be invested in other stress response strategies.[200] In a similar experiment where *E. coli* was serially passaged against BAC, Forbes *et al.* found that there was a reduced expression of genes related to motility.[201] Similar observations were found for gram-positive *Listeria monocytogenes* as BAC-adapted mutants exhibited reduced swarming motility.[202]

Wanting to build upon this knowledge, we interrogated the relationship between CB resistance of *P. aeruginosa* clinical isolates and motility. As shown in **Table 2**, we observed that generally the BAC-resistant strains exhibited less swimming behavior with the BAC-resistant mutant of MRSN6220 being markedly less motile. Though there were no apparent trends in swarming motility, it was observed that the BAC-resistant strain of MRSN6241 was less motile compared to the parent and P6P-10,10-resistant strains. These data suggests that BAC resistance can affect the swimming motility of *P. aeruginosa* and clinical isolates which reflects the findings in previous studies of motility in gram-negative and -positive bacteria.

BAC resistance results in an increase in virulence-associated pigment production.

With their characteristic greenish-blue hue, pseudomonads are easily recognizable due to their production of vibrant pigments. In *P. aeruginosa*, these colorful compounds are primarily the siderophore pyoverdine (PVD) and redox active metabolite pyocyanin (PYO).[78, 203] Both compounds are associated with *P. aeruginosa* virulence and can be used to gain an advantage over human hosts and other bacteria in its environment. The relationship between pigment production and CB resistance has been mostly overlooked; although increased expression of pyoverdine biosynthesis genes has been observed previously in BAC-adapted *P. aeruginosa* strains without further phenotypic validation noted.[124] In contrast, it has been reported that a QAC-adapted strain of *P. aeruginosa* decreased in pyocyanin production.[204] We observed a sharp increase in PYO production compared to the parental strains in BAC-resistant strains derived from PAO1 and MRSN409937 (**Fig. 3A**). In contrast, P6P-10,10 resistance appeared to have little impact on PYO production. In addition to PYO, production of the fluorescent siderophore PVD was studied. Similar to PYO, sharp increases in PVD production were observed in BAC-

resistant PAO1 and MRSN409937, and an increase was also observed in BAC-resistant MSRN6220 (**Fig. 3B**). These phenotypic shifts in pigment production are visible when culturing in solid or liquid media. Because increased PVD and PYO synthesis are known to inhibit the growth of other bacteria, we hypothesized that BAC-resistant strains might have an increased competitive advantage against other bacteria.

Lysis of S. aureus by disinfectant-resistant Pseudomonas aeruginosa PAO1.

We sought to study the effect of BAC and P6P-10,10 resistance on the inter-species relationship of *Staphylococcus aureus* and *P. aeruginosa.* Frequently isolated together, *P. aeruginosa* and *S. aureus* are important pathogens in human disease from wound infections to chronic lung infections.[205] Co-infections have been shown to be more virulent than single species infections.[206, 207] To our knowledge, no studies have been performed to assess the effect of disinfectant resistance on interbacterial competition. To interrogate the resistant *P. aeruginosa* phenotype, we conducted lysis experiments to determine whether disinfectant resistance affected the ability for PAO1 to induce lysis in *S. aureus* (**Fig. 4**). In plate assays, BAC- and P6P-10,10-resistant PAO1 strains had greater lysis zones than the parent PAO1 strain. In agreement with our previous results, the BAC-R PAO1 strain produced more bluish-green pigment than the two other PAO1 strains, correlating with the observed increase in pyocyanin production. This secreted factor production may be responsible for the increased lysis. However, despite a less substantial increase in PYO and an observed decrease in PVD, the P6P-R strain had the same lysis effects on *S. aureus*, as measured by zone of lysis. This indicates that there are other secreted virulence factors that may be primarily responsible for the causing the zone of lysis in

PAO1. Nevertheless, these data indicate that CB resistance can affect important interbacterial relationships, which may have implications for human infections.

CB-resistant mutants show competitive disadvantage compared to the PAO1 parental strain.

Bacterial competition is an important interaction that occurs between and within species and can determine which microorganism will thrive within a niche.[208] In *P. aeruginosa*, mutant isolates known as "social cheaters" take advantage of factors secreted by surrounding bacteria without the need to spend resources to produce them themselves, allowing them to thrive.[209] Similar mutants have been isolated in CF patients, highlighting the potential implications in health.[210]

Since we observed variations in fitness while growing the CB-resistant strains in different media (**Supplemental Figures S2 and S3**), we wanted to determine the competitive fitness of these isogenic mutants. To investigate this, we performed a prey recovery rate competition assay.[211, 212] This assay consists of co-incubating the strains of interest ("attackers") with a reporter strain ("prey") at a fixed ratio and using a selectable marker on the prey strain to select only this strain after competition (**Fig. 5A**). In our case, the prey strain (PAO1 pUCP30T) is gentamicin-resistant while the attacker strains (PAO1, BAC-R, and P6P-R) are gentamicin-sensitive. The PAO1 wildtype strain without the vector was included as a control. After selection, the prey recovery rate is calculated by comparing the recovered prey in selective media (output) with the prey amount at the beginning of the experiment (input). This competition assay provides additional information on the fitness of these isogenic strains under mixed culture conditions.

We observed phenotypic differences during our competition incubation period, especially with our P6P-resistant strain (**Fig. 5B**). While we observed differences suggestive of competitive

advantage of the P6P-resistant strain, the overall prey recovery rate indicated no significant difference compared to the WT control (**Fig. 5C**). The BAC-resistant strain showed an overall lower fitness compared to the WT as indicated by a higher prey recovery rate compared to the one obtained in the WT control (**Fig. 5D**). These data suggest that resistance to CBs affect competitive fitness within these isogenic strains.

Evaluation of P. aeruginosa virulence in G. mellonella larvae infection model.

To evaluate how the production of virulence factors in CB-resistant strains affect virulence *in vivo*, we utilized the *Galleria mellonella* larvae infection model. This system is used to study virulence factor production and virulence in *P. aeruginosa*.[213] As previously reported, we observed that most larvae infected with the parental strain PAO1 died within 24 hours when infected with an average dose of 5 CFU/larva, with only 13 % of the infected larvae surviving the infection.[214] The larvae infected with the P6P-resistant strain displayed similar survival probability with 17 % of the infected larvae surviving the infection after 24 hours (**Fig. 6A**). Interestingly, the larvae infected with the BAC-resistant strain showed significantly decreased morbidity with over half of the population surviving infection (60 %) throughout the experiment (**Fig. 6B**). This result is particularly interesting since the BAC-resistant strain consistently showed the highest production of different virulence factors. This suggests that the fitness cost imposed by BAC resistance development decreases virulence *in vivo*. In addition, these data indicate that CB resistance development in *P. aeruginosa* differentially affect virulence in this infection model.

Conclusion

Disinfectants are an important part of our arsenal against infections as they represent the first lines of defense against the spread of pathogens. CBs stand out as commonly used disinfectants in both household and hospital settings. In this work, we investigated the effect of CB resistance development of the widely used BAC and our next generation disinfectant P6P-10,10 on *P. aeruginosa* defensive strategies used by the bacteria to protect itself from antimicrobials and offensive strategies used to thrive against other organisms. While the changes in virulence associated with P6P mutants was modest overall, we found that resistance development to the commonly used disinfectant BAC led to increased production of virulence-associated pigments and pellicle formation, but at a fitness cost. During infection, the BAC-resistant mutant showed decreased virulence likely due to the fitness burden imposed by resistance. Nevertheless, caution should be taken when interpreting these results since compensatory mutations could remove the fitness cost imposed and allow these resistant mutants to thrive. Even though several virulence factors are increased in some of these CB-resistant strains *in vitro*, the regulation and production of these factors *in vivo* warrants further investigation. Understanding how exposure to disinfectants affect virulence in highly pathogenic bacteria such as *P. aeruginosa* is crucial for establishment of effective disinfection protocol that avoid promoting development of potentially more virulent strains.

Acknowledgements

The authors would like to thank Brandon Berryhill and members of the Bruce Levin laboratory (Emory University) for providing invaluable insights and materials for the *G. mellonella* infection model and Patrick McGann (MSRN WRAIR) for the gracious donation of bacterial strains.

Funding

Funding was provided by the NIH (GM119426 to W.M.W.; DK126467 to G.G.V.C.; T32 GM008602 to C.A.S.).

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article, its supplementary materials, and through Mendeley Data (DOI: 10.17632/rhfwf2rycs.1). MRSN strains are available directly from Dr. Patrick McGann.

Conflict of Interest

W.M.W. and K.P.C.M have invention disclosures pertaining to compound P6P and other CBs.

Materials and Methods

Bacterial strains and growth conditions. *P. aeruginosa* strains were streaked onto lysogeny broth (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) at 37 ºC overnight. Single colonies were used to inoculate liquid cultures and incubated for 18-24 hours at 37 ºC with shaking. *P. aeruginosa* clinical isolates were obtained from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN).

Growth curves were performed in 96-well flat-bottom plates (Falcon®, 351172) with shaking. Optical density was measured at 600 nm wavelength (OD_{600}) every 10 minutes and growth was monitored over 24 hours. Growth curve experiments were performed on different days with independent biological replicates with at least 6 technical replicates per strain/condition. MOPS and M63 minimal media were prepared as previously described and

supplemented with glucose as a carbon source.[215] Synthetic cystic fibrosis medium (SCFM) was prepared as previously described with addition of N-acetyl glucosamine.[79, 216]

Statistical criteria. The experimental data were analyzed using the GraphPad Prism 9.0 software (San Diego, CA). When the value of $P < 0.05$, it was considered statistically significant.

Resistance Selection. Disinfectant-resistant mutants were generated via serial passing of *P. aeruginosa* PAO1, MRSN6220, MRSN6241, MRSN409937, and MRSN5524 under increasing concentrations of either BAC or P6P-10,10 as previously reported.[125] First, an overnight culture of PAO1 in Difco™ Mueller-Hinton Broth (Sigma Aldrich, DF0757-17-6) was diluted to a concentration of 10^6 CFU/mL, according to the OD_{600} value. Six 100 µL concentrations of compound, ranging from four-fold the MIC to half the MIC were then inoculated with 100 µL of the dilute culture and incubated for 24 hours at 37 ºC. After 24 hours, a 2.0 µL aliquot from the highest concentration displaying growth was diluted 1:100 in fresh MHB (Sigma-Aldrich, DF0757-17-6) and fresh sample of compound. This process was repeated for 14 total serial passages. The experiment was performed in biological triplicates.

Cross Resistance MIC assay. To determine the MIC values, antibiotics were serially diluted two-fold from stock solutions (1.0 mM) to yield twelve 100 µL test concentrations. *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37 °C. Single colonies were used to inoculate 5 mL of Difco™ Mueller-Hinton (MHB) broth (Sigma-Aldrich, DF0757-17-6), and cultures were grown at 37 °C for 18 h with shaking. Overnight cultures were diluted 1:100 in MHB and regrown to mid-exponential phase as determined by OD_{600}. All cultures were diluted to ca. 10^4 CFU/mL in MHB

and 100 µL were inoculated into each well of a U-bottom 96-well plate (Avantor, 734-2782) containing 100 µL of antibiotic solution. Plates were incubated at 37 °C for 24 h upon which wells were evaluated visually for bacterial growth. The MIC was determined as the lowest concentration of compound resulting in no bacterial growth visible to the naked eye based on the mean in three independent experiments. MHB media and aqueous DMSO (Millipore Sigma, MX1458-6) controls were conducted for each strain. Antibiotics tested were amikacin disulfate (Alfar Aesar, J63862.14), gentamicin sulfate (Millipore Sigma, G1264-250MG), tobramycin (Millipore Sigma, PHR1079), aztreonam (TCI Chemicals, A2466), ceftazidime (Combi-Blocks, QV-7534), cefepime HCl (Chem-Impex, 15144), imipenem monohydrate (Combi-Blocks, QC-2985), meropenem trihydrate (Combi-Blocks, QH-8889), ciprofloxacin (Enzo Life Sciences, ALX-380-287-G025), levofloxacin (Alfa Aesar, J66943.06), colistin sulfate (Millipore Sigma, C4461-100MG), and polymyxin B sulfate (Oakwood Chemical, QC-8583). All antibiotics were dissolved in a 1:10 dilution of DMSO:water to create 1.0 mM solutions.

Alginate Quantification. Alginate quantification assay was performed following the procedure described by Chotirmall *et al.* with minor modifications.[217, 218] *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37 °C. Single colonies were used to inoculate 5 mL of Miller LB broth (VWR, TS61187-5000), and cultures were grown at 37 °C for 18 h with shaking. 1 M NaCl (Sigma-Aldrich, SX0420-5) was added to the overnight culture in a 1:1 ratio and vortexed. The cultures were centrifuged (Eppendorf, Enfield, CT) at 10,000 rpm for 30 minutes. 2% cetylpyridinium chloride (Sigma-Aldrich, C0732-100G) was added to the supernatant in a 2:1 ratio to allow for alginate precipitation. For collection of alginate, the mixture was centrifuged at 10,000 rpm for

10 minutes at room temperature and resuspended in 500 µL of -20 °C isopropanol (Fisher Scientific, A426P-4) for 1 hour. The mixture was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The remaining isopropanol was washed by dissolving the alginate pellet in water and lyophilized (Labconco, Kansas City, MO). The alginate pellet was resuspended in 500 µL of 1 M NaCl and heated to 60 °C. 50 µL of the alginate solution was added to 200 µL of 25 mM boric acid (Sigma-Aldrich, B0394-500G)/sulfuric acid (EMD Millipore, 258105-500ML) (2 M H_3BO_3 in sulfuric acid), and the mixture was heated to 100 °C for 10 minutes. The mixture was cooled for 15 minutes, and 50 µL of 0.125% carbazole (Sigma-Aldrich, C5132-100G) in 100% ethanol (Decon Labs, 2705SG) was added. The solution was reheated to 100 °C for 10 minutes. Once cooled, the quantification of alginate was determined spectrophotometrically at 550 nm using a BioTek Synergy H1 Hybrid plate reader (Santa Clara, CA).

Biofilm Quantification Assay. *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37 ºC. Single colonies were used to inoculate 5 mL of Miller LB broth (VWR, TS61187-5000), and cultures were grown at 37 °C for 18 h with shaking. Overnight cultures were diluted 1:100 in M63 minimal media supplemented with 2% (w/v) glucose (Sigma-Aldrich, G8270-100G).[219] Diluted cultures were added to surface-treated 96-well flat-bottom microtiter plates (Corning Incorporated, 3598). Plates were incubated at 37 °C for 24 h at which time the cell media was aspirated off. Wells were washed twice with 200 µL of phosphate buffered solution (PBS) and dried for 10 minutes. The wells were incubated for 15 minutes with 200 µL of 0.1% (w/v) crystal violet (VWR, 0528-500G) in DI H_2O. Excess crystal violet was removed by aspirating off the liquid and wells were rinsed twice with 200 µL of PBS. Crystal violet-stained biofilm was

solubilized with 200 µL of 70% (w/v) ethanol (Decon Labs, 2705SG) in DI H_2O and allowed to incubate at room temperature for 10 minutes to allow for full dissolution. Then 100 µL was transferred to a fresh flat-bottom 96-well plate (Falcon®, 351172) for absorbance measurement at 570 nm using a BioTek Synergy H1 Hybrid plate reader (Santa Clara, CA). Biological triplicates were performed with twelve technical replicates with media control.

Pellicle Quantification Assays. *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37 ºC. Single colonies were used to inoculate 5 mL of Miller LB broth (VWR, TS61187-5000), and cultures were grown at 37 °C for 18 hours with shaking. Overnight cultures were diluted 1:100 in LB and transferred onto a non-surface treated 96-well plate (Falcon®, 351172) with pegged lid (Nunc™, 445497). Plates were incubated at 37 °C for 24 h inside a secondary container to prevent undesired evaporation and processed as previously described.[197] To remove planktonic cells, the lid was carefully removed and placed into a new 96-well plate with 300 µL of PBS for 30-60 seconds prior to staining with 300 µL of 0.1% (w/v) crystal violet for 5 minutes in a different plate. After staining, excess crystal violet was removed by placing the lid in a 96-well plate with 300 µL of PBS in a new plate, and then dried for 10 minutes with pegs facing upward inside the biosafety cabinet. Finally, the pegged lid was destained in a 96-well plate containing 300 µL of 30% (v/v) acetic acid for 5 minutes. 200 µL were transferred to a new plate and absorbance was measured at a wavelength of 570 nm. Data was corrected with blank values and the average values of 3 biological replicates were analyzed.

Motility Assay. Motility assays were performed following the procedure described by Cullen *et al.* with minor modifications.[220] *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37 °C. Single

colonies were used to inoculate 5 mL of Miller LB broth (VWR, TS61187-5000), and cultures were grown at 37 °C for 18 hours with shaking. Swimming motility was assessed by inoculating the surface of LB media (Sigma-Aldrich, 1102830500) petri plate supplemented with 0.3% (w/v) Bacto™ agar (Fisher Scientific, DF0479-17-3) with overnight culture using a sterile 10 µL pipette tip. Swimming plates were incubated at 37 °C for 18 hours. Swarming motility was assessed by inoculating the surface of LB media (Sigma-Aldrich, 1102830500) supplemented with 0.5% (w/v) Bacto™ agar (Fisher Scientific, DF0479-17-3) plate with overnight culture using a sterile 10 µL pipette tip. Swarming plates were incubated at 30 °C for 18 hours. Diameters of swimming and swarming zones were calculated by averaging two perpendicular measurements. Two biological replicates per strain were performed.

***S. aureus* Lysis.** Adapted from Mashburn *et al.*, lysis of *S. aureus* on petri plates (VWR, 25384-342) was performed by swabbing a LB plate (Sigma-Aldrich, 1102830500) with an overnight culture of *S. aureus*.[221] After drying, 5 µl of an overnight culture of *P. aeruginosa* was spotted onto the petri plate, dried, and incubated at 37 °C for 24 h. Zones of lysis were subsequently measured and imaged.

Pyoverdine quantification. Pyoverdine (PVD) quantification was performed following the procedure described by Hoegy *et al*. with minor modifications.[222] Prior to completing the quantification, the parameters for the plate reader (BioTek Synergy H1 hybrid, Santa Clara, CA) were established as excitation wavelength at 400 nm and emission wavelength at 447 nm. The read height was 7.00 mm, and the temperature was set at 37 °C. Each fluorescence measurement occurred with the lid on the plate. *P. aeruginosa* strains were streaked onto LB agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 hours at 37 °C.

Single colonies were used to inoculate 5 mL of Difco™ Mueller-Hinton (MHB) broth (Sigma-Aldrich, DF0757-17-6), and cultures were grown at 37 °C for 18 hours with shaking. 100 µL of overnight culture was added to an Eppendorf tube (Fisher Scientific, 02-681-320) followed by 900 µL of Tris-HCl pH 8.0 buffer (Fisher Scientific, BP1758-100), and the solution was mixed. 100 µL of the mixture was inoculated into a well of a black flat bottom 96-well plate (Nunclon, 137101) for fluorescence measurement of apo PVD. When excited at 400 nm, the fluorescence emission of apo PVD is 447 nm; PVD in complex with iron will not be fluorescent.

Prey competitive fitness experiments. Inter-bacterial competition assays were performed as previously described with modifications.[211, 212] *P. aeruginosa* PAO1 pUCP30T (Gen^R) served as prey and readout of the experiment. Isogenic BAC-R and P6P-R strains served as attacker strains with PAO1 without the vector conferring resistance to gentamicin used as a control. A 10:1 attacker:prey ratio was used for these studies. Overnight cultures of bacterial strains were diluted 100-fold and grown to an OD_{600} of 1. Cultures were normalized to an OD_{600} of 0.4 right before the start of the experiment. The initial colony forming units (CFUs) counts were determined and used as input. Our *P. aeruginosa* pUCP30T (Gen^R) prey strain was selected in Miller LB broth (VWR, TS61187-5000) supplemented with gentamicin (Millipore Sigma, G1264-250MG) 60 µg/mL after competition and use as output. The prey recovery rate was calculated by dividing the CFUs counts of the output by the CFUs of the input.

Pyocyanin Quantification. Adapted from Taylor *et al.*, overnight cultures of *P. aeruginosa* were grown from single colonies in Miller LB medium (VWR, TS61187-5000) and grown with shaking at 37 °C for 20 hours.[223] 1 mL of each culture was subjected to centrifugation

(Eppendorf, Enfield, CT) at 13 000 rpm for 3 minutes. The clarified supernatants were collected, and the OD_{695} were measured on a BioTek Synergy H1 Hybrid plate reader (Santa Clara, CA). The pellets were resuspended in PBS, and the OD_{600} was measured to determine the cell density of each sample. Pyocyanin production was determined by normalizing OD_{695} of the clarified supernatant to the OD_{600} of the resuspended pellet.

***Galleria mellonella* infections.** Healthy *G. mellonella* 5th instar larvae (SpeedyWorm) of similar size (180 mg – 250 mg) were carefully selected for infection, and larvae displaying signs of disease (e.g. black spots) were discarded.[214] Larvae were infected with an average of 5 CFU per worm (10 µL) using 30-gauge needles (BD, 305106) and 25-µL syringe (Hamilton, 80401). Infections were monitored over a 5-day period for signs of morbidity (melanization, akinesia, inability to right itself). A total of 30 larvae were infected per group, including a PBS mock-infected control group. Two independent experiments were performed with two different batches of *G. mellonella* larvae.

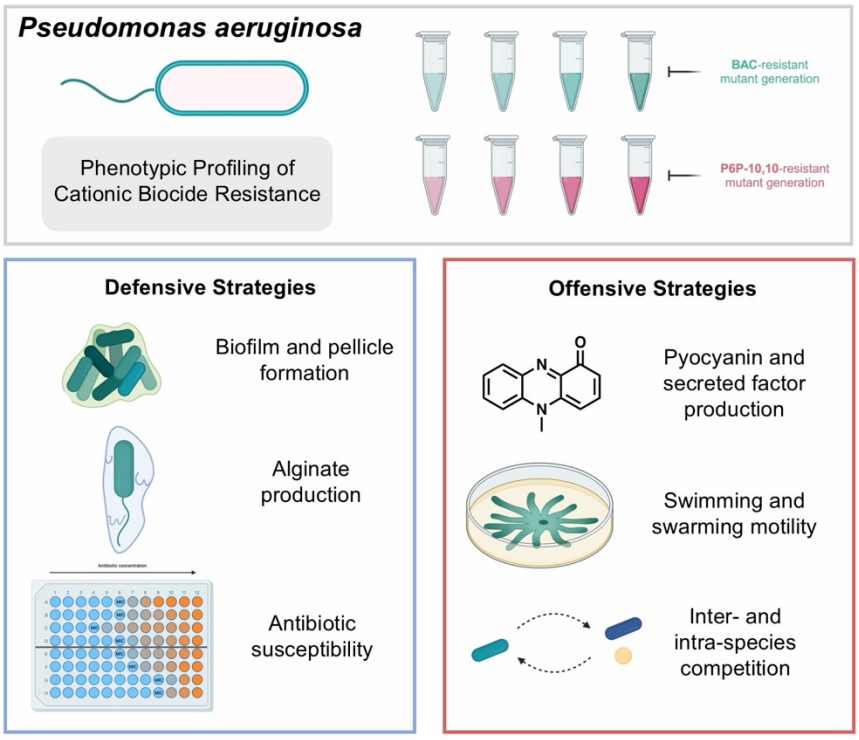

Figure 1. Schematic overview of *P. aeruginosa* virulence factors assessed after adaptation to cationic biocides (CBs). *P. aeruginosa* strains were exposed to increasing concentration of CBs over 15 days, and virulence-associated phenotypes were subsequently evaluated in these CB-resistant strains. These factors were grouped into "defensive" strategies that allow the bacterium to survive harsh conditions and external insults, and "offensive" strategies that allow the bacterium to attack, outcompete, or invade another organism. Created with BioRender.com.

Table 1. Minimum inhibitory concentrations (MIC) of parental (bold) and CB-resistant (R) strains of *P. aeruginosa*. Notable MIC differences are highlighted in red.

Cross Resistance MICs, μM					
Strain	**Aminoglycosides**			**Fluoroquinolones**	
	AMK	**GEN**	**TOB**	**CIP**	**LVX**
PAO1	4	2	0.5	2	8
PAO1 BAC^R	8	8	2	1	4
PAO1 $P6P^R$	4	4	1	4	8
6220	250	125	>250	125	250
6220 BAC^R	250	125	>250	63	125
6220 $P6P^R$	250	125	>250	125	125
6241	8	125	63	125	125
6241 BAC^R	1	16	8	8	16
6241 $P6P^R$	8	125	125	125	125
409937	16	8	4	16	63
409937 BAC^R	4	2	1	8	63
409937 $P6P^R$	1	0.25	0.25	16	63
5524	16	>250	250	4	32
5524 BAC^R	16	>250	125	2	16
5524 $P6P^R$	4	250	32	2	16

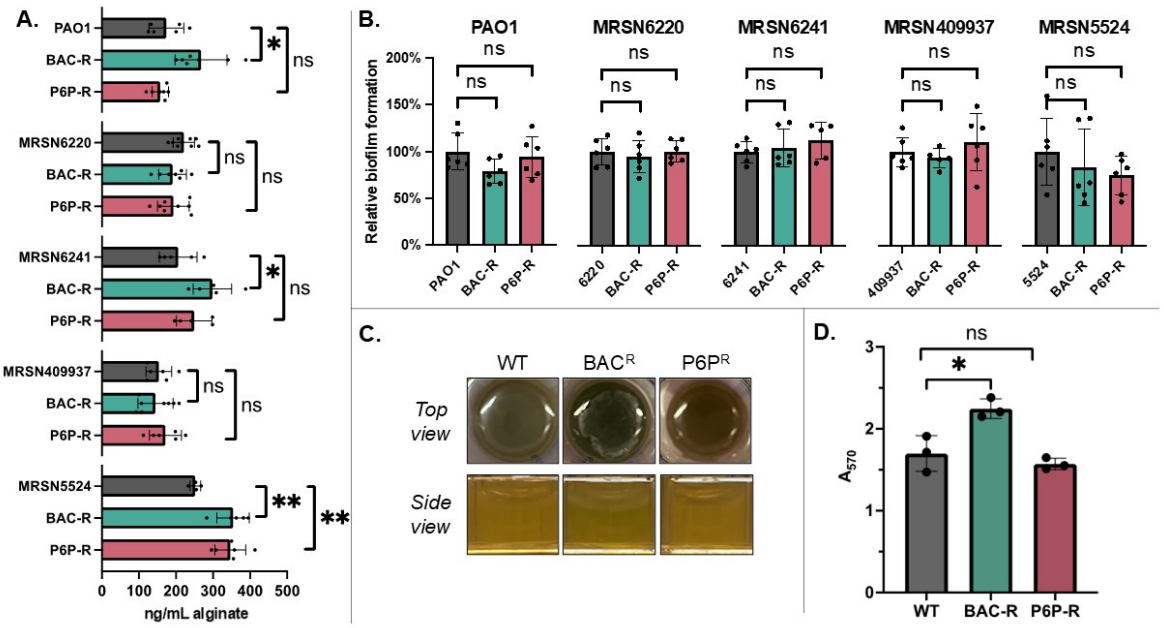

Figure 2. Evaluation of defensive strategies produced by *P. aeruginosa* CB-resistant strains. A) Alginate production was quantified in a mixture of boric acid/sulfuric acid/carbazole using a spectrophotometer (A_{550}) after alginate precipitation with 2% cetylpyridium and collection with isopropanol. The amounts of alginate produced were compared between the CB-resistant *P. aeruginosa* strains and their respective parental (WT) strain. B) Relative biofilm formation by CB-resistant and parental strains was evaluated in M63 media and quantified using crystal violet measuring absorbance at a wavelength of 570 nm. C) Pellicle formation of CB-resistant and parental PAO1 strains after overnight incubation. Differences in pellicle formation were clearly observed after overnight incubation with a thicker white film formed in the air-liquid interface (top and side view) in the BAC-resistant strain compared to the P6P-resistant or PAO1 parental strains. D) Pellicle formation was quantified using crystal violet and measuring absorbance at a wavelength of 570 nm (A_{570}). The means and individual values for 3 biological replicates are shown. Statistical significance in pellicle formation between strains was determined using the two-tailed Student's t-test comparing the A_{570} values. *, $P < 0.05$ and ns, not significant.

Statistical significance in alginate and biofilm production between strains was determined using the two-tailed Student's t-test. ns = not significant, ****P< 0.0001, ***P< 0.0005, **P< 0.005, *P< 0.05.

Table 2. Swimming and swarming behavior in parental (bold) and resistant (R) strains of *P. aeruginosa*. Diameters of swimming and swarming zones were calculated by averaging two perpendicular measurements of motility zones. Two biological replicates per strain were performed.

Strain	Swimming Diameter (mm)	Swarming Diameter (mm)
PAO1	37.0	7.5
PAO1 BAC^R	33.0	6.5
PAO1 $P6P^R$	36.5	5.0
6220	42.0	7.0
6220 BAC^R	6.0	4.0
6220 $P6P^R$	38.5	5.0
6241	35.0	21.5
6241 BAC^R	28.0	8.5
6241 $P6P^R$	30.0	18.0
409937	40.0	9.5
409937 BAC^R	24.0	5.5
409937 $P6P^R$	40.0	6.0
5524	12.0	9.5
5524 BAC^R	6.5	5.0
5524 $P6P^R$	8.5	5.0

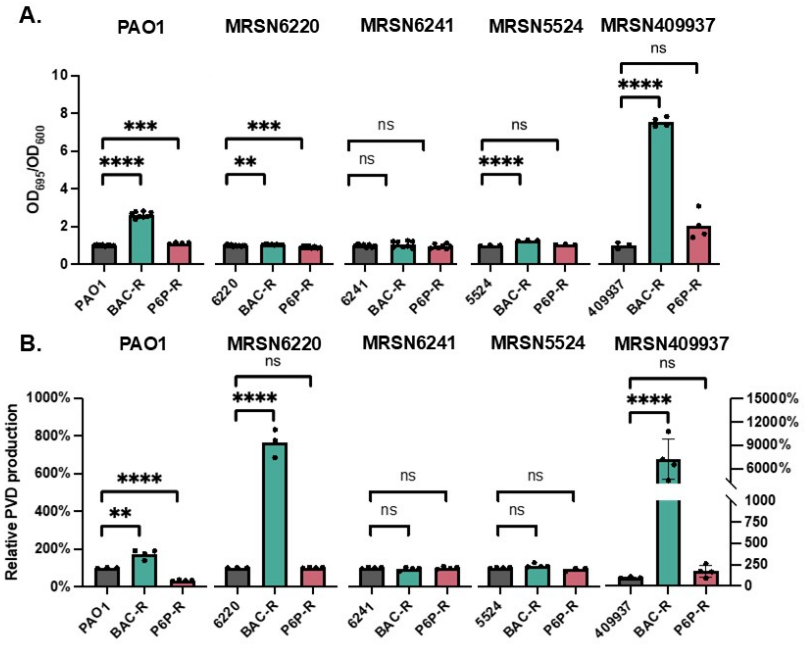

Figure 3. Evaluation of virulence-associated pigment production in *P. aeruginosa* CB-resistant strains. A) Pyocyanin (PYO) production was measured spectrophotometrically from supernatants at a wavelength of 695 nm and normalized to the cell density using OD_{600} values. The normalized values from CB-resistant strains were compared to their respective parental strains. B) Pyoverdine (PVD) production was assessed fluorometrically taking advantage of fluorescence of apo-PVD. PVD production of CB-resistant strains was compared to their respective parental strains. Statistical significance in PVD and PYO production between strains was determined using the two-tailed Student's t-test. ns = not significant, ****P< 0.0001, ***P< 0.0005, **P< 0.005, *P< 0.05

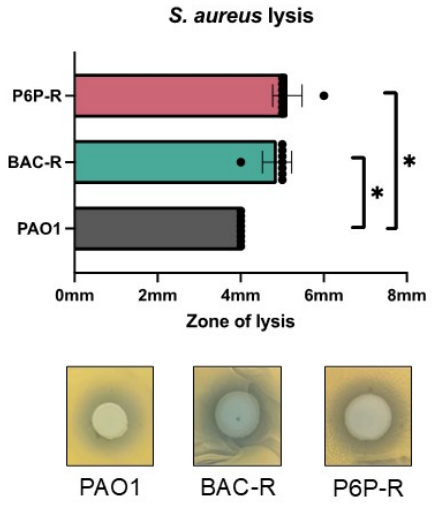

Figure 4. Lysis of *S. aureus* by *P. aeruginosa* PAO1 and CB-resistant strains. Lysis zones generated on *S. aureus* bacterial lawns by CB-resistant strains were measured and compared to the zones generated by parental isogenic strain PAO1. Top panel shows measurements of lysis zones (6 replicates analyzed/strain). Bottom panels show lysis zones on *S. aureus* produced by *P. aeruginosa* CB-resistant and PAO1 parental strains. *P< 0.05 in two-tailed Student's t-test compared to parental strain.

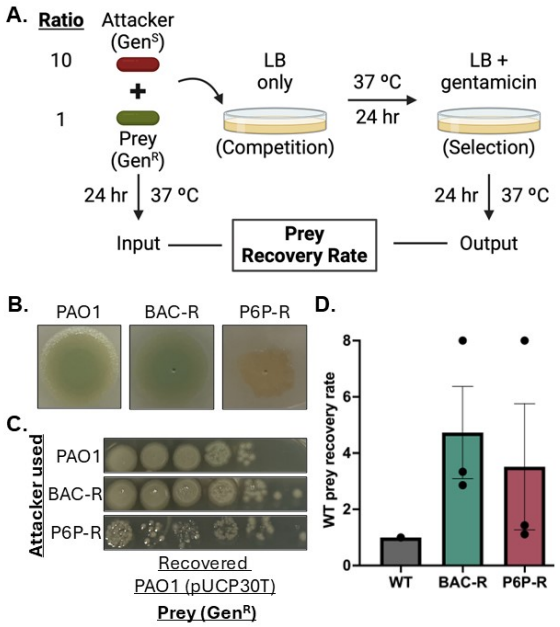

Figure 5. Bacterial competition assays of CB-resistant and PAO1 parental strain. A) Schematic of the prey recovery rate interbacterial competition assay. B) Different morphologies observed in competition spots between attacker strains PAO1 (WT control), BAC-R and P6P-R strains versus PAO1 harboring a vector conferring gentamicin resistance (prey strain; Gen^R) on LB agar media after 24 hours using an attacker to prey ratio of 10:1. C) Spot plating for recovery of prey strain in LB gentamicin (60 µg/mL) plate for prey recovery rate determination after competition. D) Prey recovery rate of competition assays between *P. aeruginosa* PAO1, BAC-resistant and P6P-resistant strains against *P. aeruginosa* PAO1 Gen^R. The mean of three replicates is shown. No statistical significance was observed by two-tailed Student's *t* test.

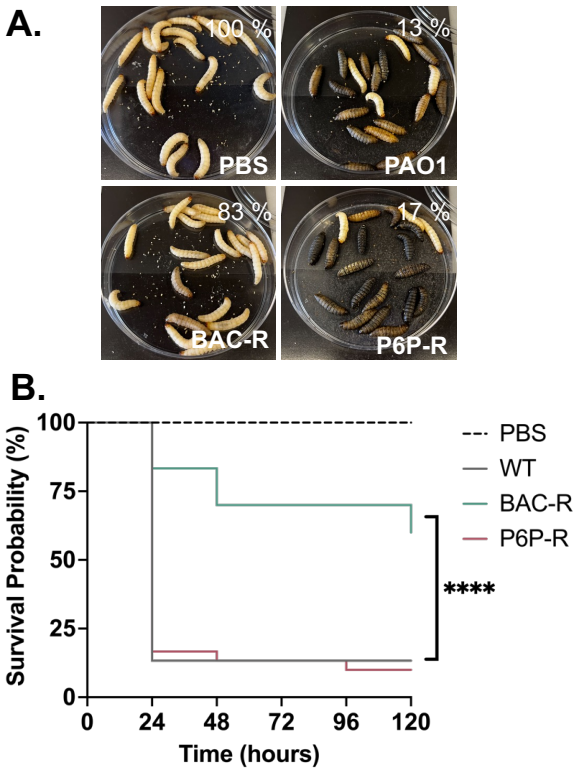

Figure 6. *Galleria mellonella* larvae infected with CB-resistant and parental PAO1 strains. A) Morbidity of *G. mellonella* larvae 24 hours post-infection. Survival (%) at 24 hours post-infection is shown in the top right corner of each picture. B) Kaplan-Meier survival curves of *G. mellonella* larvae monitored over a 5-day period. Two independent experiments were performed with a total of 120 larvae with PBS-injected larvae used as mock infection control (n = 30/group). Survival curves were compared using log-rank (Mantel-Cox) test. ****P< 0.0001.

Figure S1. Full minimum inhibitory concentrations (MIC) of parental (bold) and CB-resistant (R) strains of *P. aeruginosa*.

Cross Resistance MICs, μM												
Strain	Aminoglycosides			Monobactam	Cephalosporins		Carbapenems		Fluoroquinolones		Polymyxins	
	AMK	GEN	TOB	ATM	CAZ	FEP	IPM	MEM	CIP	LVX	CST	PMB
PAO1	4	2	0.5	16	2	1	2	1	2	8	4	0.25
PAO1 BAC^R	8	8	2	8	2	1	2	2	1	4	2	0.25
PAO1 P6P^R	4	4	1	8	4	1	8	2	4	8	2	0.25
6220	250	125	>250	>250	>250	>250	>250	>250	125	250	4	0.25
6220 BAC^R	250	125	>250	125	>250	125	>250	250	63	125	2	0.25
6220 P6P^R	250	125	>250	>250	>250	>250	>250	>250	125	125	4	0.25
6241	8	125	63	>250	>250	250	32	32	125	125	2	0.25
6241 BAC^R	1	16	8	>250	>250	250	32	32	8	16	4	0.25
6241 P6P^R	8	125	125	>250	>250	250	32	32	125	125	2	0.25
409937	16	8	4	>250	>250	250	8	32	16	63	4	0.25
409937 BAC^R	4	2	1	250	125	63	2	4	8	63	2	0.25
409937 P6P^R	1	0.25	0.25	>250	>250	125	16	16	16	63	2	0.25
5524	16	>250	250	125	16	32	16	63	4	32	2	0.25
5524 BAC^R	16	>250	125	125	16	63	16	32	2	16	4	0.5
5524 P6P^R	4	250	32	125	16	32	16	32	2	16	2	0.25

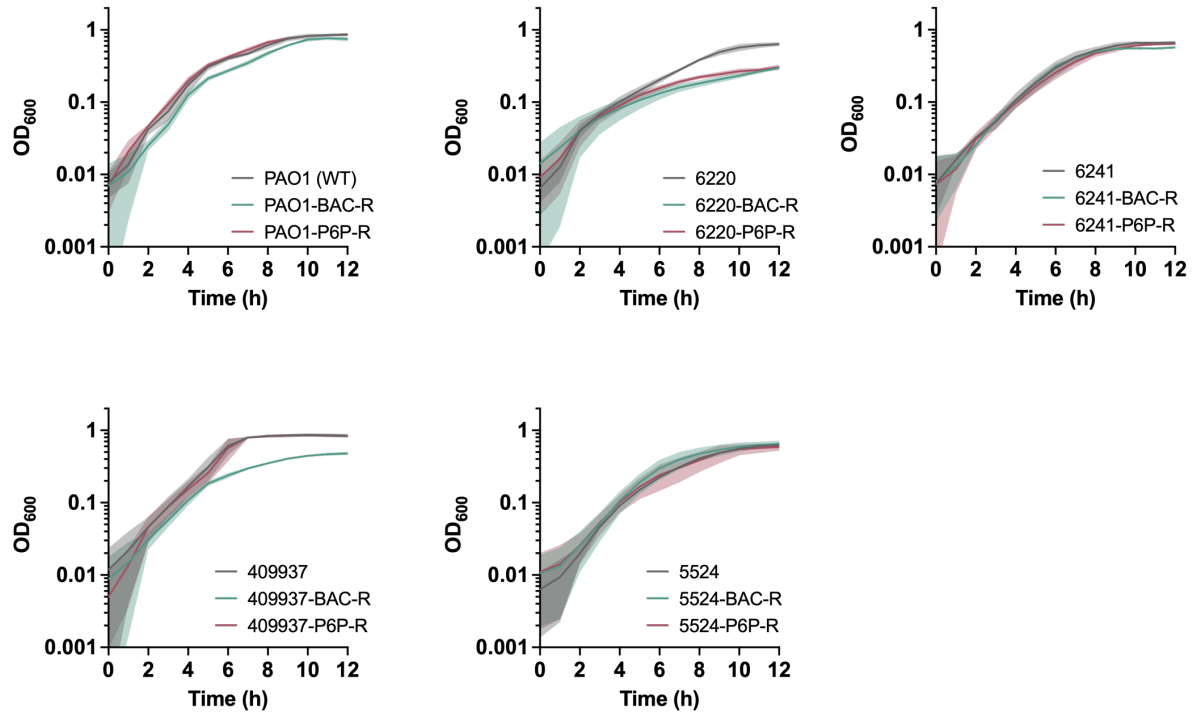

Figure S2. Growth curves performed in lysogeny broth (LB) with CB-resistant and parental strains. CB-resistant and parental strains growth was monitored over a 36-hour period in 96-well plates with shaking at 37 ºC, with OD_{600} measurements every 10 minutes. Only growth for 12 hours is shown to facilitate comparisons between strains. Each plot shows mean values of OD_{600} for the wild-type (WT) parental strain (gray), and the derived BAC-resistant (teal) and P6P-resistant (dark coral) strains. The mean value (solid lines) and standard deviation (shaded area) of 3 biological replicates for each strain is shown.

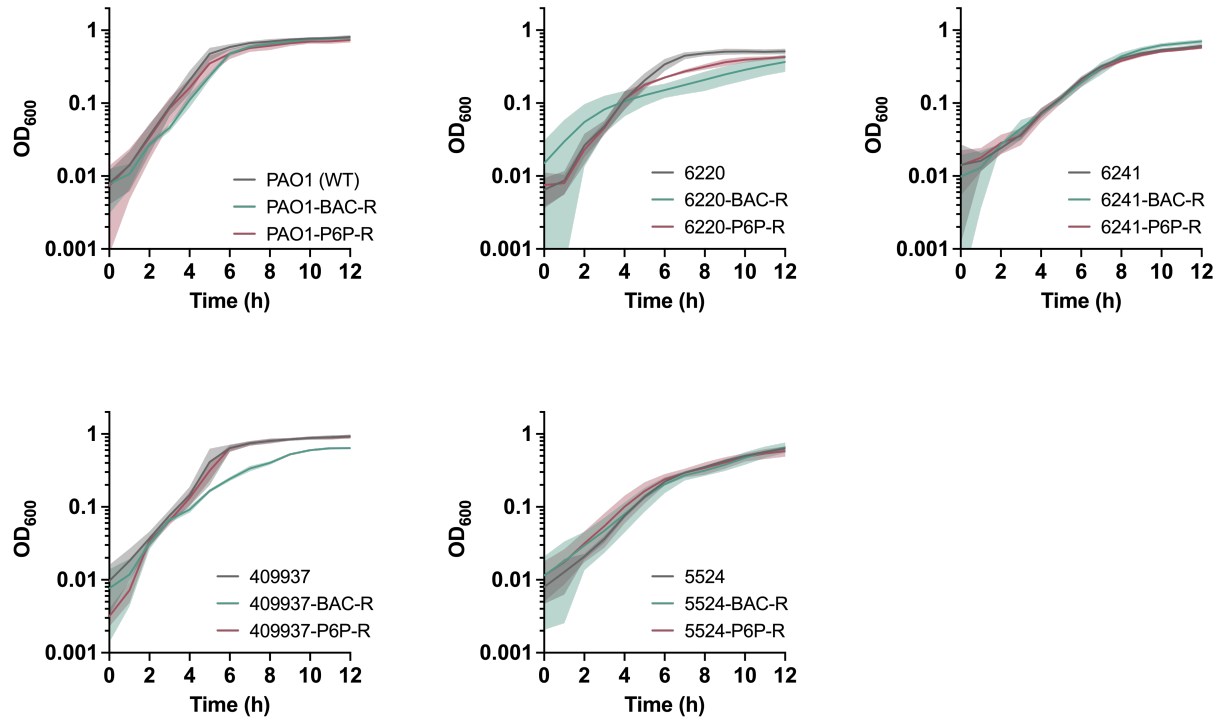

Figure S3. Growth curves performed in synthetic cystic fibrosis media (SCFM) with modifications with CB-resistant and parental strains. CB-resistant and parental strains growth was monitored over a 36-hour period in 96-well plates with shaking at 37 ºC, with OD_{600} measurements every 10 minutes. Only growth for 12 hours is shown to facilitate comparisons between strains. Each plot shows mean values of OD_{600} for the wild-type (WT) parental strain (gray), and the derived BAC-resistant (teal) and P6P-resistant (dark coral) strains. The mean value (solid lines) and standard deviation (shaded area) of 3 biological replicates for each strain is shown**.**

Chapter V

Discussion

The chain of infection is a series of six important elements that need consideration when trying to prevent the spread of infectious agents. First, the microorganism in question is pathogenic and can cause disease, either in healthy or immunocompromised individuals. The resistance profile of these microorganisms to antimicrobials is a crucial aspect as well. The second consideration is the reservoir of the pathogen where it can grow in numbers, and thirdly, the exit it uses to leave said reservoir (e.g., bodily fluids). The fourth element to consider is how this pathogen is transported from the reservoir to a host, for example, through an insect vector. The fifth consideration is what entry route(s) is used by the pathogen into the host (e.g., open wound, respiratory tract), and finally, the sixth element is the susceptibility of the host.

Antimicrobial resistance (AMR) not only jeopardizes treatment efficacy after infection but also affects other elements in the chain of infection, allowing a pathogen to continue to spread and cause more disease. While antibiotics are essential for both therapeutic and prophylactic uses to prevent or treat infections, biocides are a key component in preventing the spread of pathogens at different stages of the chain of infection. These biocides are found as food preservatives, antiseptics, in healthcare and household disinfection. Their usage improves public health by reducing or eliminating environmental pathogen reservoirs, preventing pathogen transport, and blocking the possibility of entry into the host when use as antiseptics. When appropriately used, these biocides can reduce the environmental bioburden of pathogens, consequently decreasing infection occurrences and the need of antibiotic usage.[118] Thus, biocides are a vital element in our fight against AMR.

Cationic biocides stand out as an important and versatile class of biocide in our arsenal to prevent and control infections. This class of biocide is widely used as active ingredients in disinfectants, antiseptics, household products and cosmetics, among other commercial products.

Importantly, these cationic biocides are also widely used in healthcare, including the bisbiguanides (e.g., alexidine and chlorhexidine), and quaternary ammonium compounds (QACs, e.g., benzalkonium chloride). Despite their abundant use and importance, few new cationic biocides have been introduced into the market. To address this gap, the Minbiole and Wuest groups have developed over 800 new QACs and phosphonium-containing analogs (QPCs) with a diverse range of chemical structures. However, how bacteria adapt to these chemically distinct biocides, in particular to QPCs, and how these adaptations influence virulence remain largely unexplored.

Our work focused on QACs and QPCs, and how they relate to other clinically relevant cationic biocides. First, I explored how the surface-active properties of QACs can be used to screen for QACs with better bactericidal activity. This is rather important since this class of biocide is mainly used as disinfectants, and hence they should exhibit potent bactericidal activity within short contact times. Moreover, assessing this antimicrobial activity with numerous compounds can become extremely costly and time-consuming. Strategies such as the one presented here, can provide a more cost-effective path to identifying potent QACs for disinfection. In addition, we investigated whether *P. aeruginosa* adapts differently to benzalkonium chloride (a classic QAC) compared to a novel and potent QPC, named P6P-10, 10. Finally, we assessed how these adaptations impact the production of virulence factors and pathogenicity. Using *P. aeruginosa* in these studies was advantageous because it is notoriously resistant to biocides and other antimicrobials.[224] Our results suggest that *P. aeruginosa* adapts distinctly to biocides with different chemical structure (mono-cationic vs. bis-cationic) and not composition (e.g. pnictogen), and that these differences in adaptations are reflected

phenotypically as different resistance determinants and production of virulence factors and pathogenicity.

QACs have surface-active properties that endow them with ideal characteristics for cleansing and disinfecting purposes. As such, they can reduce the surface tension of water, a property that can be readily measured as dynamic surface tension (DST). DST measures the difference in surface tension as a function of time using a tensiometer.[225] I explored whether DST values correlated with the antimicrobial activities of a panel of 10 structurally diverse QACs. I assessed the correlation between DST values with MIC values and suspension time-kill assay values, reported as log-reduction values (LRVs). We found that QACs with lower DST values (more surface-active) correlated with compounds with superior bactericidal activity (**Chapter II**). Interestingly, DST values did not correlate with inhibition of growth (MIC values), neither did MIC values of QACs with their respective LRVs. The discrepancy between these two antimicrobial activities indicates that these are dependent upon distinct factors. One potential factor that could explain these differences is the experimental conditions under which these assays are performed, with MICs using lower concentrations and longer incubations, while time-kill assays performed with significantly higher concentrations in relatively short times (0.5-5 minutes) to mimic usage in real-world disinfection conditions. It is also important to note that these measurements were performed with pure solutions of the compounds in deionized water, but most formulations of QACs also contain additional additives that are not represented in these data. In addition, even when our QACs panel had a variety of structures and cationic compositions (from mono-cationic QACs to multi-cationic QACs), there are many more structural variations that could be tested to determine if this approach is broadly applicable.

Finally, it would have been ideal to have more trials in our experiments to increase the statistical power of the correlation tests performed.

Nevertheless, my results provide a basis for using a physicochemical property to screen for bactericidal activity of disinfectants, highlight the differences observe between inhibition of growth (as measured by MIC) and killing activity (measured by suspension time-kill assays), and offer an additional method that could be employed when screening multiple QACs. Future studies should focus on expanding the panel of QACs to include additional structures and chemical compositions (e.g QPCs), measure the DST of a wide range of compounds, and test the predictive power for bactericidal activity. To circumvent the resource-intensive and time-consuming aspect of time-kill assays, we can envision applying new methods that test cell viability (e.g. geometric viability assay) to reduce the cost and labor that would go into testing this, making it more feasible for the researcher.

To interrogate the effects of exposure and adaptation to disinfectants, we exposed *P. aeruginosa* to increasing sub-inhibitory concentrations of a prototypical QAC, benzalkonium chloride (BAC), and compared it to the adaptations obtained in response to a novel QPC, P6P-10,10. This QPC became the focus of our study because it displayed remarkable activity against a panel of highly resistant *P. aeruginosa* strains (**Chapter III**). Our group had shown that this novel QPC is also highly potent against a panel of MDR *A. baumannii* strains.[125] We observed stable resistance development after 15 days of exposure to increasing sub-inhibitory concentrations of these biocides. Moreover, even when a slight increase in cross-resistance was observed (2-fold) with the biocide-resistant strains to the other biocide used in the study, the lack of this phenotype in most strain in addition to the low-level resistance observed suggested that the target and cellular effects of these biocides were different.

To fully understand the differences in adaptations to BAC and P6P-10,10, we performed whole-genome sequencing of the biocide-resistant strains and their respective parental strains. These experiments uncovered that the resistance profile to these biocides were completely different. BAC-resistant strains showed mutations in genes associated with diverse cellular functions, with the most prevalent type of mutation (3/5 strains) found in the *htrB1* gene, suggesting LPS modification. HtrB1 is a homolog of LpxL and is responsible for the addition of 2-hydroxylaurate to lipid A in *P. aeruginosa*, and lack of this modification resulted in increased resistance to colistin but not polymyxin B and other antibiotics.[226] These results obtained by Hittle *et al*. were surprising since they also observed increased membrane permeability in the *htrB1* mutant but not sufficient to permeabilize the cell to other antibiotics. In addition, mutations in DNA synthesis and repair were identified in several BAC-resistant strains. BAC has been associated with DNA damage in human corneal epithelial cells and other mammalian cells.[227, 228] If DNA damage contributes to the mechanism of action of BAC remains unknown.

On the other hand, all P6P-10,10-resistant strains (5/5) shared loss-of-function mutations in *smvR*, a negative transcriptional regulator of SmvA. To our surprise, this resistance determinant is also associated with resistance to other biocides, including octenidine and chlorhexidine.[134, 145] These compounds possess a characteristic chemical structure: hydrophilic groups (cationic heads) separated by flexible hydrocarbon chain, and are known as bolaamphiphile. To our knowledge, this is the first report of resistance to QPCs, and our data suggests that the chemical structure of P6P-10,10 (bolaamphiphile) rather than the nature of its cationic heads (P vs. N) dictates the resistance mechanisms. In addition, mutations in the phospholipid synthesis enzymes *pssA* and *pgsA* were identified, both involved in the modification of the cytoplasmic membrane phospholipid composition from a common precursor,

CDP diacylglycerol.[229] Similar mutations in *pssA* and *pgsA* have been associated with octenidine resistance in *P. aerugionsa*.[134] Importantly, these results align well with mechanistic studies performed that suggest P6P-10,10 preferentially disrupts the cytoplasmic membrane while BAC disrupts both the outer and membrane in a successive indiscriminate manner.

Two of the P6P-10,10-resistant strains isolated from clinical isolates (MRSN 409937 and MRSN 5524) showed deletions predicted to cause aberrant protein structure and premature stop codons in MexY. MexY is the cytoplasmic membrane transporter component of the tripartite RND multidrug efflux systems MexXY-OprM, and it is involved in resistance to aminoglycosides.[230] We observed collateral sensitivity to the three tested aminoglycosides (amikacin, gentamicin, tobramycin) in the P6P-resistant mutants derived from MRSN 409937 and MRSN 5524, suggesting efficient efflux of these antibiotics was compromised in these strains (**Chapter IV**). However, a null *mexY* mutant did not show any effect on P6P-10,10 susceptibility (data not shown), thus the role of these mutations in P6P-10,10 resistance remains an open question.

Further investigation is needed to fully understand how these adaptations contribute to resistance to these biocides. Are BAC-resistant mutants (which in several cases contain DNA synthesis-related mutations) more prone to continue to develop additional mutations? Indeed, increased mutational rates have been observed in *Salmonella enterica* serovar Typhimurium evolved in the presence of BAC.[231] How is the *htrB1* mutation and the expected LPS modification promoting survival to BAC? Is the increased activity the drug-proton SmvA antiporter reducing the effective concentration of the biocide by active efflux or by affecting the membrane potential and entry of the biocide into the cell in the first place?[232] Answering these questions would not only provide additional mechanistic insights about biocide resistance, but

also provide information about the changes in bacterial physiology that could translate into resistance to other antimicrobials.

In addition, these studies would benefit from further exploration of specific adaptations to other important biocides. Ideally, additional commercial biocides that are widely used, such as cetylpyridinium chloride (CPC), didecyldimethylammonium chloride (DDAC), and cetrimide could be used at sub-inhibitory concentrations to select for mutants and identify genetic adaptations that allow bacteria to survive higher concentrations of these biocides. These studies could help elucidate if similar patterns in adaptations are observed, and to what extent they might be predictable based on the chemical structure of the biocide. Even when these biocide-resistant bacteria will likely continue to be susceptible to these biocides at their in-use concentrations, the adaptations could have implications for how these bacteria respond to antibiotics.

To start exploring the potential implications of these adaptations to different biocides, we assessed how these biocide resistance adaptations influence the production of virulence factors and pathogenesis in *P. aeruginosa* (**Chapter IV**). We found that exposure and adaptation to these biocides led to completely distinct phenotypic profiles with important implications in *P. aeruginosa* virulence. Our result indicated that exposure to cationic biocides could result in increased production of virulence factors, including virulence-associated pigments, and pellicle formation (air-liquid interphase biofilm). In addition, different phenotypes were observed *in vivo*, with cationic biocide-resistant strains showing either an attenuated virulence phenotype or a wild-type phenotype. These results indicate that different biocides can influence virulence to varying degrees, and that these potential effects on virulence should not be overlooked.

Biocide-resistant strains displayed collateral sensitivity, resulting in increased susceptibility to several antibiotics. This is contrary to previously findings were decreased

susceptibility (or higher resistance) to antibiotics was observed in *P. aeruginosa* strains exposed to BAC.[57, 120, 233] Additionally, the majority of BAC-resistant strains had two obvious phenotypes that could be detected with the naked eye: i) intense blue/green color due to increased pigment production, and ii) increased pellicle formation. Our results indicated that production of pyocyanin and pyoverdine was increased in several BAC-resistant strains, two pigments associated with virulence in *P. aeruginosa*. Is important to note that previous reports have shown that decreased integrity of the outer membrane is associated with increased pyocyanin production.[234] If this increase in pigment production is associated with or due to outer membrane changes in the BAC-resistant strains remains unresolved.

It was apparent that BAC resistance imposed a fitness cost, as measured by growth curves and competition assays. However, the specific genetic variation responsible for this fitness cost remain unclear. As mentioned above, several BAC-resistant strains had a loss-of-function mutation in *htrB1*, indicating a likely modification to the lipid A moiety of LPS. Hittle and coworkers observed a perceivable fitness disadvantage associated with the loss of *htrB2* but not *htrB1*.[226] When our data is contrasted with that of Hittle *et al.*, it suggests that the fitness cost observed may be due to the presence of additional mutations in each genetic background.

To evaluate pathogenicity, we optimized and used the *Galleria mellonella* larvae infection model. Data collected from independent experiments indicated that adaptations to BAC resulted in reduced pathogenicity. In contrast, P6P-10,10-adapted strains displayed wild-type level pathogenesis on this infection model. However, not all virulence factors could be assessed (e.g. elastase) and these differences in virulence could be due to differential expression of these additional factors not tested. Our current hypothesis lies on the fitness cost differences observed to explain the lower pathogenesis of the BAC-resistant strain during *in vivo* experiments.

Exploring the transcriptional changes involved in transient adaptation to these and other biocides, and how these relate to the establishment of genetically distinct populations in *P. aeruginosa* would provide a fuller picture of biocide resistance development. Finally, evaluation of the impact of disinfectants on virulence and pathogenesis of other important pathogens that are frequently exposed to biocides is needed.

In conclusion, my dissertation work explores different stages of these quaternized compounds (QACs and QPCs): from the physicochemical properties that may help QAC screening during development, to the mechanistic nuances related to them that lead to phenotypic differences associated with resistance development. Throughout these studies, we have uncovered key differences in the mechanism of action between BAC, a widely used and prototypical QAC, and P6P-10,10, a novel QPC with potent antimicrobial activity against *P. aeruginosa*, and other gram-negatives pathogens. The structure-activity relationships unveiled through these research efforts set the stage to better understand how the chemical structure of biocides dictates their targets, which in turn results in different adaptations with potential implications for virulence and pathogenesis. Further studies would help guide rational design of new biocides by taking into account their expected cellular targets and potential resistance mechanisms based on their chemical structure.

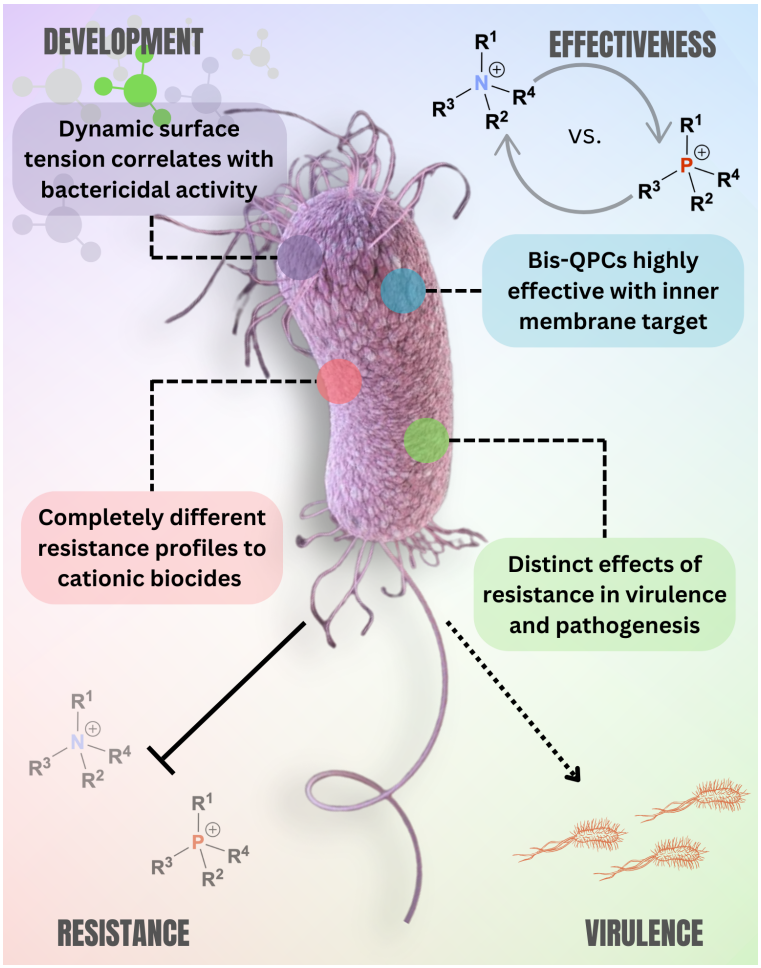

Fig 1. Dissertation research findings summary. This dissertation research work uncovered how dynamic surface tension values of QACs correlated with their bactericidal activity in disinfection-like conditions, the mechanistic differences that made bolaamphiphile QPCs more effective against *P. aeruginosa*, the distinct genetic variations that made *P. aeruginosa* resistant to these biocides, and how these adaptations influenced virulence and pathogenesis.

Chapter VI

Bibliography

1. Salam MA, Al-Amin MY, Salam MT, Pawar JS, Akhter N, Rabaan AA, Alqumber MAA. Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. Healthcare (Basel). 2023;11(13). Epub 20230705. doi: 10.3390/healthcare11131946. PubMed PMID: 37444780; PMCID: PMC10340576.

2. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrob Resist Infect Control. 2017;6:47. Epub 20170515. doi: 10.1186/s13756-017-0208-x. PubMed PMID: 28515903; PMCID: PMC5433038.

3. Penicillin's finder assays its future. New York Times. 1945.

4. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-83. PubMed PMID: 25859123; PMCID: PMC4378521.

5. Price R. O'Neill report on antimicrobial resistance: funding for antimicrobial specialists should be improved. Eur J Hosp Pharm. 2016;23(4):245-7. doi: 10.1136/ejhpharm-2016-001013. PubMed PMID: 31156859; PMCID: PMC6451500.

6. Antimicrobial Resistance C. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399(10325):629-55. Epub 20220119. doi: 10.1016/S0140-6736(21)02724-0. PubMed PMID: 35065702; PMCID: PMC8841637.

7. Thorpe KE, Joski P, Johnston KJ. Antibiotic-Resistant Infection Treatment Costs Have Doubled Since 2002, Now Exceeding $2 Billion Annually. Health Aff (Millwood). 2018;37(4):662-9. Epub 20180321. doi: 10.1377/hlthaff.2017.1153. PubMed PMID: 29561692.

8. Nelson RE, Hatfield KM, Wolford H, Samore MH, Scott RD, Reddy SC, Olubajo B, Paul P, Jernigan JA, Baggs J. National Estimates of Healthcare Costs Associated With Multidrug-

Resistant Bacterial Infections Among Hospitalized Patients in the United States. Clin Infect Dis. 2021;72(Suppl 1):S17-S26. doi: 10.1093/cid/ciaa1581. PubMed PMID: 33512523.

9. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health. 2015;109(7):309-18. Epub 20150907. doi: 10.1179/2047773215Y.0000000030. PubMed PMID: 26343252; PMCID: PMC4768623.

10. Darby EM, Trampari E, Siasat P, Gaya MS, Alav I, Webber MA, Blair JMA. Molecular mechanisms of antibiotic resistance revisited. Nat Rev Microbiol. 2023;21(5):280-95. Epub 20221121. doi: 10.1038/s41579-022-00820-y. PubMed PMID: 36411397.

11. CDC. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention. 2019.

12. World Health Organization. WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. 2024.

13. Best M, Neuhauser D. Ignaz Semmelweis and the birth of infection control. Qual Saf Health Care. 2004;13(3):233-4. doi: 10.1136/qhc.13.3.233. PubMed PMID: 15175497; PMCID: PMC1743827.

14. Pittet D, Allegranzi B. Preventing sepsis in healthcare - 200 years after the birth of Ignaz Semmelweis. Euro Surveill. 2018;23(18). doi: 10.2807/1560-7917.ES.2018.23.18.18-00222. PubMed PMID: 29741152; PMCID: PMC6053623.

15. Spaulding EH. Recommendations for chemical disinfection of medical and surgical materials. Environmental Aspects of the Hospital. 1966;1:65.

16. William A. Rutala DJW, Healthcare, Committee ICPA. Guideline for Disinfection and Sterilization in Healthcare Facilities2008:[7-45 pp.]. Available from: https://www.cdc.gov/infection-control/hcp/disinfection-and-sterilization/index.html.

17. Rutala WA, Weber DJ. Disinfection, sterilization, and antisepsis: An overview. Am J Infect Control. 2016;44(5 Suppl):e1-6. doi: 10.1016/j.ajic.2015.10.038. PubMed PMID: 27131128.

18. Maillard JY, Pascoe M. Disinfectants and antiseptics: mechanisms of action and resistance. Nat Rev Microbiol. 2024;22(1):4-17. Epub 20230830. doi: 10.1038/s41579-023-00958-3. PubMed PMID: 37648789.

19. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev. 1999;12(1):147-79. doi: 10.1128/CMR.12.1.147. PubMed PMID: 9880479; PMCID: PMC88911.

20. Beauchamp RO, Jr., St Clair MB, Fennell TR, Clarke DO, Morgan KT, Kari FW. A critical review of the toxicology of glutaraldehyde. Crit Rev Toxicol. 1992;22(3-4):143-74. doi: 10.3109/10408449209145322. PubMed PMID: 1388704.

21. Toxicological Profile for Glutaraldehyde. Atlanta (GA)2017.

22. Rogers CA, Gaskin SE, Thredgold LD, Pukala TL. An approach to quantify ortho-phthalaldehyde contamination on work surfaces. Ann Work Expo Health. 2023;67(7):886-94. doi: 10.1093/annweh/wxad039. PubMed PMID: 37436000; PMCID: PMC10410490.

23. Guidelines for Drinking-Water Quality: Fourth Edition Incorporating the First Addendum. Geneva2017.

24. von Gunten U. The basics of oxidants in water treatment. Part B: ozone reactions. Water Sci Technol. 2007;55(12):25-9. doi: 10.2166/wst.2007.382. PubMed PMID: 17674823.

25. Bigliardi PL, Alsagoff SAL, El-Kafrawi HY, Pyon JK, Wa CTC, Villa MA. Povidone iodine in wound healing: A review of current concepts and practices. Int J Surg. 2017;44:260-8. Epub 20170623. doi: 10.1016/j.ijsu.2017.06.073. PubMed PMID: 28648795.

26. Monstrey SJ, Govaers K, Lejuste P, Lepelletier D, Ribeiro de Oliveira P. Evaluation of the role of povidone-iodine in the prevention of surgical site infections. Surg Open Sci. 2023;13:9-17. Epub 20230316. doi: 10.1016/j.sopen.2023.03.005. PubMed PMID: 37034245; PMCID: PMC10074992.

27. Boyce JM. Alcohols as Surface Disinfectants in Healthcare Settings. Infect Control Hosp Epidemiol. 2018;39(3):323-8. Epub 20180128. doi: 10.1017/ice.2017.301. PubMed PMID: 29374503.

28. Weatherly LM, Gosse JA. Triclosan exposure, transformation, and human health effects. J Toxicol Environ Health B Crit Rev. 2017;20(8):447-69. doi: 10.1080/10937404.2017.1399306. PubMed PMID: 29182464; PMCID: PMC6126357.

29. Shrestha P, Zhang Y, Chen WJ, Wong TY. Triclosan: antimicrobial mechanisms, antibiotics interactions, clinical applications, and human health. J Environ Sci Health C Toxicol Carcinog. 2020;38(3):245-68. doi: 10.1080/26896583.2020.1809286. PubMed PMID: 32955413.

30. Fukuzaki S. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sci. 2006;11(4):147-57. doi: 10.4265/bio.11.147. PubMed PMID: 17190269.

31. Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol. 2005;99(4):703-15. doi: 10.1111/j.1365-2672.2005.02664.x. PubMed PMID: 16162221.

32. Leeming JP. Principles and Practice of Disinfection, Preservation and Sterilization, 3rd edn. Journal of Antimicrobial Chemotherapy. 2000;45(1):135-. doi: 10.1093/jac/45.1.135.

33. Toxic Use Reduction Institute. Quaternary Ammonium Compounds (QACs). UMass Lowell2022.

34. Ghafoor D, Khan Z, Khan A, Ualiyeva D, Zaman N. Excessive use of disinfectants against COVID-19 posing a potential threat to living beings. Curr Res Toxicol. 2021;2:159-68. Epub 20210304. doi: 10.1016/j.crtox.2021.02.008. PubMed PMID: 33688633; PMCID: PMC7931675.

35. Arnold WA, Blum A, Branyan J, Bruton TA, Carignan CC, Cortopassi G, Datta S, DeWitt J, Doherty AC, Halden RU, Harari H, Hartmann EM, Hrubec TC, Iyer S, Kwiatkowski CF, LaPier J, Li D, Li L, Muniz Ortiz JG, Salamova A, Schettler T, Seguin RP, Soehl A, Sutton R, Xu L, Zheng G. Quaternary Ammonium Compounds: A Chemical Class of Emerging Concern. Environ Sci Technol. 2023;57(20):7645-65. Epub 20230508. doi: 10.1021/acs.est.2c08244. PubMed PMID: 37157132; PMCID: PMC10210541.

36. Schrank CL, Minbiole KPC, Wuest WM. Are Quaternary Ammonium Compounds, the Workhorse Disinfectants, Effective against Severe Acute Respiratory Syndrome-Coronavirus-2? ACS Infect Dis. 2020;6(7):1553-7. Epub 20200515. doi: 10.1021/acsinfecdis.0c00265. PubMed PMID: 32412231; PMCID: PMC10464937.

37. Hora PI, Pati SG, McNamara PJ, Arnold WA. Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications. Environ Sci Technol Lett. 2020;7(9):622-31. Epub 20200626. doi: 10.1021/acs.estlett.0c00437. PubMed PMID: 37566314.

38. Davidson PM, Branden AL. Antimicrobial Activity of Non-Halogenated Phenolic Compounds. J Food Prot. 1981;44(8):623-32. doi: 10.4315/0362-028X-44.8.623. PubMed PMID: 30836539.

39. Russell AD. Whither triclosan? J Antimicrob Chemother. 2004;53(5):693-5. Epub 20040408. doi: 10.1093/jac/dkh171. PubMed PMID: 15073159.

40. Gold NA, Mirza TM, Avva U. Alcohol Sanitizer. StatPearls. Treasure Island (FL)2024.

41. Rutala WA, Weber DJ. Disinfection and Sterilization in Health Care Facilities: An Overview and Current Issues. Infect Dis Clin North Am. 2016;30(3):609-37. doi: 10.1016/j.idc.2016.04.002. PubMed PMID: 27515140; PMCID: PMC7134755.

42. Zaman SB, Siddique AB, Ruysen H, Kc A, Peven K, Ameen S, Thakur N, Rahman QS, Salim N, Gurung R, Tahsina T, Rahman AE, Coffey PS, Rawlins B, Day LT, Lawn JE, Arifeen SE, Group E-BS. Chlorhexidine for facility-based umbilical cord care: EN-BIRTH multi-country validation study. BMC Pregnancy Childbirth. 2021;21(Suppl 1):239. Epub 20210326. doi: 10.1186/s12884-020-03338-4. PubMed PMID: 33765947; PMCID: PMC7995704.

43. Lakhi NA, Tricorico G, Osipova Y, Moretti ML. Vaginal cleansing with chlorhexidine gluconate or povidone-iodine prior to cesarean delivery: a randomized comparator-controlled trial. Am J Obstet Gynecol MFM. 2019;1(1):2-9. Epub 20190319. doi: 10.1016/j.ajogmf.2019.03.004. PubMed PMID: 33319753.

44. Denyer SP, Hugo WB. The mode of action of tetradecyltrimethyl ammonium bromide (CTAB) on Staphylococcus aureus [proceedings]. J Pharm Pharmacol. 1977;29 Suppl:66P. doi: 10.1111/j.2042-7158.1977.tb11534.x. PubMed PMID: 22699.

45. Nakashima AK, McCarthy MA, Martone WJ, Anderson RL. Epidemic septic arthritis caused by Serratia marcescens and associated with a benzalkonium chloride antiseptic. J Clin

Microbiol. 1987;25(6):1014-8. doi: 10.1128/jcm.25.6.1014-1018.1987. PubMed PMID: 3298308; PMCID: PMC269126.

46. Hsueh PR, Teng LJ, Yang PC, Pan HL, Ho SW, Luh KT. Nosocomial pseudoepidemic caused by Bacillus cereus traced to contaminated ethyl alcohol from a liquor factory. J Clin Microbiol. 1999;37(7):2280-4. doi: 10.1128/JCM.37.7.2280-2284.1999. PubMed PMID: 10364598; PMCID: PMC85137.

47. Romero-Gomez MP, Quiles-Melero MI, Pena Garcia P, Gutierrez Altes A, Garcia de Miguel MA, Jimenez C, Valdezate S, Saez Nieto JA. Outbreak of Burkholderia cepacia bacteremia caused by contaminated chlorhexidine in a hemodialysis unit. Infect Control Hosp Epidemiol. 2008;29(4):377-8. doi: 10.1086/529032. PubMed PMID: 18462153.

48. Lee CS, Lee HB, Cho YG, Park JH, Lee HS. Hospital-acquired Burkholderia cepacia infection related to contaminated benzalkonium chloride. J Hosp Infect. 2008;68(3):280-2. Epub 20080304. doi: 10.1016/j.jhin.2008.01.002. PubMed PMID: 18289727.

49. Ko S, An HS, Bang JH, Park SW. An outbreak of Burkholderia cepacia complex pseudobacteremia associated with intrinsically contaminated commercial 0.5% chlorhexidine solution. Am J Infect Control. 2015;43(3):266-8. Epub 20150101. doi: 10.1016/j.ajic.2014.11.010. PubMed PMID: 25557770.

50. Jackson M. The mycobacterial cell envelope-lipids. Cold Spring Harb Perspect Med. 2014;4(10). Epub 20140807. doi: 10.1101/cshperspect.a021105. PubMed PMID: 25104772; PMCID: PMC4200213.

51. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harb Perspect Biol. 2010;2(5):a000414. Epub 20100414. doi: 10.1101/cshperspect.a000414. PubMed PMID: 20452953; PMCID: PMC2857177.

52. Wesgate R, Grasha P, Maillard JY. Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage. Am J Infect Control. 2016;44(4):458-64. Epub 20160122. doi: 10.1016/j.ajic.2015.11.009. PubMed PMID: 26810885.

53. Swanson S, Fu TJ. Effect of Water Hardness on Efficacy of Sodium Hypochlorite Inactivation of Escherichia coli O157:H7 in Water. J Food Prot. 2017;80(3):497-501. doi: 10.4315/0362-028X.JFP-16-112. PubMed PMID: 28207312.

54. COUSINS CM, CLEGG LFL. THE EFFECT OF WATER HARDNESS AND TEMPERATURE ON WATER STERILIZATION BY MIXTURES OF DETERGENTS AND QUATERNARY AMMONIUM COMPOUNDS. Journal of Applied Bacteriology. 1956;19(2):250-5. doi: 10.1111/j.1365-2672.1956.tb00075.x.

55. William A. Rutala PD, M.P.H., David J. Weber, M.D., M.P.H., Healthcare Infection Control Practices Advisory Committee (HICPAC),. Factors Affecting the Efficacy of Disinfection and Sterilization2008:[1-45 pp.].

56. Fernando DM, Xu W, Loewen PC, Zhanel GG, Kumar A. Triclosan can select for an AdeIJK-overexpressing mutant of Acinetobacter baumannii ATCC 17978 that displays reduced susceptibility to multiple antibiotics. Antimicrob Agents Chemother. 2014;58(11):6424-31. Epub 20140818. doi: 10.1128/AAC.03074-14. PubMed PMID: 25136007; PMCID: PMC4249441.

57. Mc Cay PH, Ocampo-Sosa AA, Fleming GTA. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. Microbiology (Reading). 2010;156(Pt 1):30-8. Epub 20091008. doi: 10.1099/mic.0.029751-0. PubMed PMID: 19815578.

58. Centers for Disease Control and Prevention. ANTIMICROBIAL RESISTANCE THREATS in the United States, 2021-2022. 2024.

59. Royo-Cebrecos C, Laporte-Amargos J, Pena M, Ruiz-Camps I, Puerta-Alcalde P, Abdala E, Oltolini C, Akova M, Montejo M, Mikulska M, Martin-Davila P, Herrera F, Gasch O, Drgona L, Morales HMP, Brunel AS, Garcia E, Isler B, Kern WV, Palacios-Baena ZR, de la Calle GM, Montero MM, Kanj SS, Sipahi OR, Calik S, Marquez-Gomez I, Marin JI, Gomes MZR, Hemmatti P, Araos R, Peghin M, Del Pozo JL, Yanez L, Tilley R, Manzur A, Novo A, Carratala J, Gudiol C, group Is. Pseudomonas aeruginosa Bloodstream Infections in Patients with Cancer: Differences between Patients with Hematological Malignancies and Solid Tumors. Pathogens. 2022;11(10). Epub 20220930. doi: 10.3390/pathogens11101132. PubMed PMID: 36297188; PMCID: PMC9610728.

60. Qin S, Xiao W, Zhou C, Pu Q, Deng X, Lan L, Liang H, Song X, Wu M. Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct Target Ther. 2022;7(1):199. Epub 20220625. doi: 10.1038/s41392-022-01056-1. PubMed PMID: 35752612; PMCID: PMC9233671.

61. Mc Gann PT, Lebreton F, Jones BT, Dao HD, Martin MJ, Nelson MJ, Luo T, Wyatt AC, Smedberg JR, Kettlewell JM, Cohee BM, Hawley-Molloy JS, Bennett JW. Six Extensively Drug-Resistant Bacteria in an Injured Soldier, Ukraine. Emerg Infect Dis. 2023;29(8):1692-5. Epub 20230715. doi: 10.3201/eid2908.230567. PubMed PMID: 37406356; PMCID: PMC10370857.

62. Li Y, Roberts JA, Walker MM, Aslan AT, Harris PNA, Sime FB. The global epidemiology of ventilator-associated pneumonia caused by multi-drug resistant Pseudomonas aeruginosa: A systematic review and meta-analysis. Int J Infect Dis. 2024;139:78-85. Epub 20231126. doi: 10.1016/j.ijid.2023.11.023. PubMed PMID: 38013153.

63. Hultqvist LD, Andersen JB, Nilsson CM, Jansen CU, Rybtke M, Jakobsen TH, Nielsen TE, Qvortrup K, Moser C, Graz M, Qvortrup K, Tolker-Nielsen T, Givskov M. High efficacy treatment of murine Pseudomonas aeruginosa catheter-associated urinary tract infections using the c-di-GMP modulating anti-biofilm compound Disperazol in combination with ciprofloxacin. Antimicrob Agents Chemother. 2024:e0148123. Epub 20240508. doi: 10.1128/aac.01481-23. PubMed PMID: 38717093.

64. Bisht K, Baishya J, Wakeman CA. Pseudomonas aeruginosa polymicrobial interactions during lung infection. Curr Opin Microbiol. 2020;53:1-8. Epub 20200212. doi: 10.1016/j.mib.2020.01.014. PubMed PMID: 32062024; PMCID: PMC7244363.

65. Galdino ACM, Branquinha MH, Santos ALS, Viganor L. Pseudomonas aeruginosa and Its Arsenal of Proteases: Weapons to Battle the Host. In: Chakraborti S, Dhalla NS, editors. Pathophysiological Aspects of Proteases. Singapore: Springer Singapore; 2017. p. 381-97.

66. Cowell BA, Twining SS, Hobden JA, Kwong MSF, Fleiszig SMJ. Mutation of lasA and lasB reduces Pseudomonas aeruginosa invasion of epithelial cells. Microbiology (Reading). 2003;149(Pt 8):2291-9. doi: 10.1099/mic.0.26280-0. PubMed PMID: 12904569.

67. Hoge R, Pelzer A, Rosenau F, Wilhelm S, Duesseldorf H-H-U, editors. "Weapons of a pathogen: Proteases and their role in virulence of Pseudomonas aeruginosa"2010.

68. Voynow JA, Fischer BM, Zheng S. Proteases and cystic fibrosis. Int J Biochem Cell Biol. 2008;40(6-7):1238-45. Epub 20080314. doi: 10.1016/j.biocel.2008.03.003. PubMed PMID: 18395488; PMCID: PMC2431113.

69. Kessler E, Safrin M, Olson JC, Ohman DE. Secreted LasA of Pseudomonas aeruginosa is a staphylolytic protease. J Biol Chem. 1993;268(10):7503-8. PubMed PMID: 8463280.

70. Horvat RT, Parmely MJ. Pseudomonas aeruginosa alkaline protease degrades human gamma interferon and inhibits its bioactivity. Infect Immun. 1988;56(11):2925-32. doi: 10.1128/iai.56.11.2925-2932.1988. PubMed PMID: 3139565; PMCID: PMC259672.

71. Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW. Proteolytic inactivation of cytokines by Pseudomonas aeruginosa. Infect Immun. 1990;58(9):3009-14. doi: 10.1128/iai.58.9.3009-3014.1990. PubMed PMID: 2117578; PMCID: PMC313603.

72. Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ. Protease IV, a unique extracellular protease and virulence factor from Pseudomonas aeruginosa. J Biol Chem. 1998;273(27):16792-7. doi: 10.1074/jbc.273.27.16792. PubMed PMID: 9642237.

73. Malloy JL, Veldhuizen RA, Thibodeaux BA, O'Callaghan RJ, Wright JR. Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. Am J Physiol Lung Cell Mol Physiol. 2005;288(2):L409-18. Epub 20041029. doi: 10.1152/ajplung.00322.2004. PubMed PMID: 15516485.

74. Hauser AR. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol. 2009;7(9):654-65. doi: 10.1038/nrmicro2199. PubMed PMID: 19680249; PMCID: PMC2766515.

75. Shaver CM, Hauser AR. Relative contributions of Pseudomonas aeruginosa ExoU, ExoS, and ExoT to virulence in the lung. Infect Immun. 2004;72(12):6969-77. doi: 10.1128/IAI.72.12.6969-6977.2004. PubMed PMID: 15557619; PMCID: PMC529154.

76. Wood SJ, Goldufsky JW, Seu MY, Dorafshar AH, Shafikhani SH. Pseudomonas aeruginosa Cytotoxins: Mechanisms of Cytotoxicity and Impact on Inflammatory Responses. Cells. 2023;12(1). Epub 20230103. doi: 10.3390/cells12010195. PubMed PMID: 36611990; PMCID: PMC9818787.

77. DeBritto S, Gajbar TD, Satapute P, Sundaram L, Lakshmikantha RY, Jogaiah S, Ito SI. Isolation and characterization of nutrient dependent pyocyanin from Pseudomonas aeruginosa and its dye and agrochemical properties. Sci Rep. 2020;10(1):1542. Epub 20200131. doi: 10.1038/s41598-020-58335-6. PubMed PMID: 32005900; PMCID: PMC6994680.

78. Hall S, McDermott C, Anoopkumar-Dukie S, McFarland AJ, Forbes A, Perkins AV, Davey AK, Chess-Williams R, Kiefel MJ, Arora D, Grant GD. Cellular Effects of Pyocyanin, a Secreted Virulence Factor of Pseudomonas aeruginosa. Toxins (Basel). 2016;8(8). Epub 20160809. doi: 10.3390/toxins8080236. PubMed PMID: 27517959; PMCID: PMC4999852.

79. Meirelles LA, Perry EK, Bergkessel M, Newman DK. Bacterial defenses against a natural antibiotic promote collateral resilience to clinical antibiotics. PLoS Biol. 2021;19(3):e3001093. Epub 20210310. doi: 10.1371/journal.pbio.3001093. PubMed PMID: 33690640; PMCID: PMC7946323.

80. Kang D, Kirienko DR, Webster P, Fisher AL, Kirienko NV. Pyoverdine, a siderophore from Pseudomonas aeruginosa, translocates into C. elegans, removes iron, and activates a distinct host response. Virulence. 2018;9(1):804-17. doi: 10.1080/21505594.2018.1449508. PubMed PMID: 29532717; PMCID: PMC5955448.

81. Hunter RC, Newman DK. A putative ABC transporter, hatABCDE, is among molecular determinants of pyomelanin production in Pseudomonas aeruginosa. J Bacteriol. 2010;192(22):5962-71. Epub 20100924. doi: 10.1128/JB.01021-10. PubMed PMID: 20870774; PMCID: PMC2976449.

82. Kandela SA, al-Shibib AS, al-Khayat BH. A study of purified pyorubrin produced by local Pseudomonas aeruginosa. Acta Microbiol Pol. 1997;46(1):37-43. PubMed PMID: 9271846.

83. Rajyaguru N, Velez A, Sandin RL, Yacoub AT, Greene JN. Pyorubin Producing Pseudomonas Scalp Infection. Infectious Diseases in Clinical Practice. 2014;22(4):e91-e2. doi: 10.1097/ipc.0000000000000151. PubMed PMID: 00019048-201407000-00039.

84. Vereshchagin AN, Frolov NA, Egorova KS, Seitkalieva MM, Ananikov VP. Quaternary Ammonium Compounds (QACs) and Ionic Liquids (ILs) as Biocides: From Simple Antiseptics to Tunable Antimicrobials. Int J Mol Sci. 2021;22(13). Epub 20210624. doi: 10.3390/ijms22136793. PubMed PMID: 34202677; PMCID: PMC8268321.

85. Dan W, Gao J, Qi X, Wang J, Dai J. Antibacterial quaternary ammonium agents: Chemical diversity and biological mechanism. Eur J Med Chem. 2022;243:114765. Epub 20220913. doi: 10.1016/j.ejmech.2022.114765. PubMed PMID: 36116235.

86. Rutala WA, Weber DJ. Selection of the ideal disinfectant. Infect Control Hosp Epidemiol. 2014;35(7):855-65. Epub 20140529. doi: 10.1086/676877. PubMed PMID: 24915214.

87. Boyce JM. Quaternary ammonium disinfectants and antiseptics: tolerance, resistance and potential impact on antibiotic resistance. Antimicrob Resist Infect Control. 2023;12(1):32. Epub 20230413. doi: 10.1186/s13756-023-01241-z. PubMed PMID: 37055844; PMCID: PMC10099023.

88. Coombs K, Rodriguez-Quijada C, Clevenger JO, Sauer-Budge AF. Current Understanding of Potential Linkages between Biocide Tolerance and Antibiotic Cross-Resistance. Microorganisms. 2023;11(8). Epub 20230803. doi: 10.3390/microorganisms11082000. PubMed PMID: 37630560; PMCID: PMC10459251.

89. Maillard JY. Resistance of Bacteria to Biocides. Microbiol Spectr. 2018;6(2). doi: 10.1128/microbiolspec.ARBA-0006-2017. PubMed PMID: 29676244.

90. Morrison KR, Allen RA, Minbiole KPC, Wuest WM. More QACs, more questions: Recent advances in structure activity relationships and hurdles in understanding resistance mechanisms. Tetrahedron Lett. 2019;60(37). Epub 20190726. doi: 10.1016/j.tetlet.2019.07.026. PubMed PMID: 32296251; PMCID: PMC7158862.

91. Brayton SR, Toles ZEA, Sanchez CA, Michaud ME, Thierer LM, Keller TM, Risener CJ, Quave CL, Wuest WM, Minbiole KPC. Soft QPCs: Biscationic Quaternary Phosphonium Compounds as Soft Antimicrobial Agents. ACS Infect Dis. 2023;9(4):943-51. Epub 20230316. doi: 10.1021/acsinfecdis.2c00624. PubMed PMID: 36926876; PMCID: PMC10111419.

92. Sommers KJ, Michaud ME, Hogue CE, Scharnow AM, Amoo LE, Petersen AA, Carden RG, Minbiole KPC, Wuest WM. Quaternary Phosphonium Compounds: An Examination of Non-Nitrogenous Cationic Amphiphiles That Evade Disinfectant Resistance. ACS Infect Dis. 2022;8(2):387-97. Epub 20220125. doi: 10.1021/acsinfecdis.1c00611. PubMed PMID: 35077149; PMCID: PMC8996050.

93. Feliciano JA, Leitgeb AJ, Schrank CL, Allen RA, Minbiole KPC, Wuest WM, Carden RG. Trivalent sulfonium compounds (TSCs): Tetrahydrothiophene-based amphiphiles exhibit similar antimicrobial activity to analogous ammonium-based amphiphiles. Bioorg Med Chem Lett. 2021;37:127809. Epub 20210128. doi: 10.1016/j.bmcl.2021.127809. PubMed PMID: 33516911; PMCID: PMC7965331.

94. Toles ZEA, Wu A, Sanchez CA, Michaud ME, Thierer LM, Wuest WM, Minbiole KPC. Double BAC and Triple BAC: A Systematic Analysis of the Disinfectant Properties of Multicationic Derivatives of Benzalkonium Chloride (BAC). ChemMedChem. 2023;18(10):e202300018. Epub 20230313. doi: 10.1002/cmdc.202300018. PubMed PMID: 36823400; PMCID: PMC10192024.

95. Kontos RC, Schallenhammer SA, Bentley BS, Morrison KR, Feliciano JA, Tasca JA, Kaplan AR, Bezpalko MW, Kassel WS, Wuest WM, Minbiole KPC. An Investigation into Rigidity-Activity Relationships in BisQAC Amphiphilic Antiseptics. ChemMedChem. 2019;14(1):83-7. Epub 20181218. doi: 10.1002/cmdc.201800622. PubMed PMID: 30358105; PMCID: PMC6440202.

96. Belanger CR, Hancock REW. Testing physiologically relevant conditions in minimal inhibitory concentration assays. Nat Protoc. 2021;16(8):3761-74. Epub 20210702. doi: 10.1038/s41596-021-00572-8. PubMed PMID: 34215865.

97. Alves J, Sargison FA, Stawarz H, Fox WB, Huete SG, Hassan A, McTeir B, Pickering AC. A case report: insights into reducing plastic waste in a microbiology laboratory. Access Microbiol. 2021;3(3):000173. Epub 20201014. doi: 10.1099/acmi.0.000173. PubMed PMID: 34151149; PMCID: PMC8209715.

98. Thieme L, Hartung A, Tramm K, Graf J, Spott R, Makarewicz O, Pletz MW. Adaptation of the Start-Growth-Time Method for High-Throughput Biofilm Quantification. Front Microbiol. 2021;12:631248. Epub 20210826. doi: 10.3389/fmicb.2021.631248. PubMed PMID: 34512560; PMCID: PMC8428173.

99. Laatiris A, El Achouri M, Infante MR, Bensouda Y. Antibacterial activity, structure and CMC relationships of alkanediyl alpha,omega-bis(dimethylammonium bromide) surfactants. Microbiol Res. 2008;163(6):645-50. doi: 10.1016/j.micres.2006.09.006. PubMed PMID: 17207986.

100. Tobe S, Majima T, Tadenuma H, Suekuni T, Sakai K, Sakai H, Abe M. Nonionic surfactants enhancing bactericidal activity at their critical micelle concentrations. J Oleo Sci. 2015;64(1):61-8. Epub 20141210. doi: 10.5650/jos.ess14159. PubMed PMID: 25492231.

101. Viscardi G, Quagliotto P, Barolo C, Savarino P, Barni E, Fisicaro E. Synthesis and surface and antimicrobial properties of novel cationic surfactants. J Org Chem. 2000;65(24):8197-203. doi: 10.1021/jo0006425. PubMed PMID: 11101373.

102. Bakhshi N, Soleimanian-Zad S, Sheikh-Zeinoddin M. Dynamic surface tension measurement for the screening of biosurfactants produced by Lactobacillus plantarum subsp. plantarum PTCC 1896. Enzyme Microb Technol. 2017;101:1-8. Epub 20170221. doi: 10.1016/j.enzmictec.2017.02.010. PubMed PMID: 28433185.

103. Frobisher M. Studies Upon the Relationship between Surface Tension and the Action of Disinfectants, with Special Reference to Hexylresorcinol. J Bacteriol. 1927;13(3):163-82. doi: 10.1128/jb.13.3.163-182.1927. PubMed PMID: 16559238; PMCID: PMC374927.

104. Paniak TJ, Jennings MC, Shanahan PC, Joyce MD, Santiago CN, Wuest WM, Minbiole KPC. The antimicrobial activity of mono-, bis-, tris-, and tetracationic amphiphiles derived from simple polyamine platforms. Bioorg Med Chem Lett. 2014;24(24):5824-8. Epub 20141023. doi: 10.1016/j.bmcl.2014.10.018. PubMed PMID: 25455498.

105. Mitchell MA, Iannetta AA, Jennings MC, Fletcher MH, Wuest WM, Minbiole KP. Scaffold-Hopping of Multicationic Amphiphiles Yields Three New Classes of Antimicrobials. Chembiochem. 2015;16(16):2299-303. Epub 20150911. doi: 10.1002/cbic.201500381. PubMed PMID: 26316312.

106. William M. Wuest KPCM, inventorPolycationic Amphiphiles as Antimicrobial Agents and Methods Using Same. US2021.

107. Forman ME, Jennings MC, Wuest WM, Minbiole KP. Building a Better Quaternary Ammonium Compound (QAC): Branched Tetracationic Antiseptic Amphiphiles. ChemMedChem. 2016;11(13):1401-5. Epub 20160601. doi: 10.1002/cmdc.201600176. PubMed PMID: 27245743.

108. International A. Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure. 2016. p. 5.

109. Tezel U, Pavlostathis SG. Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. Curr Opin Biotechnol. 2015;33:296-304. Epub 20150410. doi: 10.1016/j.copbio.2015.03.018. PubMed PMID: 25864173.

110. International A. Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents. 2013.

111. Dey BP, Engley FB, Jr. Methodology for recovery of chemically treated Staphylococcus aureus with neutralizing medium. Appl Environ Microbiol. 1983;45(5):1533-7. doi: 10.1128/aem.45.5.1533-1537.1983. PubMed PMID: 6870240; PMCID: PMC242496.

112. Clifton LA, Skoda MW, Le Brun AP, Ciesielski F, Kuzmenko I, Holt SA, Lakey JH. Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models. Langmuir. 2015;31(1):404-12. Epub 20141219. doi: 10.1021/la504407v. PubMed PMID: 25489959; PMCID: PMC4295546.

113. Kula N, Lamch L, Futoma-Koloch B, Wilk KA, Oblak E. The effectiveness of newly synthesized quaternary ammonium salts differing in chain length and type of counterion against priority human pathogens. Sci Rep. 2022;12(1):21799. Epub 20221216. doi: 10.1038/s41598-022-24760-y. PubMed PMID: 36526659; PMCID: PMC9757636.

114. Oblak E, Piecuch A, Krasowska A, Luczynski J. Antifungal activity of gemini quaternary ammonium salts. Microbiol Res. 2013;168(10):630-8. Epub 20130701. doi: 10.1016/j.micres.2013.06.001. PubMed PMID: 23827647.

115. Mahoney AR, Safaee MM, Wuest WM, Furst AL. The silent pandemic: Emergent antibiotic resistances following the global response to SARS-CoV-2. iScience.

2021;24(4):102304. Epub 20210313. doi: 10.1016/j.isci.2021.102304. PubMed PMID: 33748695; PMCID: PMC7955580.

116. Humphries RM, Ambler J, Mitchell SL, Castanheira M, Dingle T, Hindler JA, Koeth L, Sei K, Development CM, Standardization Working Group of the Subcommittee on Antimicrobial Susceptibility T. CLSI Methods Development and Standardization Working Group Best Practices for Evaluation of Antimicrobial Susceptibility Tests. J Clin Microbiol. 2018;56(4). Epub 20180326. doi: 10.1128/JCM.01934-17. PubMed PMID: 29367292; PMCID: PMC5869819.

117. Maillard JY. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. Ther Clin Risk Manag. 2005;1(4):307-20. PubMed PMID: 18360573; PMCID: PMC1661639.

118. Fox LJ, Kelly PP, Humphreys GJ, Waigh TA, Lu JR, McBain AJ. Assessing the risk of resistance to cationic biocides incorporating realism-based and biophysical approaches. J Ind Microbiol Biotechnol. 2022;49(1). doi: 10.1093/jimb/kuab074. PubMed PMID: 34718634; PMCID: PMC9113109.

119. Gilbert P, McBain AJ. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin Microbiol Rev. 2003;16(2):189-208. doi: 10.1128/CMR.16.2.189-208.2003. PubMed PMID: 12692093; PMCID: PMC153147.

120. Kim M, Weigand MR, Oh S, Hatt JK, Krishnan R, Tezel U, Pavlostathis SG, Konstantinidis KT. Widely Used Benzalkonium Chloride Disinfectants Can Promote Antibiotic Resistance. Appl Environ Microbiol. 2018;84(17). Epub 20180817. doi: 10.1128/AEM.01201-18. PubMed PMID: 29959242; PMCID: PMC6102991.

121. Short FL, Lee V, Mamun R, Malmberg R, Li L, Espinosa MI, Venkatesan K, Paulsen IT. Benzalkonium chloride antagonises aminoglycoside antibiotics and promotes evolution of

resistance. EBioMedicine. 2021;73:103653. Epub 20211028. doi: 10.1016/j.ebiom.2021.103653. PubMed PMID: 34717227; PMCID: PMC8577336.

122. Stribling W, Hall LR, Powell A, Harless C, Martin MJ, Corey BW, Snesrud E, Ong A, Maybank R, Stam J, Bartlett K, Jones BT, Preston LN, Lane KF, Thompson B, Young LM, Kwak YI, Barsoumian AE, Markelz A-E, Kiley JL, Cybulski RJ, Bennett JW, Mc Gann PT, Lebreton F. Detecting, mapping, and suppressing the spread of a decade-long *Pseudomonas aeruginosa* nosocomial outbreak with genomics. Cold Spring Harbor Laboratory; 2023.

123. Miller SI. Antibiotic Resistance and Regulation of the Gram-Negative Bacterial Outer Membrane Barrier by Host Innate Immune Molecules. mBio. 2016;7(5). Epub 20160927. doi: 10.1128/mBio.01541-16. PubMed PMID: 27677793; PMCID: PMC5040116.

124. Kim M, Hatt JK, Weigand MR, Krishnan R, Pavlostathis SG, Konstantinidis KT. Genomic and Transcriptomic Insights into How Bacteria Withstand High Concentrations of Benzalkonium Chloride Biocides. Appl Environ Microbiol. 2018;84(12). Epub 20180531. doi: 10.1128/AEM.00197-18. PubMed PMID: 29654181; PMCID: PMC5981084.

125. Michaud ME, Allen RA, Morrison-Lewis KR, Sanchez CA, Minbiole KPC, Post SJ, Wuest WM. Quaternary Phosphonium Compound Unveiled as a Potent Disinfectant against Highly Resistant Acinetobacter baumannii Clinical Isolates. ACS Infect Dis. 2022;8(11):2307-14. Epub 20221027. doi: 10.1021/acsinfecdis.2c00382. PubMed PMID: 36301313.

126. Lebreton F, Snesrud E, Hall L, Mills E, Galac M, Stam J, Ong A, Maybank R, Kwak YI, Johnson S, Julius M, Ly M, Swierczewski B, Waterman PE, Hinkle M, Jones A, Lesho E, Bennett JW, McGann P. A panel of diverse Pseudomonas aeruginosa clinical isolates for research and development. JAC Antimicrob Resist. 2021;3(4):dlab179. Epub 20211210. doi: 10.1093/jacamr/dlab179. PubMed PMID: 34909689; PMCID: PMC8664536.

127. Longshaw C, Santerre Henriksen A, Dressel D, Malysa M, Silvestri C, Takemura M, Yamano Y, Baba T, Slover CM. Heteroresistance to cefiderocol in carbapenem-resistant Acinetobacter baumannii in the CREDIBLE-CR study was not linked to clinical outcomes: a post hoc analysis. Microbiol Spectr. 2023;11(6):e0237123. Epub 20231115. doi: 10.1128/spectrum.02371-23. PubMed PMID: 37966262; PMCID: PMC10714777.

128. Roy PH, Tetu SG, Larouche A, Elbourne L, Tremblay S, Ren Q, Dodson R, Harkins D, Shay R, Watkins K, Mahamoud Y, Paulsen IT. Complete genome sequence of the multiresistant taxonomic outlier Pseudomonas aeruginosa PA7. PLoS One. 2010;5(1):e8842. Epub 20100122. doi: 10.1371/journal.pone.0008842. PubMed PMID: 20107499; PMCID: PMC2809737.

129. Hancock RE, Carey AM. Outer membrane of Pseudomonas aeruginosa: heat- 2-mercaptoethanol-modifiable proteins. J Bacteriol. 1979;140(3):902-10. doi: 10.1128/jb.140.3.902-910.1979. PubMed PMID: 118160; PMCID: PMC216732.

130. Boaro A, Ageitos L, Torres MT, Blasco EB, Oztekin S, de la Fuente-Nunez C. Structure-function-guided design of synthetic peptides with anti-infective activity derived from wasp venom. Cell Rep Phys Sci. 2023;4(7). doi: 10.1016/j.xcrp.2023.101459. PubMed PMID: 38239869; PMCID: PMC10795512.

131. McGrath DM, Barbu EM, Driessen WH, Lasco TM, Tarrand JJ, Okhuysen PC, Kontoyiannis DP, Sidman RL, Pasqualini R, Arap W. Mechanism of action and initial evaluation of a membrane active all-D-enantiomer antimicrobial peptidomimetic. Proc Natl Acad Sci U S A. 2013;110(9):3477-82. Epub 20130123. doi: 10.1073/pnas.1221924110. PubMed PMID: 23345420; PMCID: PMC3587187.

132. Klobucar K, Cote JP, French S, Borrillo L, Guo ABY, Serrano-Wu MH, Lee KK, Hubbard B, Johnson JW, Gaulin JL, Magolan J, Hung DT, Brown ED. Chemical Screen for Vancomycin

Antagonism Uncovers Probes of the Gram-Negative Outer Membrane. ACS Chem Biol. 2021;16(5):929-42. Epub 20210511. doi: 10.1021/acschembio.1c00179. PubMed PMID: 33974796.

133. Bonvin E, Personne H, Paschoud T, Reusser J, Gan BH, Luscher A, Kohler T, van Delden C, Reymond JL. Antimicrobial Peptide-Peptoid Hybrids with and without Membrane Disruption. ACS Infect Dis. 2023;9(12):2593-606. Epub 20231121. doi: 10.1021/acsinfecdis.3c00421. PubMed PMID: 38062792; PMCID: PMC10714400.

134. Bock LJ, Ferguson PM, Clarke M, Pumpitakkul V, Wand ME, Fady PE, Allison L, Fleck RA, Shepherd MJ, Mason AJ, Sutton JM. Pseudomonas aeruginosa adapts to octenidine via a combination of efflux and membrane remodelling. Commun Biol. 2021;4(1):1058. Epub 20210909. doi: 10.1038/s42003-021-02566-4. PubMed PMID: 34504285; PMCID: PMC8429429.

135. Hoque J, Akkapeddi P, Yarlagadda V, Uppu DS, Kumar P, Haldar J. Cleavable cationic antibacterial amphiphiles: synthesis, mechanism of action, and cytotoxicities. Langmuir. 2012;28(33):12225-34. Epub 20120808. doi: 10.1021/la302303d. PubMed PMID: 22838496.

136. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-localized spermidine protects the Pseudomonas aeruginosa outer membrane from antibiotic treatment and oxidative stress. J Bacteriol. 2012;194(4):813-26. Epub 20111209. doi: 10.1128/JB.05230-11. PubMed PMID: 22155771; PMCID: PMC3272965.

137. Hasan CM, Pottenger S, Green AE, Cox AA, White JS, Jones T, Winstanley C, Kadioglu A, Wright MH, Neill DR, Fothergill JL. Pseudomonas aeruginosa utilizes the host-derived polyamine spermidine to facilitate antimicrobial tolerance. JCI Insight. 2022;7(22). Epub 20221122. doi: 10.1172/jci.insight.158879. PubMed PMID: 36194492; PMCID: PMC9746822.

138. Kwon DH, Lu CD. Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in Pseudomonas aeruginosa PAO1. Antimicrob Agents Chemother. 2006;50(5):1615-22. doi: 10.1128/AAC.50.5.1615-1622.2006. PubMed PMID: 16641426; PMCID: PMC1472189.

139. Ikonomidis A, Tsakris A, Kanellopoulou M, Maniatis AN, Pournaras S. Effect of the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on Pseudomonas aeruginosa biofilm development. Lett Appl Microbiol. 2008;47(4):298-302. doi: 10.1111/j.1472-765x.2008.02430.x. PubMed PMID: 19241523.

140. Spindler EC, Hale JD, Giddings TH, Jr., Hancock RE, Gill RT. Deciphering the mode of action of the synthetic antimicrobial peptide Bac8c. Antimicrob Agents Chemother. 2011;55(4):1706-16. Epub 20110131. doi: 10.1128/AAC.01053-10. PubMed PMID: 21282431; PMCID: PMC3067151.

141. Li L, Short FL, Hassan KA, Naidu V, Pokhrel A, Nagy SS, Prity FT, Shah BS, Afrin N, Baker S, Parkhill J, Cain AK, Paulsen IT. Systematic analyses identify modes of action of ten clinically relevant biocides and antibiotic antagonism in Acinetobacter baumannii. Nat Microbiol. 2023;8(11):1995-2005. Epub 20231009. doi: 10.1038/s41564-023-01474-z. PubMed PMID: 37814070.

142. Dashtbani-Roozbehani A, Brown MH. Efflux Pump Mediated Antimicrobial Resistance by Staphylococci in Health-Related Environments: Challenges and the Quest for Inhibition. Antibiotics (Basel). 2021;10(12). Epub 20211207. doi: 10.3390/antibiotics10121502. PubMed PMID: 34943714; PMCID: PMC8698293.

143. Majumder P, Ahmed S, Ahuja P, Athreya A, Ranjan R, Penmatsa A. Cryo-EM structure of antibacterial efflux transporter QacA from Staphylococcus aureus reveals a novel extracellular

loop with allosteric role. EMBO J. 2023;42(16):e113418. Epub 20230717. doi: 10.15252/embj.2023113418. PubMed PMID: 37458117; PMCID: PMC10425836.

144. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. Construction of improved Escherichia-Pseudomonas shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in Pseudomonas aeruginosa. Gene. 1994;148(1):81-6. doi: 10.1016/0378-1119(94)90237-2. PubMed PMID: 7926843.

145. Guerin F, Gravey F, Plesiat P, Aubourg M, Beyrouthy R, Bonnet R, Cattoir V, Giard JC. The Transcriptional Repressor SmvR Is Important for Decreased Chlorhexidine Susceptibility in Enterobacter cloacae Complex. Antimicrob Agents Chemother. 2019;64(1). Epub 20191220. doi: 10.1128/AAC.01845-19. PubMed PMID: 31685460; PMCID: PMC7187565.

146. Wand ME, Jamshidi S, Bock LJ, Rahman KM, Sutton JM. SmvA is an important efflux pump for cationic biocides in Klebsiella pneumoniae and other Enterobacteriaceae. Sci Rep. 2019;9(1):1344. Epub 20190204. doi: 10.1038/s41598-018-37730-0. PubMed PMID: 30718598; PMCID: PMC6362122.

147. O'Toole GA, Wathier M, Zegans ME, Shanks RM, Kowalski R, Grinstaff MW. Diphosphonium ionic liquids as broad-spectrum antimicrobial agents. Cornea. 2012;31(7):810-6. doi: 10.1097/ICO.0b013e31823f0a86. PubMed PMID: 22236790; PMCID: PMC3336019.

148. Zeng H, Johnson ME, Oldenhuis NJ, Tiambeng TN, Guan Z. Structure-Based Design of Dendritic Peptide Bolaamphiphiles for siRNA Delivery. ACS Cent Sci. 2015;1(6):303-12. Epub 20150814. doi: 10.1021/acscentsci.5b00233. PubMed PMID: 26436138; PMCID: PMC4582325.

149. Di Blasio S, Clarke M, Hind CK, Asai M, Laurence L, Benvenuti A, Hassan M, Semenya D, Man DK, Horrocks V, Manzo G, Van Der Lith S, Lam C, Gentile E, Annette C, Bosse J, Li Y, Panaretou B, Langford PR, Robertson BD, Lam JKW, Sutton JM, McArthur M, Mason AJ.

Bolaamphiphile Analogues of 12-bis-THA Cl(2) Are Potent Antimicrobial Therapeutics with Distinct Mechanisms of Action against Bacterial, Mycobacterial, and Fungal Pathogens. mSphere. 2023;8(1):e0050822. Epub 20221213. doi: 10.1128/msphere.00508-22. PubMed PMID: 36511707; PMCID: PMC9942557.

150. Cameron LM, Fyles TM, Hu CW. Synthesis and membrane activity of a bis(metacyclophane)bolaamphiphile. J Org Chem. 2002;67(5):1548-53. doi: 10.1021/jo0160930. PubMed PMID: 11871885.

151. Fyles TM, Hu C, Knoy R. Transmembrane ion conductance by an acyclic bolaamphiphile. Org Lett. 2001;3(9):1335-7. doi: 10.1021/ol015713g. PubMed PMID: 11348228.

152. O'Neil EJ, DiVittorio KM, Smith BD. Phosphatidylcholine-derived bolaamphiphiles via click chemistry. Org Lett. 2007;9(2):199-202. doi: 10.1021/ol062557a. PubMed PMID: 17217264; PMCID: PMC2851100.

153. Hughes JR, Miller AS, Wallace CE, Vemuri GN, Iovine PM. Biomedically Relevant Applications of Bolaamphiphiles and Bolaamphiphile-Containing Materials. Front Chem. 2020;8:604151. Epub 20210120. doi: 10.3389/fchem.2020.604151. PubMed PMID: 33553103; PMCID: PMC7855593.

154. Li J, Liu S, Koh JJ, Zou H, Lakshminarayanan R, Bai Y, Pervushin K, Zhou L, Verma C, Beuerman RW. A novel fragment based strategy for membrane active antimicrobials against MRSA. Biochim Biophys Acta. 2015;1848(4):1023-31. Epub 20150110. doi: 10.1016/j.bbamem.2015.01.001. PubMed PMID: 25582665.

155. Alkhalifa S, Jennings MC, Granata D, Klein M, Wuest WM, Minbiole KPC, Carnevale V. Analysis of the Destabilization of Bacterial Membranes by Quaternary Ammonium Compounds:

A Combined Experimental and Computational Study. Chembiochem. 2020;21(10):1510-6. Epub 20200218. doi: 10.1002/cbic.201900698. PubMed PMID: 31859426; PMCID: PMC7237276.

156. Jennings MC, Forman ME, Duggan SM, Minbiole KPC, Wuest WM. Efflux Pumps Might Not Be the Major Drivers of QAC Resistance in Methicillin-Resistant Staphylococcus aureus. Chembiochem. 2017;18(16):1573-7. Epub 20170627. doi: 10.1002/cbic.201700233. PubMed PMID: 28510317; PMCID: PMC5664203.

157. Grkovic S, Brown MH, Roberts NJ, Paulsen IT, Skurray RA. QacR is a repressor protein that regulates expression of the Staphylococcus aureus multidrug efflux pump QacA. J Biol Chem. 1998;273(29):18665-73. doi: 10.1074/jbc.273.29.18665. PubMed PMID: 9660841.

158. Grkovic S, Brown MH, Schumacher MA, Brennan RG, Skurray RA. The staphylococcal QacR multidrug regulator binds a correctly spaced operator as a pair of dimers. J Bacteriol. 2001;183(24):7102-9. doi: 10.1128/JB.183.24.7102-7109.2001. PubMed PMID: 11717268; PMCID: PMC95558.

159. Gellatly SL, Hancock RE. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-73. Epub 20130315. doi: 10.1111/2049-632X.12033. PubMed PMID: 23620179.

160. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, Group WHOPPLW. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-27. Epub 20171221. doi: 10.1016/S1473-3099(17)30753-3. PubMed PMID: 29276051.

161. WHO E. Antimicrobial resistance surveillance in Europe 2022 – 2020 data. . World Health Organization Regional Office for Europe. 2022.

162. Thierer LM, Petersen AA, Michaud ME, Sanchez CA, Brayton SR, Wuest WM, Minbiole KPC. Atom Economical QPCs: Phenyl-Free Biscationic Quaternary Phosphonium Compounds as Potent Disinfectants. ACS Infect Dis. 2023;9(3):609-16. Epub 20230209. doi: 10.1021/acsinfecdis.2c00575. PubMed PMID: 36757826; PMCID: PMC10032568.

163. Brand S, Schlusener MP, Albrecht D, Kunkel U, Strobel C, Grummt T, Ternes TA. Quaternary (triphenyl-) phosphonium compounds: Environmental behavior and toxicity. Water Res. 2018;136:207-19. Epub 20180302. doi: 10.1016/j.watres.2018.02.032. PubMed PMID: 29518585.

164. Lei Q, Lai X, Zhang Y, Li Z, Li R, Zhang W, Ao N, Zhang H. PEGylated Bis-Quaternary Triphenyl-Phosphonium Tosylate Allows for Balanced Antibacterial Activity and Cytotoxicity. ACS Appl Bio Mater. 2020;3(9):6400-7. Epub 20200826. doi: 10.1021/acsabm.0c00837. PubMed PMID: 35021771.

165. Ermolaev VV, Arkhipova DM, Miluykov VA, Lyubina AP, Amerhanova SK, Kulik NV, Voloshina AD, Ananikov VP. Sterically Hindered Quaternary Phosphonium Salts (QPSs): Antimicrobial Activity and Hemolytic and Cytotoxic Properties. Int J Mol Sci. 2021;23(1). Epub 20211222. doi: 10.3390/ijms23010086. PubMed PMID: 35008507; PMCID: PMC8744835.

166. Leatherbury MS, Thierer L, Sanchez CA, Vargas-Cuebas G, Petersen AA, Amoo LE, Bezold EL, Washington KC, Mistrot MB, Zdilla MJ, Wuest WM, Minbiole KPC. Chimeric Amphiphilic Disinfectants: Quaternary Ammonium / Quaternary Phosphonium Hybrid Structures. ChemMedChem. 2024:e202300718. Epub 20240228. doi: 10.1002/cmdc.202300718. PubMed PMID: 38416542.

167. Ibrahim MK, Haria A, Mehta NV, Degani MS. Antimicrobial potential of quaternary phosphonium salt compounds: a review. Future Med Chem. 2023;15(22):2113-41. Epub 20231106. doi: 10.4155/fmc-2023-0188. PubMed PMID: 37929337.

168. Jones IA, Joshi LT. Biocide Use in the Antimicrobial Era: A Review. Molecules. 2021;26(8). Epub 20210414. doi: 10.3390/molecules26082276. PubMed PMID: 33919993; PMCID: PMC8071000.

169. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. Methods Mol Biol. 2014;1151:165-88. doi: 10.1007/978-1-4939-0554-6_12. PubMed PMID: 24838886; PMCID: PMC4239701.

170. Coppens L, Lavigne R. SAPPHIRE: a neural network based classifier for sigma70 promoter prediction in Pseudomonas. BMC Bioinformatics. 2020;21(1):415. Epub 20200922. doi: 10.1186/s12859-020-03730-z. PubMed PMID: 32962628; PMCID: PMC7510298.

171. Choi KH, Kumar A, Schweizer HP. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods. 2006;64(3):391-7. Epub 20050628. doi: 10.1016/j.mimet.2005.06.001. PubMed PMID: 15987659.

172. Varga JJ, Barbier M, Mulet X, Bielecki P, Bartell JA, Owings JP, Martinez-Ramos I, Hittle LE, Davis MR, Jr., Damron FH, Liechti GW, Puchalka J, dos Santos VA, Ernst RK, Papin JA, Alberti S, Oliver A, Goldberg JB. Genotypic and phenotypic analyses of a Pseudomonas aeruginosa chronic bronchiectasis isolate reveal differences from cystic fibrosis and laboratory strains. BMC Genomics. 2015;16:883. Epub 20151030. doi: 10.1186/s12864-015-2069-0. PubMed PMID: 26519161; PMCID: PMC4628258.

173. Sobhanipoor MH, Ahmadrajabi R, Nave HH, Saffari F. Determination of efflux activity in Enterococci by Hoechst accumulation assay and the role of zinc oxide nanoparticles in inhibition of this activity. BMC Microbiol. 2022;22(1):195. Epub 20220809. doi: 10.1186/s12866-022-02595-x. PubMed PMID: 35941529; PMCID: PMC9361545.

174. Collaborators GBDAR. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2022;400(10369):2221-48. Epub 20221121. doi: 10.1016/S0140-6736(22)02185-7. PubMed PMID: 36423648; PMCID: PMC9763654.

175. Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. Resistance of bacterial biofilms to disinfectants: a review. Biofouling. 2011;27(9):1017-32. doi: 10.1080/08927014.2011.626899. PubMed PMID: 22011093.

176. Malhotra S, Hayes D, Jr., Wozniak DJ. Cystic Fibrosis and Pseudomonas aeruginosa: the Host-Microbe Interface. Clin Microbiol Rev. 2019;32(3). Epub 20190529. doi: 10.1128/CMR.00138-18. PubMed PMID: 31142499; PMCID: PMC6589863.

177. Rozov SM, Deineko EV. Bacterial intracellular pathogens: Offense and defense strategies. Biology Bulletin Reviews. 2016;6(2):189-201. doi: 10.1134/S2079086416020067.

178. Nitzan M, Fechter P, Peer A, Altuvia Y, Bronesky D, Vandenesch F, Romby P, Biham O, Margalit H. A defense-offense multi-layered regulatory switch in a pathogenic bacterium. Nucleic Acids Res. 2015;43(3):1357-69. Epub 20150127. doi: 10.1093/nar/gkv001. PubMed PMID: 25628364; PMCID: PMC4330369.

179. Pachori P, Gothalwal R, Gandhi P. Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. Genes Dis. 2019;6(2):109-19. Epub 20190417. doi: 10.1016/j.gendis.2019.04.001. PubMed PMID: 31194018; PMCID: PMC6545445.

180. Damiano P, Silago V, Nyawale HA, Mushi MF, Mirambo MM, Kimaro EE, Mshana SE. Efficacy of disinfectants on control and clinical bacteria strains at a zonal referral hospital in Mwanza, Tanzania: a cross sectional hospital-based study. Sci Rep. 2023;13(1):17998. Epub 20231021. doi: 10.1038/s41598-023-45228-7. PubMed PMID: 37865710; PMCID: PMC10590406.

181. Loughlin MF, Jones MV, Lambert PA. Pseudomonas aeruginosa cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrob Chemother. 2002;49(4):631-9. doi: 10.1093/jac/49.4.631. PubMed PMID: 11909837.

182. Grobe S, Wingender J, Flemming HC. Capability of mucoid Pseudomonas aeruginosa to survive in chlorinated water. Int J Hyg Environ Health. 2001;204(2-3):139-42. doi: 10.1078/1438-4639-00085. PubMed PMID: 11759157.

183. Oliver AM, Weir DM. The effect of Pseudomonas alginate on rat alveolar macrophage phagocytosis and bacterial opsonization. Clin Exp Immunol. 1985;59(1):190-6. PubMed PMID: 3918817; PMCID: PMC1577170.

184. Araujo PA, Lemos M, Mergulhao F, Melo L, Simoes M. The Influence of Interfering Substances on the Antimicrobial Activity of Selected Quaternary Ammonium Compounds. Int J Food Sci. 2013;2013:237581. Epub 20130914. doi: 10.1155/2013/237581. PubMed PMID: 26904590; PMCID: PMC4745498.

185. Boucher JC, Yu H, Mudd MH, Deretic V. Mucoid Pseudomonas aeruginosa in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun. 1997;65(9):3838-46. doi: 10.1128/iai.65.9.3838-3846.1997. PubMed PMID: 9284161; PMCID: PMC175548.

186. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol. 2016;14(9):563-75. doi: 10.1038/nrmicro.2016.94. PubMed PMID: 27510863.

187. Rather MA, Gupta K, Mandal M. Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. Braz J Microbiol. 2021;52(4):1701-18. Epub 20210923. doi: 10.1007/s42770-021-00624-x. PubMed PMID: 34558029; PMCID: PMC8578483.

188. Flemming HC, van Hullebusch ED, Neu TR, Nielsen PH, Seviour T, Stoodley P, Wingender J, Wuertz S. The biofilm matrix: multitasking in a shared space. Nat Rev Microbiol. 2023;21(2):70-86. Epub 20220920. doi: 10.1038/s41579-022-00791-0. PubMed PMID: 36127518.

189. Henly EL, Dowling JAR, Maingay JB, Lacey MM, Smith TJ, Forbes S. Biocide Exposure Induces Changes in Susceptibility, Pathogenicity, and Biofilm Formation in Uropathogenic Escherichia coli. Antimicrob Agents Chemother. 2019;63(3). Epub 20190226. doi: 10.1128/AAC.01892-18. PubMed PMID: 30642923; PMCID: PMC6395906.

190. Ghafoor A, Hay ID, Rehm BH. Role of exopolysaccharides in Pseudomonas aeruginosa biofilm formation and architecture. Appl Environ Microbiol. 2011;77(15):5238-46. Epub 20110610. doi: 10.1128/AEM.00637-11. PubMed PMID: 21666010; PMCID: PMC3147449.

191. Jennings LK, Dreifus JE, Reichhardt C, Storek KM, Secor PR, Wozniak DJ, Hisert KB, Parsek MR. Pseudomonas aeruginosa aggregates in cystic fibrosis sputum produce exopolysaccharides that likely impede current therapies. Cell Rep. 2021;34(8):108782. doi: 10.1016/j.celrep.2021.108782. PubMed PMID: 33626358; PMCID: PMC7958924.

192. Franklin MJ, Nivens DE, Weadge JT, Howell PL. Biosynthesis of the Pseudomonas aeruginosa Extracellular Polysaccharides, Alginate, Pel, and Psl. Front Microbiol. 2011;2:167.

Epub 20110822. doi: 10.3389/fmicb.2011.00167. PubMed PMID: 21991261; PMCID: PMC3159412.

193. Armitano J, Mejean V, Jourlin-Castelli C. Gram-negative bacteria can also form pellicles. Environ Microbiol Rep. 2014;6(6):534-44. doi: 10.1111/1758-2229.12171. PubMed PMID: 25756106.

194. Pang XY, Yang YS, Yuk HG. Biofilm formation and disinfectant resistance of Salmonella sp. in mono- and dual-species with Pseudomonas aeruginosa. J Appl Microbiol. 2017;123(3):651-60. Epub 20170731. doi: 10.1111/jam.13521. PubMed PMID: 28644912.

195. Shemesh M, Kolter R, Losick R. The biocide chlorine dioxide stimulates biofilm formation in Bacillus subtilis by activation of the histidine kinase KinC. J Bacteriol. 2010;192(24):6352-6. Epub 20101022. doi: 10.1128/JB.01025-10. PubMed PMID: 20971918; PMCID: PMC3008516.

196. Friedman L, Kolter R. Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. J Bacteriol. 2004;186(14):4457-65. doi: 10.1128/JB.186.14.4457-4465.2004. PubMed PMID: 15231777; PMCID: PMC438632.

197. Doucet AN, Slipski CJ, Golding GR, Mulvey MR, Bay DC. Generation of Greater Bacterial Biofilm Biomass using PCR-Plate Deep Well Microplate Devices. J Vis Exp. 2022(182). Epub 20220422. doi: 10.3791/63069. PubMed PMID: 35532279.

198. Khan F, Pham DTN, Oloketuyi SF, Kim YM. Regulation and controlling the motility properties of Pseudomonas aeruginosa. Appl Microbiol Biotechnol. 2020;104(1):33-49. Epub 20191125. doi: 10.1007/s00253-019-10201-w. PubMed PMID: 31768614.

199. Nordholt N, Kanaris O, Schmidt SBI, Schreiber F. Persistence against benzalkonium chloride promotes rapid evolution of tolerance during periodic disinfection. Nat Commun.

2021;12(1):6792. Epub 20211123. doi: 10.1038/s41467-021-27019-8. PubMed PMID: 34815390; PMCID: PMC8611074.

200. Merchel Piovesan Pereira B, Wang X, Tagkopoulos I. Short- and Long-Term Transcriptomic Responses of Escherichia coli to Biocides: a Systems Analysis. Appl Environ Microbiol. 2020;86(14). Epub 20200702. doi: 10.1128/AEM.00708-20. PubMed PMID: 32385082; PMCID: PMC7357472.

201. Forbes S, Morgan N, Humphreys GJ, Amezquita A, Mistry H, McBain AJ. Loss of Function in Escherichia coli Exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride. Appl Environ Microbiol. 2019;85(4). Epub 20190206. doi: 10.1128/AEM.02417-18. PubMed PMID: 30530708; PMCID: PMC6365820.

202. Jiang X, Jiang C, Yu T, Jiang X, Ren S, Kang R, Qiu S. Benzalkonium Chloride Adaptation Increases Expression of the Agr System, Biofilm Formation, and Virulence in Listeria monocytogenes. Front Microbiol. 2022;13:856274. Epub 20220223. doi: 10.3389/fmicb.2022.856274. PubMed PMID: 35283841; PMCID: PMC8905296.

203. Ghssein G, Ezzeddine Z. A Review of Pseudomonas aeruginosa Metallophores: Pyoverdine, Pyochelin and Pseudopaline. Biology (Basel). 2022;11(12). Epub 20221125. doi: 10.3390/biology11121711. PubMed PMID: 36552220; PMCID: PMC9774294.

204. Washam CJ, Sandine WE, Elliker PR. A Strain of Pseudomonas aeruginosa Resistant to a Quaternary Ammonium Compound. Journal of Food Protection. 1976;39(2):101-6. doi: https://doi.org/10.4315/0022-2747-39.2.101.

205. DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. Synergistic interactions of Pseudomonas aeruginosa and Staphylococcus aureus in an in vitro wound model.

Infect Immun. 2014;82(11):4718-28. Epub 20140825. doi: 10.1128/IAI.02198-14. PubMed PMID: 25156721; PMCID: PMC4249327.

206. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection. Proc Natl Acad Sci U S A. 2013;110(3):1059-64. Epub 20121231. doi: 10.1073/pnas.1214550110. PubMed PMID: 23277552; PMCID: PMC3549110.

207. Shoaib A, Lone NA, Yi X. Co-infection with Pseudomonas aeruginosa impacts virulence of Staphylococcus aureus and intensifies the severity of infection. Pak J Pharm Sci. 2020;33(5):1933-7. PubMed PMID: 33824098.

208. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol. 2010;8(1):15-25. doi: 10.1038/nrmicro2259. PubMed PMID: 19946288; PMCID: PMC2879262.

209. Sandoz KM, Mitzimberg SM, Schuster M. Social cheating in Pseudomonas aeruginosa quorum sensing. Proc Natl Acad Sci U S A. 2007;104(40):15876-81. Epub 20070926. doi: 10.1073/pnas.0705653104. PubMed PMID: 17898171; PMCID: PMC2000394.

210. Chen R, Deziel E, Groleau MC, Schaefer AL, Greenberg EP. Social cheating in a Pseudomonas aeruginosa quorum-sensing variant. Proc Natl Acad Sci U S A. 2019;116(14):7021-6. Epub 20190307. doi: 10.1073/pnas.1819801116. PubMed PMID: 30846553; PMCID: PMC6452681.

211. Hespanhol JT, Sanchez-Limache DE, Nicastro GG, Mead L, Llontop EE, Chagas-Santos G, Farah CS, de Souza RF, Galhardo RDS, Lovering AL, Bayer-Santos E. Antibacterial T6SS effectors with a VRR-Nuc domain are structure-specific nucleases. Elife. 2022;11. Epub 20221013. doi: 10.7554/eLife.82437. PubMed PMID: 36226828; PMCID: PMC9635880.

212. Bayer-Santos EaS-L, D.E. . Interbacterial competition assay. Bio-protocol Preprint. 2023.

213. Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. J Bacteriol. 2000;182(13):3843-5. doi: 10.1128/JB.182.13.3843-3845.2000. PubMed PMID: 10851003; PMCID: PMC94559.

214. Andrea A, Krogfelt KA, Jenssen H. Methods and Challenges of Using the Greater Wax Moth (Galleria mellonella) as a Model Organism in Antimicrobial Compound Discovery. Microorganisms. 2019;7(3). Epub 20190319. doi: 10.3390/microorganisms7030085. PubMed PMID: 30893839; PMCID: PMC6463077.

215. LaBauve AE, Wargo MJ. Growth and laboratory maintenance of Pseudomonas aeruginosa. Curr Protoc Microbiol. 2012;Chapter 6:Unit 6E 1. doi: 10.1002/9780471729259.mc06e01s25. PubMed PMID: 22549165; PMCID: PMC4296558.

216. Palmer KL, Aye LM, Whiteley M. Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. J Bacteriol. 2007;189(22):8079-87. Epub 20070914. doi: 10.1128/JB.01138-07. PubMed PMID: 17873029; PMCID: PMC2168676.

217. Chotirmall SH, Smith SG, Gunaratnam C, Cosgrove S, Dimitrov BD, O'Neill SJ, Harvey BJ, Greene CM, McElvaney NG. Effect of estrogen on pseudomonas mucoidy and exacerbations in cystic fibrosis. N Engl J Med. 2012;366(21):1978-86. Epub 20120520. doi: 10.1056/NEJMoa1106126. PubMed PMID: 22607135.

218. Cross AR, Goldberg JB. Remodeling of O Antigen in Mucoid Pseudomonas aeruginosa via Transcriptional Repression of wzz2. mBio. 2019;10(1). Epub 20190219. doi: 10.1128/mBio.02914-18. PubMed PMID: 30782665; PMCID: PMC6381286.

219. Tseng CL, Leng CH. Influence of medium components on the expression of recombinant lipoproteins in Escherichia coli. Appl Microbiol Biotechnol. 2012;93(4):1539-52. Epub 20110806. doi: 10.1007/s00253-011-3516-8. PubMed PMID: 21822641.

220. Cullen L, Weiser R, Olszak T, Maldonado RF, Moreira AS, Slachmuylders L, Brackman G, Paunova-Krasteva TS, Zarnowiec P, Czerwonka G, Reilly J, Drevinek P, Kaca W, Melter O, De Soyza A, Perry A, Winstanley C, Stoitsova SR, Lavigne R, Mahenthiralingam E, Sa-Correia I, Coenye T, Drulis-Kawa Z, Augustyniak D, Valvano MA, McClean S. Phenotypic characterization of an international Pseudomonas aeruginosa reference panel: strains of cystic fibrosis (CF) origin show less in vivo virulence than non-CF strains. Microbiology (Reading). 2015;161(10):1961-77. Epub 20150806. doi: 10.1099/mic.0.000155. PubMed PMID: 26253522.

221. Mashburn LM, Jett AM, Akins DR, Whiteley M. Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. J Bacteriol. 2005;187(2):554-66. doi: 10.1128/JB.187.2.554-566.2005. PubMed PMID: 15629927; PMCID: PMC543556.

222. Hoegy F, Mislin GL, Schalk IJ. Pyoverdine and pyochelin measurements. Methods Mol Biol. 2014;1149:293-301. doi: 10.1007/978-1-4939-0473-0_24. PubMed PMID: 24818914.

223. Taylor IR, Paczkowski JE, Jeffrey PD, Henke BR, Smith CD, Bassler BL. Inhibitor Mimetic Mutations in the Pseudomonas aeruginosa PqsE Enzyme Reveal a Protein-Protein Interaction with the Quorum-Sensing Receptor RhlR That Is Vital for Virulence Factor Production. ACS Chem Biol. 2021;16(4):740-52. Epub 20210401. doi: 10.1021/acschembio.1c00049. PubMed PMID: 33793200; PMCID: PMC8056388.

224. Sagripanti JL, Bonifacino A. Resistance of Pseudomonas aeruginosa to liquid disinfectants on contaminated surfaces before formation of biofilms. J AOAC Int. 2000;83(6):1415-22. PubMed PMID: 11128146.

225. Qazi MJ, Schlegel SJ, Backus EHG, Bonn M, Bonn D, Shahidzadeh N. Dynamic Surface Tension of Surfactants in the Presence of High Salt Concentrations. Langmuir. 2020;36(27):7956-64. Epub 20200702. doi: 10.1021/acs.langmuir.0c01211. PubMed PMID: 32545966; PMCID: PMC7366510.

226. Hittle LE, Powell DA, Jones JW, Tofigh M, Goodlett DR, Moskowitz SM, Ernst RK. Site-specific activity of the acyltransferases HtrB1 and HtrB2 in Pseudomonas aeruginosa lipid A biosynthesis. Pathog Dis. 2015;73(8):ftv053. Epub 20150729. doi: 10.1093/femspd/ftv053. PubMed PMID: 26223882; PMCID: PMC4626592.

227. Ye J, Wu H, Zhang H, Wu Y, Yang J, Jin X, Shi X. Role of benzalkonium chloride in DNA strand breaks in human corneal epithelial cells. Graefes Arch Clin Exp Ophthalmol. 2011;249(11):1681-7. Epub 20110813. doi: 10.1007/s00417-011-1755-0. PubMed PMID: 21842132.

228. Ferk F, Misik M, Hoelzl C, Uhl M, Fuerhacker M, Grillitsch B, Parzefall W, Nersesyan A, Micieta K, Grummt T, Ehrlich V, Knasmuller S. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. Mutagenesis. 2007;22(6):363-70. Epub 20070726. doi: 10.1093/mutage/gem027. PubMed PMID: 17656635.

229. Dowhan W. A retrospective: use of Escherichia coli as a vehicle to study phospholipid synthesis and function. Biochim Biophys Acta. 2013;1831(3):471-94. Epub 20120814. doi: 10.1016/j.bbalip.2012.08.007. PubMed PMID: 22925633; PMCID: PMC3513495.

230. Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plesiat P. MexXY-OprM efflux pump is necessary for a adaptive resistance of Pseudomonas aeruginosa to

aminoglycosides. Antimicrob Agents Chemother. 2003;47(4):1371-5. doi: 10.1128/AAC.47.4.1371-1375.2003. PubMed PMID: 12654672; PMCID: PMC152483.

231. Wu-Chen RA, Feng J, Elhadidy M, Nambiar RB, Liao X, Yue M, Ding T. Benzalkonium chloride forces selective evolution of resistance towards antibiotics in Salmonella enterica serovar Typhimurium. J Infect Public Health. 2023;16 Suppl 1:225-35. Epub 20231028. doi: 10.1016/j.jiph.2023.10.022. PubMed PMID: 37935604.

232. Krulwich TA, Hicks DB, Ito M. Cation/proton antiporter complements of bacteria: why so large and diverse? Mol Microbiol. 2009;74(2):257-60. Epub 20090811. doi: 10.1111/j.1365-2958.2009.06842.x. PubMed PMID: 19682259; PMCID: PMC2765581.

233. Tandukar M, Oh S, Tezel U, Konstantinidis KT, Pavlostathis SG. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. Environ Sci Technol. 2013;47(17):9730-8. Epub 20130819. doi: 10.1021/es401507k. PubMed PMID: 23924280.

234. Guest RL, Lee MJ, Wang W, Silhavy TJ. A periplasmic phospholipase that maintains outer membrane lipid asymmetry in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2023;120(30):e2302546120. Epub 20230718. doi: 10.1073/pnas.2302546120. PubMed PMID: 37463202; PMCID: PMC10374164.

Appendix

PDF files of:

1. M. S. Leatherbury, L. M. Thierer, C. A. Sanchez, **G. G. Vargas-Cuebas**, A. A. Petersen, L. E. Amoo, E. L. Bezold, K. C. Washington, M. B. Mistrot, M. J. Zdilla, W. M. Wuest, K. P. C. Minbiole. Chimeric Amphiphilic Disinfectants: Quaternary Ammonium/Quaternary Phosphonium Hybrid Structures. *ChemMedChem* 2024, 19, e202300718.

2. Z. E. A. Toles, L. M. Thierer, A. Wu, E. L. Bezold, D. Rachii, C. A. Sanchez, **G. G. Vargas-Cuebas**, T. M. Keller, P. J. Carroll, W. M. Wuest, K. P. C. Minbiole. Bushy-Tailed QACs: The Development of Multicationic Quaternary Ammonium Compounds with a High Degree of Alkyl Chain Substitution. *ChemMedChem* 2024, 19, e202400301.

Contribution(s):

For both articles, I contributed to the evaluation of the biological activity of the synthetized compounds for both manuscripts, alongside C.A.S. and E.L.B. These data are presented in Table 1 of each article.

ChemMedChem

Research Article
doi.org/10.1002/cmdc.202300718

Chemistry Europe
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Chimeric Amphiphilic Disinfectants: Quaternary Ammonium/Quaternary Phosphonium Hybrid Structures

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Cationic biocides play a crucial role in the disinfection of domestic and healthcare surfaces. Due to the rise of bacterial resistance towards common cationic disinfectants like quaternary ammonium compounds (QACs), the development of novel actives is necessary for effective infection prevention and control. Toward this end, a series of 15 chimeric biscationic amphiphilic compounds, bearing both ammonium and phosphonium residues, were prepared to probe the structure and efficacy of mixed cationic ammonium-phosphonium structures. Compounds were obtained in two steps and good yields, with straightforward and chromatography-free purifications. Antibacterial activity evaluation of these compounds against a panel of seven bacterial strains, including two MRSA strains as well as opportunistic pathogen *A. baumannii*, were encouraging, as low micromolar inhibitory activity was observed for multiple structures. Alkyl chain length on the ammonium group was, as expected, a major determinant of bioactivity. In addition, high therapeutic indexes (up to 125-fold) for triphenyl phosphonium-bearing amphiphiles were observed when comparing antimicrobial activity to mammalian cell lysis activity.

Introduction

There has been a resurgence of interest in the development of novel amphiphilic disinfectants in recent years,[1,2] inspired by the diminishing efficacy of commonplace quaternary ammonium compound (QAC) structures[3] as well as increased concerns about the health hazards of currently used QACs.[4,5] Bacterial resistance to QACs, first identified in the 1990s,[6] is understood to result from a combination of mechanisms, such as efflux pumps, enzymatic degradation, biofilm formation, and compositional changes to the bacterial cell membrane.[7] Benzalkonium chloride (BAC, Figure 1) is a ubiquitous QAC used in home, healthcare, and industrial settings. With a 9-month half-life in environmental conditions, sub-inhibitory concentrations accumulate in the environment, resulting in increasing levels of bacterial resistance.[8,9]

Research into developing novel QAC structures to increase activity and to overcome concerning resistance mechanisms is warranted.[1,10] These campaigns have included broad architectural variations,[1,11] pushing the envelope of amphiphilic architecture by varying alkyl chain count and length, number and nature of cationic residues, and molecular platform. The development of "soft" antimicrobials,[12–14] designed to decompose upon environmental exposure and thus impede resistance development, has also been pursued, and shows promise to deliver both efficacy and diminished long-term environmental impacts.

A particularly promising development has been the renewed interest in quaternary phosphonium compounds (QPCs), which had been pioneered in a systematic investigation in the 1990s by Endo and coworkers (Figure 1, center).[15] While QPCs have found industrial applications and utility in enhancing the activity and transport of select bioactive agents,[16–17] the development of QPCs as standalone disinfectants or sanitizing agents is still in its infancy. Fortunately, multicationic QPCs are being actively explored by our group[18–21] and others.[22–23] Recently, these QPCs have been shown to have privileged properties against otherwise pan-drug resistant strains of *Acinetobacter baumannii*,[24] outperforming a variety of established disinfectants and potent antibiotics. QPCs hold the promise to significantly change the field of cationic disinfectants, presenting structural novelty to forestall bacterial resistance.

One tried-and-true strategy for the development of disinfectants in the face of struggles with resistance is a combination approach. For example, benzalkonium chloride is prepared as a mixture, similar to antibiotic combinations like Augmentin (amoxicillin/clavulanic acid) and Bactrim (sulfamethoxazole/trimethoprim) and antiviral cocktails, which take advantage of a multifold approach to pathogen eradication. We observed little

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202300718

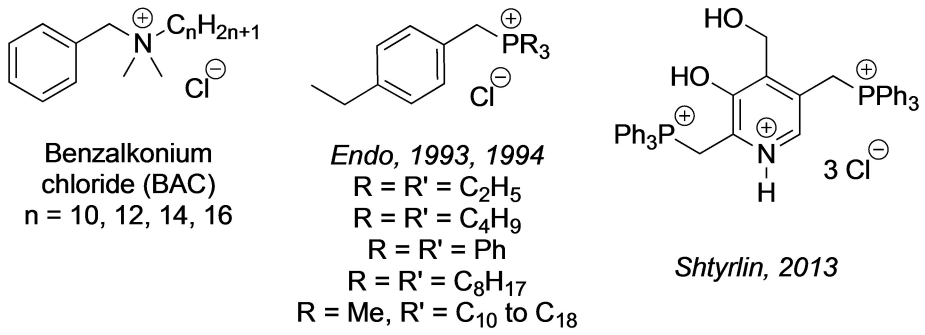
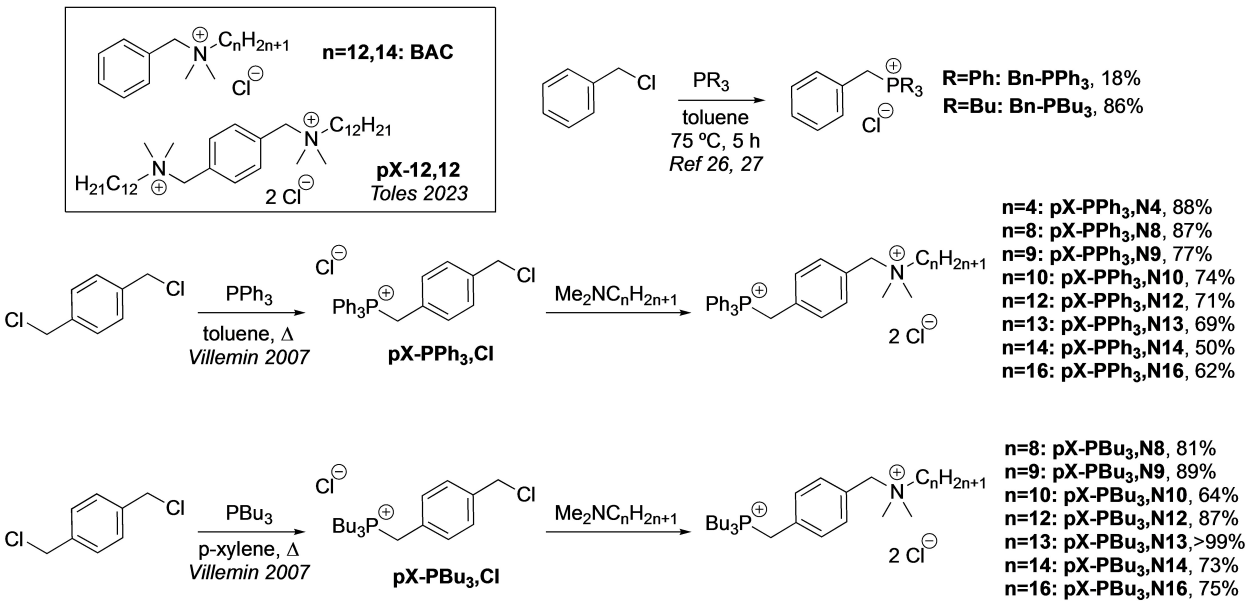


Figure 1. Key structures in quaternary cationic disinfectants.

precedent for discrete disinfectant amphiphiles that possess both an ammonium group and a quaternary phosphonium group, thus representing a QAC/QPC chimera; one literature inspiration from Shtyrlin and coworkers is shown in Figure 1.[25] Chimeric compounds could take advantage of the bioactivity of both the QPC and QAC families, by presenting bacteria with two distinct "warhead" groups to stymie resistance mechanisms. Even subtle bioactivity differences between these two cationic groups, which we suspect to arise from previously-unmeasured charge density differences,[21] could exploit weaknesses in bacterial defenses. Accordingly, we set out to develop a series of chimeric molecules, amphiphiles that include both quaternary ammonium and quaternary phosphonium residues, in order to examine their antimicrobial capacities in comparison to amphiphiles of a single cationic element. Thus, starting with a bifunctional electrophile core (α,α′-dichloro-p-xylene), we aimed to install a variety of functionality to probe the utility of chimeric QAC-QPC structures.

Results and Discussion

After setting out to prepare two known monocationic control compounds (Bn-PPh_3[26] and Bn-PBu_3,[27] Scheme 1 top), and informed by a biscationic QAC (bisQAC, with two ammonium groups) analog pX-12,12,[28] a series of biscationic chimeric amphiphiles was prepared. Accordingly, α,α′-dichloro-p-xylene was exposed to an excess of either triphenylphosphine or tributylphosphine, modifying a precedent of Villemin,[29] to afford pX-PPh_3,Cl and pX-PBu_3,Cl respectively (Scheme 1). The use of two common phosphine nucleophiles ensures a level of structural diversity around the phosphorus center, and reaction conditions exploit the poor solubility of prepared salts in toluene or p-xylene to ensure monoalkylation. Subsequent exposure of the intermediate mono benzyl chloride to a dimethylalkyl amine of varied chain lengths at elevated temperatures led to the preparation of a series of 16 chimeric biscationic amphiphiles in good yields (50–99%). Compounds built on this p-xylene scaffold were dubbed either pX-PPh_3,N# or pX-Pbu_3,N#, according to the number (#) of carbons in the long-chained alkyl amine.



Scheme 1. Synthetic strategy for formation of chimeric amphiphiles.

Purification was accomplished via simple trituration, affording spectroscopically pure compounds in relatively short order.

Structural analysis of a sample chimeric biscationic amphiphile, the short-chained pX-PPh_3N_4, was accomplished via X-ray crystallography, which identified the dihemihydrate of the chloride salt, [pX-PPh_3N_4]$Cl_2 \cdot 2.5\ H_2O$, and provides insight into the differentiation of the phosphonium and ammonium centers in the same amphiphilic compound. While the packed structure, bond lengths, and other structural details are presented in the Supporting Information, a thermal ellipsoid plot is shown in Figure 2, and displays an extended structure. We investigated charge distribution via a highly accurate structure refinement using non-spherical formfactors calculated using the program NoSpherA2[30] based on the single-point electronic structure calculated at the B3LYP[31]/6-311G(d,p)[32] level of theory. Hirshfeld charges of the atoms from the refined structure (Table S5) reveal that the charge of the phosphonium moiety is almost entirely relegated to the phosphorus atom, which has a charge of +0.397. Some of this charge is delocalized onto the neighboring methylene group (e.g., C19 and associated H atoms, +0.063). Very little charge is delocalized onto the three phenyl groups, whose charge sums to only +0.081 across all three phenyl groups. In contrast, the ammonium moiety delocalizes more of its charge onto the neighboring methyl and methylene groups. The nitrogen atom bears the largest charge of any individual atom (+0.091), though its methylene neighbors sum to a charge of +0.252 collectively, and the methyl groups sum to +0.332. The findings suggest that, while the heteroatoms share positive charge predominantly with their α neighbors through a very localized inductive effect, the localization of the cationic charge differs greatly from phosphorus to nitrogen. This is consistent with previous NMR chemical shift observations.[21]

The bioactivities of the prepared cationic amphiphilic compounds were assessed via minimum inhibitory concentration (MIC) and red blood cell (RBC) hemolysis ($lysis_{20}$) assays, wherein the latter was used as a proxy for cytotoxicity. A representative commercial preparation of BAC (70% benzyldimethyldodecylammonium chloride and 30% benzyldimethyltetradecylammonium chloride) was tested as a control; the previously reported pX-12,12 served as a bisQAC reference for these chimeric compounds. To determine the MIC values, the compounds were each screened against a panel of seven bacterial strains, including four Gram-positive strains (methicillin-susceptible *Staphylococcus aureus* [MSSA; SH1000], community-acquired methicillin-resistant *S. aureus* [CA-MRSA; USA 300–0114], hospital-acquired methicillin-resistant *S. aureus* [HA-MRSA; ATCC 33591], and *Enterococcus faecalis* [OG1RF]), as well as three Gram-negative strains (*Escherichia coli* [MC4100], *Acinetobacter baumannii* [ATCC 17978], and *Pseudomonas aeruginosa* [PAO1]). To evaluate the hemolysis activities, the compound concentrations resulting in 20% RBC lysis ($lysis_{20}$) were determined. The results of the MIC and hemolysis assays are presented in Table 1.

Several key themes emerged in our bioactivity analysis. Of primary importance, about half of the chimeric compounds produced in this effort showed inhibitory activity superior to that of BAC, whose MIC was only below 16 μM in Gram-positive MSSA and CA-MRSA. In contrast, the synthesized chimeric compounds bearing alkyl chains of at least 12 carbons on the ammonium residue showed promising low-micromolar activity. Of the compounds that showed single-digit micromolar activity against

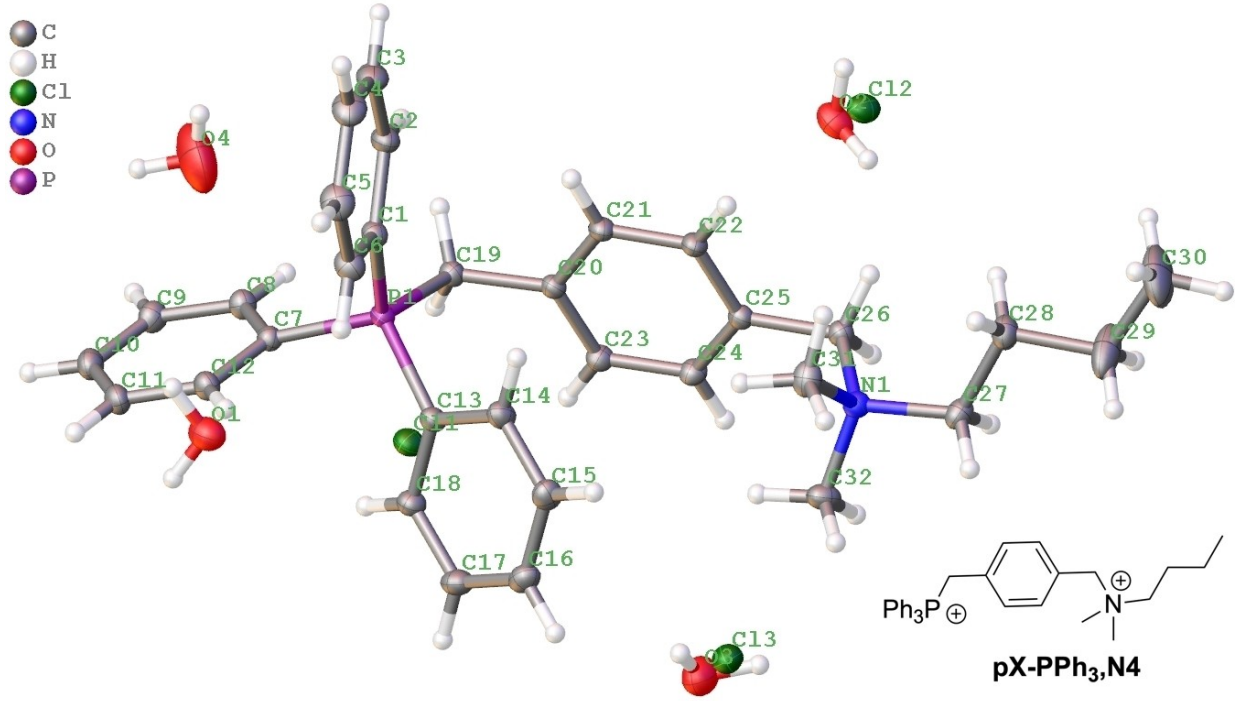


Figure 2. Thermal ellipsoid plot of [pX-PPh_3N_4]$Cl_2 \cdot 2.5\ H_2O$ with both well-ordered and occupationally disordered Cl^- and water sites. Ellipsoids set at 50% probability level. Calculations indicate that the phosphorus atom bears a Hirshfeld charge of +0.397, and the nitrogen atom bears a charge of +0.091.

Table 1. Biological activity and lysis$_{20}$ data of synthesized compounds and BAC control. Darker colors indicate preferential performance. Therapeutic indices are presented in the Supporting Information.

	MIC (μM)							
Compound	**MSSA**	**CA-MRSA**	**HA-MRSA**	***E. facaelis***	***E. coli***	***A. baumannii***	***P. aeruginosa***	**Lysis$_{20}$**
BAC	2	2	32	32	32	16	125	63
pX-12,12	1	16	2	4	2	4	4	8
Bn-PPh$_3$	125	63	>250	>250	>250	>250	>250	250
Bn-PBu$_3$	>250	63	>250	>250	>250	>250	>250	250
pX-PBu$_3$Cl	>250	>250	>250	>250	>250	>250	>250	250
pX-PPh$_3$Cl	>250	>250	>250	>250	>250	>250	>250	250
pX-PPh$_3$,N8	125	125	>250	>250	>250	>250	>250	250
pX-PPh$_3$,N9	32	63	>250	250	250	>250	>250	250
pX-PPh$_3$,N10	16	32	125	125	125	250	>250	250
pX-PPh$_3$,N12	2	4	16	8	8	63	32	250
pX-PPh$_3$,N13	2	2	4	4	4	32	32	32
pX-PPh$_3$,N14	1	1	2	2	2	8	16	125
pX-PPh$_3$,N16	1	1	2	2	2	2	8	8
pX-PBu$_3$,N8	250	>250	>250	>250	>250	>250	>250	250
pX-PBu$_3$,N9	125	250	>250	>250	>250	>250	>250	250
pX-PBu$_3$,N10	63	63	>250	>250	>250	>250	>250	250
pX-PBu$_3$,N12	4	8	32	32	32	250	63	250
pX-PBu$_3$,N13	2	8	16	8	8	63	32	125
pX-PBu$_3$,N14	1	8	4	4	4	16	16	63
pX-PBu$_3$,N16	1	16	2	2	2	4	8	8

most of the bacterial panel, three novel structures (pX-PPh$_3$-N14, pX-PPh$_3$-N16, and pX-PBu$_3$-N16) were comparable to the previously reported non-chimeric, bisQAC pX-12,12 (Figure 1). The strongest overall activity was observed for pX-PPh$_3$-N16, with MIC values of 1–2 μM in six strains and an MIC of 8 μM in *A. baumannii*. Surprisingly, the increase in chain length was the greatest determinant for bioactivity, and the hexadecyl chain represents an optimum in amphiphilicity for this series. Most precedents have favored chains of ~10–12 carbons in length and noted a decrease in performance at these longer chain lengths, which is observed with the bisQAC pX-12,12,[28] as well as monoQPC[15] and bisQPC[18] structures. Impedance to the formation of micelles or other supramolecular structures, brought about by this mixed QAC/QPC system, may allow longer-chained compounds to exist in solution as monomers and thus preserve their antimicrobial potency despite their longer chains.

When comparing the triphenyl phosphine series and the tributyl phosphine series, the triphenyl compounds are observed to have roughly a two-fold advantage in activity over tributyl analogs. This observation is more pronounced when focusing on bacteria with resistance mechanisms; the triphenyl phosphine analogs display minimal loss of activity when comparing MSSA to the resistant CA-MRSA and HA-MRSA analogs. In contrast, the tributyl analogs demonstrate an ~8-fold decline in activity against the MRSA strains. This is consistent with the small antimicrobial activity observed for BnPPh$_3$ versus the inactive BnPBu$_3$, and might be a reflection of variations of the significant charge density on the cationic phosphorus center discussed above. More specifically, ^{31}P NMR data for the tributyl analogs display a phosphorus chemical shift of ~32.5 ppm, in contrast with an observed shift of ~24.4 ppm for the triphenyl compounds. This might reflect the small calculated spread of cationic charge throughout the aromatic systems, which may in turn lead to increased resistance-to-resistance, although a phenyl advantage is not consistent with our previous reports of highly potent alkyl-based QPCs.[21]

Red blood lysis values also provide insight into the selective bioactivity of these antimicrobial amphiphiles. Lysis$_{20}$ values were observed to be a modest 250 μM for all compounds with chains of 12 carbons or shorter. While the strongest antimicrobial activity is observed for the hexadecylammonium compounds, the therapeutic index (TI) for the 14-carbon chains is compelling. The therapeutic index, which is defined as the ratio of RBC lysis$_{20}$ divided by MIC values, ranges from 8 to 125 for pX-PPh$_3$-N14, which displays a favorable lysis$_{20}$ value of 125 μM. This compares well to the observed therapeutic index of BAC, whose moderate lysis$_{20}$ value (63 μM) leads to TI ratios of 2 to 32. A full table of therapeutic indices are presented in the Supporting Information.

In conclusion, a variety of chimeric biscationic amphiphilic compounds bearing both ammonium and phosphonium residues were synthesized to explore the molecular structure and efficacy of mixed cationic amphiphilic structures. These chimeric compounds present strong (low micromolar) inhibitory activity against highly resistant bacteria, and promising lysis$_{20}$-based

therapeutic indexes were observed. While no particular advantages were seen in the MIC values for these chimeric QAC/QPC structures compared to our biscationic QACs control, we are encouraged that the best performance was observed with the longer hexadecyl chain compounds. This marked deviation in optimal chain length from many previously reported bisQAC and our bisQPC compounds is a tantalizing indication that these chimeric compounds are structurally unique, and may suggest unanticipated supramolecular effects. Therefore, we aim to continue to interrogate the role of chimeric quaternary cationic amphiphiles in the inhibition of highly resistant strains of bacteria.

Funding Sources

Funding for this work was provided by the National Institute of General Medical Sciences (R35GM119426 to W.M.W.; T32GM008602 to C.A.S.; F31 F31DK126467 to G.G.V.-C.), and from the National Science Foundation (Major Research Instrumentation grants CHE-1827930 and CHE-2018399 to K.P.C.M., and CHE-2215854 to M.J.Z.)

Acknowledgements

The authors wish to thank Florian Kleemis for advice on the use of NoSpherA2.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Cationic Biocide · Amphiphiles · Chimera · Disinfectants · Quaternary Phosphonium Compounds · QPC

[1] W. Dan, J. Gao, X. Qi, J. Wang, J. Dai, *Eur. J. Med. Chem.* **2022**, *243*, 114765.
[2] N. Frolov, E. Detusheva, N. Fursova, I. Ostashevskaya, A. Vereshchagin, *Pharmaceuticals* **2022**, *15*, 514.
[3] J. M. Boyce, *Antimicrob. Resist. Infect. Control* **2023**, *12*, 1–14.
[4] S. Mohapatra, L. Yutao, S. G. Goh, C. Ng, Y. Luhua, N. H. Tran, K. Y. H. Gin, *J. Hazard. Mater.* **2023**, *445*, 130393.
[5] W. A. Arnold, A. Blum, J. Branyan, T. A. Bruton, C. C. Carignan, G. Cortopassi, S. Datta, J. DeWitt, A. C. Doherty, R. U. Halden, H. Harari, *Environ. Sci. Technol.* **2023**, *57*, 7645–7665.
[6] a) I. T. Paulsen, M. H. Brown, R. A. Skurray, *Microbiol. Rev.* **1996**, *60*, 575–608; b) B. A. Mitchell, M. H. Brown, R. A. Skurray, *Antimicrob. Agents Chemother.* **1998**, *42*, 475–477; c) M. H. Brown, R. A. Skurray, *J. Mol. Microbiol. Biotechnol.* **2001**, *3*, 163–170.
[7] M. C. Jennings, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2015**, *1*, 288–303.
[8] C. Zhang, F. Cui, G. M. Zeng, M. Jiang, Z. Z. Yang, Z. G. Yu, M. Y. Zhu, L. Q. Shen, *Sci. Total Environ.* **2015**, *518*, 352–362.
[9] Y. Jia, H. Lu, L. Zhu, *Sci. Total Environ.* **2022**, *832*, 155090.
[10] E. A. Saverina, N. A. Frolov, O. A. Kamanina, V. A. Arlyapov, A. N. Vereshchagin, V. P. Ananikov, *ACS Infect. Dis.* **2023**, *9*, 394–422.
[11] K. R. Morrison, R. A. Allen, K. P. C. Minbiole, W. M. Wuest, *Tetrahedron Lett.* **2019**, *60*, 150935.
[12] N. Bodor, J. Kaminski, S. Selk, *J. Med. Chem.* **1980**, *23*, 469–474.
[13] J. Haldar, P. Kondaiah, S. Bhattacharya, *J. Med. Chem.* **2005**, *48*, 3823–3831.
[14] R. A. Allen, M. C. Jennings, M. A. Mitchell, S. E. Al-Khalifa, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2017**, *27*, 2107–2112.
[15] a) A. Kanazawa, T. Ikeda, T. Endo, *J. Polym. Sci. Part A* **1993**, *31*, 335–343; b) A. Kanazawa, T. Ikeda, T. Endo, *Antimicrob. Agents Chemother.* **1994**, *38*, 945–952.
[16] J. Wang, J. Li, Y. Xiao, B. Fu, Z. Qin, *RSC Med. Chem.* **2020**, *11*, 858– 875.
[17] M. P. Murphy, R. A. Smith, *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 629–656.
[18] K. J. Sommers, M. E. Michaud, C. E. Hogue, A. M. Scharnow, L. E. Amoo, A. A. Petersen, R. G. Carden, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2022**, *8*, 387–397.
[19] A. C. Spahr, M. E. Michaud, L. E. Amoo, C. A. Sanchez, C. E. Hogue, L. M. Thierer, M. R. Gau, W. M. Wuest, K. P. C. Minbiole, *ChemMedChem* **2022**, *17*, e202200224.
[20] S. R. Brayton, Z. E. A. Toles, C. A. Sanchez, M. E. Michaud, L. M. Thierer, T. M. Keller, C. J. Risener, C. L. Quave, W. M. Wuest, K. P. C. Minbiole. *ACS Infectious Disease*, **2023**, *9*, 943–951.
[21] L. M. Thierer, A. A. Petersen, M. E. Michaud, C. A. Sanchez, S. R. Brayton, W. M. Wuest,K. P. C. Minbiole* *ACS Infectious Disease*, **2023**, *9*, 609–616.
[22] V. V. Ermolaev, D. M. Arkhipova, V. A. Miluykov, A. P. Lyubina, S. K. Amerhanova, N. V. Kulik, A. D. Voloshina, V. P. Ananikov, *Int. J. Mol. Sci.* **2021**, *23*, 86.
[23] L. Li, H. Zhou, F. Gai, X. Chi, Y. Zhao, F. Zhang, Z. Zhao, *RSC Adv.* **2017**, *7*, 13244–13249.
[24] M. E. Michaud, R. A. Allen, K. R. Morrison-Lewis, C. A. Sanchez, K. P. Minbiole, S. J. Post, W. M. Wuest, *ACS Infect. Dis.* **2022**, *8*, 2307–2314.
[25] M. V. Pugachev, N. V. Shtyrlin, L. P. Sysoeva, E. V. Nikitina, T. I. Abdullin, A. G. Iksanova, A. A. Ilaeva, R. Z. Musin, E. A. Berdnikov, Y. G. Shtyrlin, *Bioorg. Med. Chem.* **2013**, *21*, 4388–4395.
[26] B. De Filippis, P. Linciano, A. Ammazzalorso, C. Di Giovanni, M. Fantacuzzi, L. Giampietro, A. Laghezza, C. Maccallini, P. Tortorella, A. Lavecchia, F. Loiodice, R. Amoroso, *Eur. J. Med. Chem.* **2015**, *89*, 817–825.
[27] T. Yan, K. R. Babu, Y. Wu, Y. Li, Y. Tang, S. Xu, *Org. Lett.* **2021**, *23*, 4570–4574.
[28] Z. E. A. Toles, A. Wu, C. A. Sanchez, M. E. Michaud, L. M. Thierer, W. M. Wuest, K. P. C. Minbiole. *ChemMedChem*, **2023**, *18*, e202300018.
[29] D. Villemin, M. A. Didi, B. Makhoukhi, *Lett. Org. Chem.* **2007**, *4*, 509–511.
[30] F. Kleemiss, O. V. Dolomanov, M. Bodensteiner, N. Peyerimhoff, L. Midgley, L. J. Bourhis, A. Genoni, L. A. Malaspina, D. Jayatilaka, J. L. Spencer, F. White, *Chem. Sci.* **2021** *12*, 1675–1692.
[31] A. D. Becke, *J. Chem. Phys.* **1992** *96*, 2155–2160.
[32] a) J. P. Blaudeau, M. P. McGrath, L. A. Curtiss, L. Radom, *J. Chem. Phys.* **1997**, *107*, 5016–5021; b) R. B. J. S. Krishnan, J. S. Binkley, R. Seeger, J. A. Pople, *J. Chem. Phys.* **1980**, *72*, 650–654.

Manuscript received: December 20, 2023
Revised manuscript received: February 22, 2024
Accepted manuscript online: February 28, 2024
Version of record online: March 14, 2024

ChemMedChem

Research Article
doi.org/10.1002/cmdc.202400301

Chemistry Europe
European Chemical Societies Publishing

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Bushy-Tailed QACs: The Development of Multicationic Quaternary Ammonium Compounds with a High Degree of Alkyl Chain Substitution

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Quaternary ammonium compounds have served as a first line of protection for human health as surface disinfectants and sanitizers for nearly a century. However, increasing levels of bacterial resistance have spurred the development of novel QAC architectures. In light of the observed reduction in eukaryotic cell toxicity when the alkyl chains on QACs are shorter in nature ($\leq$10 C), we prepared 47 QAC architectures that bear multiple short alkyl chains appended to up to three cationic groups, thus rendering them "bushy-tailed" multiQACs. Antibacterial activity was strong (often ~1–4 μM) in a varied set of bushy-tailed architectures, though observed therapeutic indices were not significantly improved over QAC structures bearing fewer and longer alkyl chains.

Introduction

Quaternary ammonium compounds have served as disinfectants for nearly a century, and appear in a wide variety of household products, as well as in industrial and medical applications. Benzalkonium chloride or BAC (Figure 1), first registered in 1947, is commonly found in wipes and spray disinfectants, as well as in oral canker sore medications.[1,2,3] Dimethyl didecyl ammonium chloride (DDAC) was approved in 1962[4] and remains a lead compound in disinfection; cetyl trimethyl ammonium (cetrimonium) chloride and its bromide analog have similarly straightforward alkyl structures. Benzethonium chloride, akin to BAC but with a significantly substituted non-polar region, is used as a disinfectant in food processing plants. Cetylpyridinium chloride, which adds a modest structural modification to include a pyridyl substitution, is commonplace in mouthwashes. All of these QAC structures enjoy straightforward synthetic routes and good molecular stability, giving them advantages in the marketplace.[5]

Unfortunately, the relatively uniform structural palette of modern QACs, which generally rely on a single cationic center and a single nonpolar chain to attach to and subsequently disrupt bacterial cell membranes, have allowed for an unfortunate outcome – the development of bacterial resistance.[6,7] An increasing fraction of human pathogens being encountered in hospitals are found to possess resistance mechanisms, such as efflux pumps[8,9,10] modification of membrane composition,[11] and biodegradation,[12] that minimize the efficacy of these disinfectants. More alarmingly, co-resistance to antibiotics is often conferred with these genetic elements. For example, increased expression of TolC efflux pumps has been associated with QAC and antibiotic cross-resistance in gram-negative bacterial species.[13] In gram-positive species, *qac* disinfectant resistance genes have been identified on plasmids also harboring beta-lactam, aminoglycoside, trimethoprim, and bleomycin resistance genes.[14] The current state of QAC resistance highlights the need for innovation in the field in order to remain a potent class of disinfectants.

The historical tendency towards comparable monocationic structures in commercially available QAC disinfectants has spurred a number of synthetic campaigns in the last decade or so into significantly varied QAC structures, which often employ multicationic QACs, or multiQACs. These multiQACs have shown

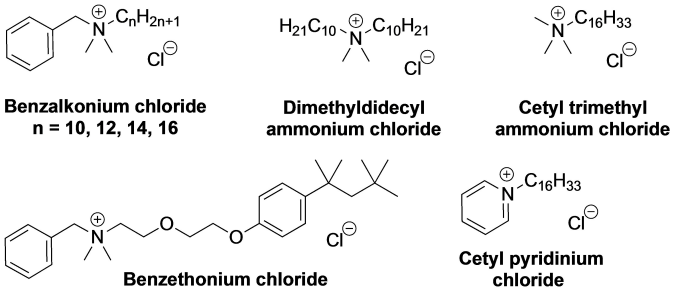

Figure 1. Commonplace quaternary ammonium compounds.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202400301

promise to overcome bacterial resistance mechanisms. Increasingly frequent reports have investigated the structure-activity relationships of compounds having two to four cationic centers, the molecular architecture separating those centers, and the non-polar groups that are attached to them. A small sampling of such novel QAC structures from a variety of research groups is presented in Figure 2,[15-21] and readers are pointed to more through reviews on this topic.[22-27] A number of these novel QAC structures, as well as their phosphonium analogs, show excellent bioactivity, and oftentimes, resistance to resistance. Thus, despite a relative dearth of novel QAC structures entering the marketplace in recent years, long-term development prospects seem strong.

Through dedicated efforts in our own laboratory, where we have constructed ~850 novel QAC and quaternary phosphonium compound (QPC) structures, several key findings have emerged. First, the utilization of QAC structures bearing two cationic centers shows superior bioactivity, particularly against resistant bacteria, as compared to monocationic amphiphiles, but diminishing effects are noted in adding more than two ammonium centers.[23,26] Secondly, the use of a phosphonium residue in place of (and at times, in addition to)[28] an ammonium center can broaden bacterial inhibition. Further, observed optimal bioactivity tends to correlate with 10- to 12-carbon alkyl chains on the non-polar tails of these amphiphilic structures. However, the therapeutic index (measured as a ratio of toxicity/MIC) seems to be optimized when chains are somewhat shorter, often 8–10 carbons in length. We thus hypothesized that there exists an optimal set of compounds with *multiple* shorter chains that could be significantly bioactive, yet exhibit reduced toxicity. A conceptual presentation of representative multi-armed QACs, which we refer to as "bushy-tailed," is illustrated in Figure 3.

Modest literature precedent supports this inquiry. Potent bioactivity for tributylphosphonium-based amphiphiles was reported by Endo as early as 1993 (Figure 4),[29] with rapid eradication of strains of *Staphylococcus aureus* and *Escherichia coli* observed within 30 minutes of contact at only 10 μg/mL.

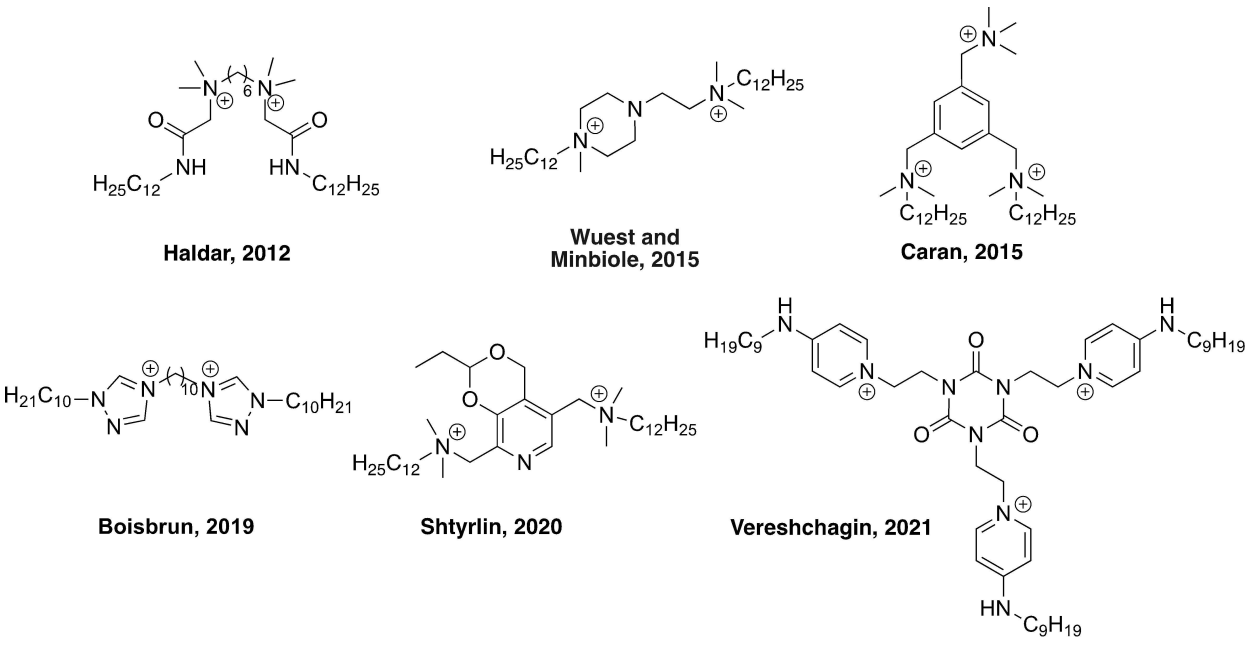


Figure 2. Samples of recent QAC architectural developments.

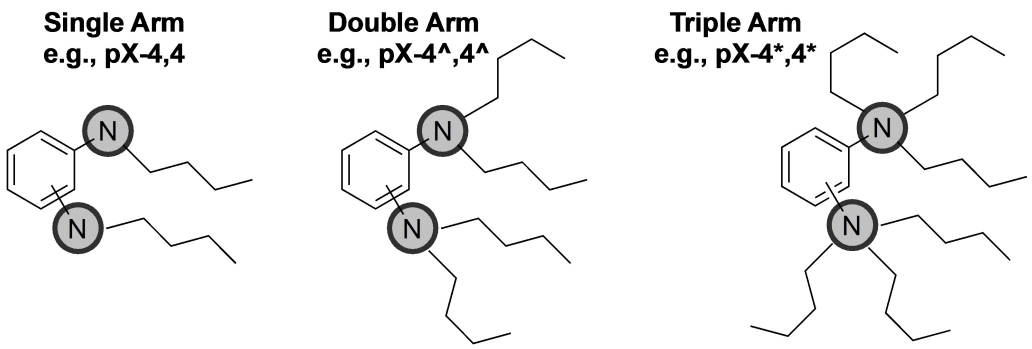


Figure 3. Conceptual presentation of multi-arm/bushy tailed QACs, where a circled N represents an ammonium residue. The ^ symbol represents two longer-chained alkyl substituents, and the * represents three.

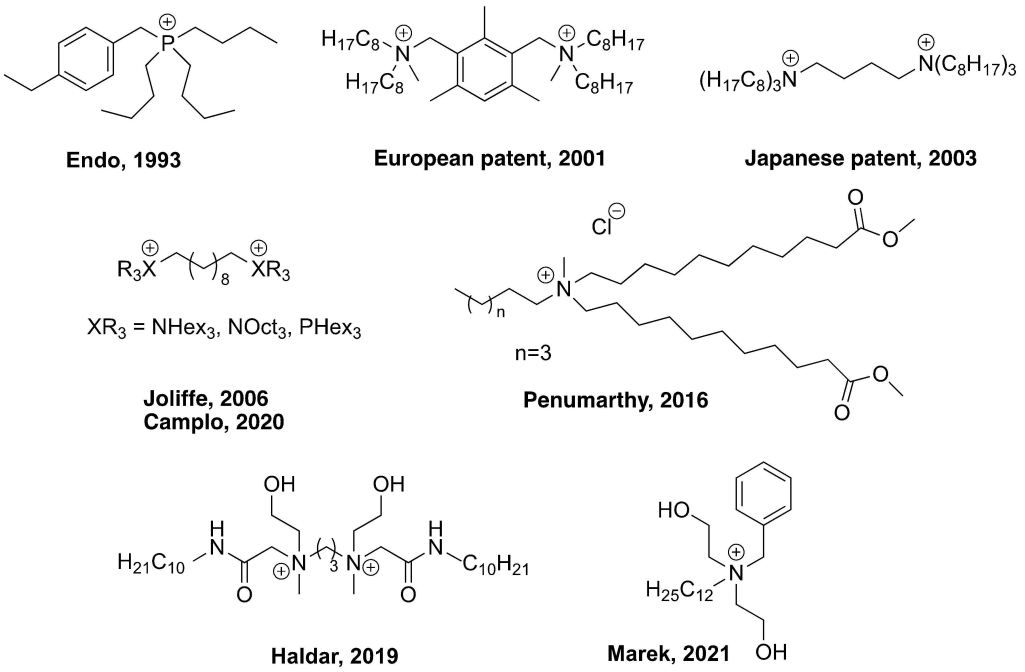


Figure 4. Relevant multi-armed QAC and QPC precedents.

Bushy bisQAC structures have been presented in multiple patents,[30,31] as well as in similar reports from Joliffe (2006)[32] and Camplo (2020)[33] regarding antifungal and antibacterial applications, respectively. Some of the compounds developed by Camplo and colleagues exhibited high antimicrobial activities (MIC = 0.5 mg L^{-1}) against several bacterial strains from the *ESKAPE* group of pathogens. Penumarthy and co-workers synthesized esterquat compounds that showed excellent antimicrobial activity with MIC values ranging between 1.9 and 7.8 μg mL^{-1} against *Micrococcus luteus*, *S. aureus*, and *B. subtilis* strains.[34] In a more recent study by Haldar et al., incorporation of two multi-armed quaternary ammonium groups into a new class of small antibacterial molecules helped to achieve high activity against MRSA and vancomycin-resistant *S. aureus* (VRSA) with MIC values in the range of 1–4 μg mL^{-1}.[35] Lastly, Marek and co-workers reported a series of benzalkonium-like salts with alkyl chain length C_{10}-C_{18} and identified the C_{12} homolog with the highest biocidal activity against *Francisella tularensis* and *S. aureus* in biofilm form.[36]

Taking inspiration from compounds developed for use as phase transfer agents and ionic liquids for separation applications[37,38,39] and the commercial availability of a variety of analogous amines with one, two, or three long alkyl chains, a library of "bushy-tailed" amphiphilic molecules was envisioned. Varying the number of cations within the molecule could be achieved by selecting a relatively reactive set of benzylic halides – specifically benzyl chloride or bromide, xylylene dichloride, and mesitylene tribromide – to allow for mono-, bis-, or triscationic structures, respectively. The availability of all three isomers of xylylene dichloride would allow further interrogation of structure-activity relationships within a biscationic framework. To describe the compounds in this library, we created a shorthand (Figure 3) where they are categorized as "single arm," "double arm" (noted by "^"), and "triple arm" (noted by "*") according to the number of long-chained substitutions; methyl groups represent the implied short-chained alternative that ensures quaternization. Interestingly, the bioactivity of this library seems largely unexplored, with only the Bn-n^ compounds having been investigated for antimicrobial activity by Marek and coworkers.[40]

We were pleased to find that most of the compounds reported herein could be synthesized by addition of the relevant tertiary amine to the corresponding electrophile followed by heating in acetonitrile under standard S_N2 conditions, as shown in Figure 5 and outlined in our previous report.[41] The volume of acetonitrile used in the reactions varied according to empirical observations of the relative miscibility of the amine with the solvent. Yields varied significantly, mainly due to inconsistent outcomes of the purification steps (generally, trituration). In addition, syntheses of the longest chained xylyl (**pX-8*8***, **pX-10*10***, **pX-12*12***) and all "triple arm" mesityl compounds (**Mes-4*,4*,4***, **Mes-6*,6*,6***, **Mes- 8*,8*,8*)** benefitted from the addition of isopropanol as a co-solvent, to facilitate miscibility of the reactants. Further, these compounds required significantly longer reaction times, likely due to steric bulk of the nucleophile hampering the rate of nucleophilic attack. The three long-chained triple-arm xylyl compounds were also more challenging to purify than the others, resulting in the need for either passage over a silica gel plug or silica gel column chromatography to isolate pure compounds. Perhaps owing to even greater steric hindrance, compounds in the oX-n*,n* and mX-n*,n* series were not successfully prepared in reasonable purity. Steric bulk of the tertiary amines also influenced the synthesis of the Bn-n* compounds. Yields and

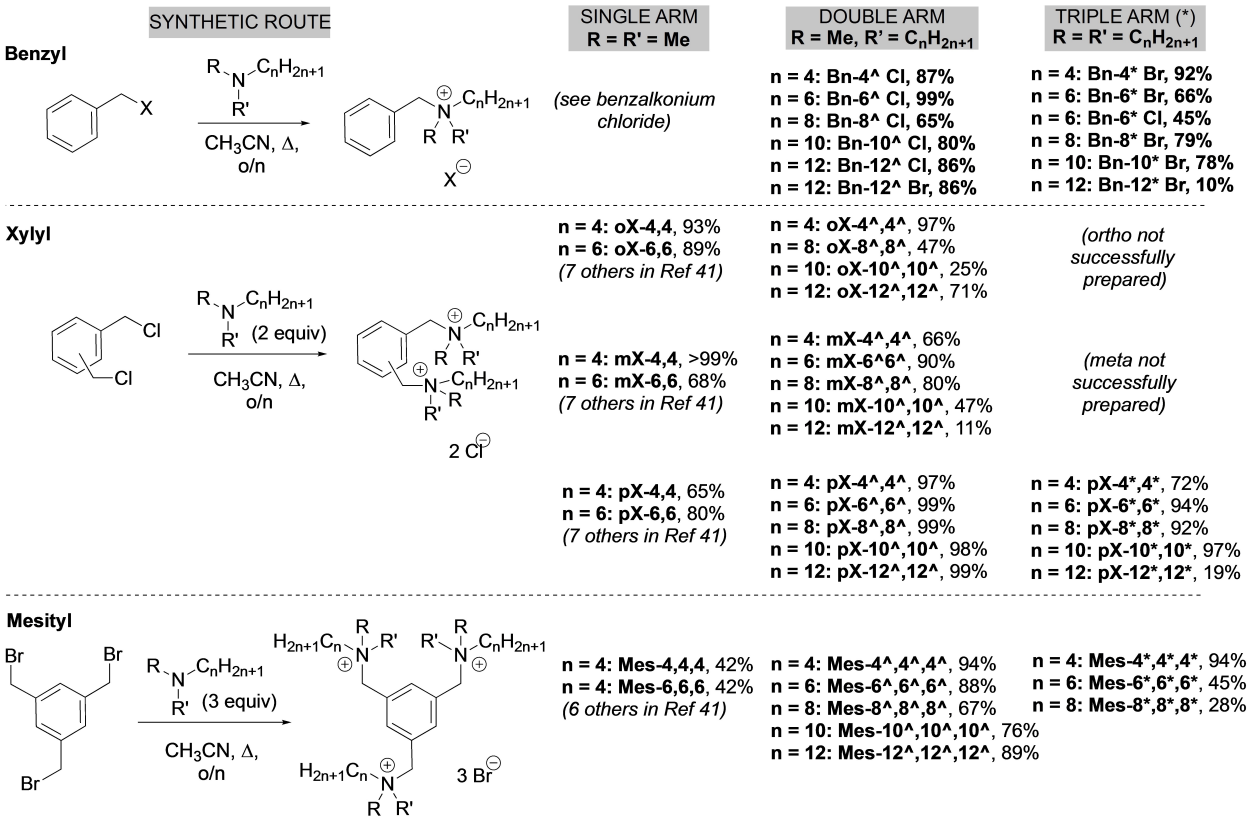


Figure 5. Synthetic campaign for the construction of 47 quaternary ammonium compounds with a bushy-tailed architecture.

purity were greatly improved with the use of benzyl bromide instead of the benzyl chloride used for the preparation of the Bn-n and Bn-n^ compounds. It is hypothesized that the change to the less nucleophilic bromide counterion disfavors the reverse reaction where the halide attacks to displace the amine, although this may simply reflect an increase in the electrophilicity of benzyl bromide. Additionally, the Bn-n* syntheses were found to proceed in the absence of a secondary solvent, as the benzyl halide served well in both roles of solvent and electrophile, thereby avoiding issues of miscibility of acetonitrile with the more highly substituted amines. To provide examples for the potential influence of this change in counterion on observed bioactivity, **Bn-6*-Br** was prepared alongside **Bn-6*-Cl** (the latter in lower yield), and **Bn-12^-Br** was constructed with its **Bn-12^-Cl** counterpart. In sum, a total of 47 compounds were prepared with yields ranging from 10 to >99%; complete synthetic and characterization details for all compounds are presented in the Supporting Information.

As we moved towards structural assessment, we employed single crystal X-ray crystallography for compounds representative of the structural variety herein. Accordingly, crystallographic data was obtained for **pX-4,4**, **pX-4^,4^**, and **pX-4*4*** as three representative compounds within this series to explore the structural impact of increasing the degree of substitution surrounding the ammonium cation. An ORTEP image for each of these compounds is presented in Figure 6. The asymmetric unit of the **pX-4*4*** crystal contains two crystallographically unique molecules due to minor shifts in position of the carbon atoms at the end of the alkyl chains. However, the relative orientation of the ammonium cations to the benzyl ring is the same for the co-crystallized **pX-4*4*** molecules. Summaries of the collection data for each compound and the CCDC numbers can be found in the Supporting Information.

Geometry around each of the ammonium nitrogen atoms is best described for all structures as a slightly distorted tetrahedral (C–N–C ∠109.5°±2.1°). Slightly more variability in bond angles can be observed in the more sterically hindered **pX-4*4*** compared to **pX-4,4**, but the average bond angle for all three molecules is the same. Carbon-nitrogen bond lengths in the ammonium cations are similar across the structures (C–N 1.519 Å±0.013 Å) and any differences in the averages for the structures do not appear to be statistically significant. Detailed bond length and bond angle data for the ammonium cations can be found in the Supporting Information. (Table S3).

Perhaps the most interesting aspect in comparing these bisQAC structures is the relative orientation of the ammonium arms relative to the central benzene ring. In the solid state, the **pX-4,4** structure orients the two ammonium cations to the same face relative to the benzyl ring. This orientation remains for **pX-4^,4^** despite the addition of another alkyl chain to each arm. The steric bulk from this second longer chain arm is accommodated by rotating the ammonium cations such that one of the alkyl chains on each ammonium cation points away from the center benzyl ring of the molecule. The relative positioning of the ammonium cations changes for **pX-4*,4***, with the addition of the third alkyl chain; the ammonium

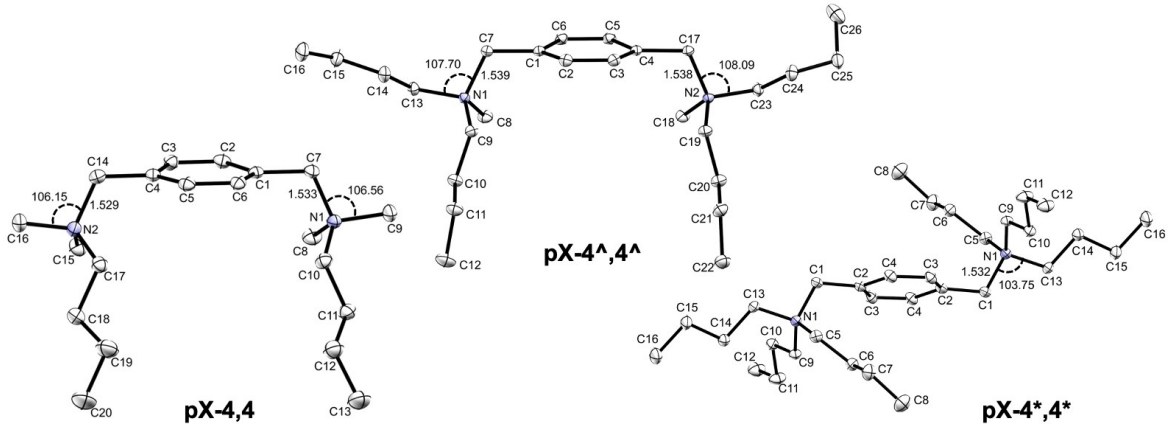


Figure 6. ORTEP diagrams for **pX-4,4** (left), **pX-4^,4^** (middle) and **pX-4*4*** (right) as representative complexes of the bisQAC series. Thermal ellipsoids are shown at the 50% probability level. Hydrogen atoms, counter anions, and any co-crystalized solvents or disordered atoms are omitted for clarity. Nitrogen atoms are presented as light blue to distinguish them from the white carbon atoms. One representative C–N–C bond angle (°) and N–C bond length (Å) are noted for each crystallographically unique ammonium cation.

groups orient to opposite faces of the benzyl ring and create a point of symmetry in the crystal structure solution. It is presumed that the added steric constraint stemming from the third alkyl chain on each of the ammonium cations drives this change in orientation observed in this solid state analysis, and by extension, likely represents a preferred relative orientation of the alkyl chains in the solution state as well.

The bioactivities of the prepared cationic amphiphilic compounds were assessed via minimum inhibitory concentration (MIC) and red blood cell (RBC) hemolysis ($lysis_{20}$) assays, wherein the latter was used as a proxy for cytotoxicity. A representative commercial preparation of BAC (70% benzyldimethyldodecylammonium chloride and 30% benzyldimethyltetradecylammonium chloride) and DDAC were tested as control compounds; the previously reported pX-12,12[41] served as a reference for this investigation. To determine MIC values, the compounds were each screened against a panel of seven bacterial strains, including four gram-positive strains (methicillin-susceptible *Staphylococcus aureus* [MSSA; SH1000], community-acquired methicillin-resistant *S. aureus* [CA-MRSA; USA 300–0114], hospital-acquired methicillin-resistant *S. aureus* [HA-MRSA; ATCC 33591], and *Enterococcus faecalis* [OG1RF]), as well as three gram-negative strains (*Escherichia coli* [MC4100], *Acinetobacter baumannii* [ATCC 17978], and *Pseudomonas aeruginosa* [PAO1]). To evaluate the hemolysis activities, the compound concentrations resulting in 20% RBC lysis ($lysis_{20}$) were determined. Poor water solubility, as well as initial purification challenges, led to the exclusion of some compounds (**mX-4,4**, **mX-6,6**, **oX-4,4**, **oX-6,6**, **oX-10^10^**) from the bioanalysis. The results of the MIC and hemolysis assays for 42 compounds plus three controls are presented in Table 1.

Several bioactivity trends are apparent from these results. First, while our previously developed QAC structures have generally indicated that monocationic species are less potent and susceptible to bacterial resistance than bis- and triscationic analogs, there are some significant exceptions in the compounds reported herein, particularly for **Bn-10^-Cl** as well as **Bn-8*-Br**. Commercially available didecyl dimethyl ammonium chloride (DDAC) is highly analogous to **Bn-10^-Cl** (benzyl didecyl methyl ammonium chloride) except for the phenyl substitution; interestingly, DDAC and **Bn-10^-Cl** displayed precisely the same bioactivity against the entire panel of seven tested bacteria. Differently put, switching one of the methyl groups in DDAC to a benzyl group had no effect on the bioactivity observed, and in both cases, bioactivity against the panel of bacteria tested was strong.

In a similar structural comparison, **Bn-12^-Cl** (benzyl didodecyl methyl ammonium chloride) can nominally be described as the addition of a second dodecyl chain to BAC in replacement of a methyl group, though BAC shows superior bioactivity. The strongly decreased activity of **Bn-12^-Br** versus **Bn-12^-Cl** suggests that bioactivity is likely connected to differences in water solubility, perhaps due to the relative ability to hydrate the anion. Relative water solubility may also explain the decrease in bioactivity in both Bn-12 structures in comparison to **BAC**. Otherwise, counterion identity was not observed to influence activity; for example, **Bn-6*-Br** and **Bn-6*-Cl** display identical (albeit weak) bioactivity.

It is of note, and perhaps not a surprise, that optimal bioactivity observed in the 42 compounds tested was generally dictated by chain length; however, that preferred length varied by the number of cations. For monocationic species, octyl and decyl chains led to optimal activity. For biscationic species, octyl chains were optimal, and for the triscationic mesityl series, hexyl chains had superior activity. This, again, likely reflects the decreasing water solubility for compounds with up to nine long alkyl chains.

The three most potent compounds tested, which unlike DDAC demonstrate inhibition at 1–4 μM against all seven bacterial strains including *P. aeruginosa*, were three structural isomers: **oX-8^,8^**, **mX-8^,8^**, and **pX-8^,8^**. This supports a previous observation that the geometric isomers around the xylene core have negligible variation in bioactivity;[41] indeed, these three structures display MIC values within 2x of each

Table 1. Biological activity and $lysis_{20}$ data of 42 of the synthesized compounds, as well as commercially available controls and a previously reported analog. Darker colors indicate preferential performance.

Compound	MIC (μM) MSSA	CA-MRSA	HA-MRSA	*E. faecalis*	*E. coli*	*A. baumannii*	*P. aeruginosa*	$Lysis_{20}$
pX-12,12	1	16	2	4	2	4	4	8
BAC	2	2	32	32	32	16	125	63
DDAC	1	1	4	4	4	4	16	32
Bn-4^-Cl	>250	>250	>250	>250	>250	>250	>250	250
Bn-6^-Cl	250	250	>250	>250	>250	>250	>250	250
Bn-8^-Cl	2	2	125	63	63	63	250	125
Bn-10^-Cl	1	1	4	4	4	4	16	16
Bn-12^-Cl	2	16	125	125	63	32	63	16
Bn-12^-Br	32	32	>250	>250	>250	>250	125	16
Bn-4*-Br	>250	>250	>250	>250	>250	>250	>250	250
Bn-6*-Br	16	32	>250	>250	>250	>250	>250	250
Bn-6*-Cl	16	32	>250	>250	>250	>250	>250	250
Bn-8*-Br	1	1	4	4	4	8	16	32
Bn-10*-Br	16	32	>250	>250	>250	>250	250	8
Bn-12*-Br	16	32	>250	>250	>250	>250	>250	63
pX-4,4	>250	>250	>250	>250	>250	>250	>250	250
pX-6,6	>250	>250	>250	>250	>250	>250	>250	250
pX-4^,4^	>250	>250	>250	>250	>250	>250	>250	250
pX-6^,6^	32	32	125	125	125	>250	250	250
pX-8^,8^	1	1	4	2	4	2	2	8
pX-10^,10^	4	4	63	63	32	16	125	16
pX-12^,12^	32	16	>250	250	>250	250	>250	32
pX-4*,4*	250	>250	>250	>250	>250	>250	>250	250
pX-6*,6*	1	1	8	8	8	32	32	125
pX-8*,8*	2	8	63	63	32	>250	63	16
pX-10*,10*	32	16	>250	>250	>250	>250	>250	125
pX-12*,12*	16	16	250	125	>250	125	250	125
mX-8^,8^	2	1	2	8	2	2	2	8
mX-4^,4^	>250	>250	>250	>250	>250	250	>250	250
mX-6^,6^	16	32	125	125	63	>250	250	250
mX-10^,10^	4	2	>250	63	63	16	63	16
mx-12^,12^	32	16	>250	>250	>250	>250	>250	16
oX-4^,4^	>250	>250	>250	>250	>250	>250	>250	250
oX-8^,8^	1	1	4	4	4	2	4	8
oX-12^,12^	8	8	125	125	63	63	250	32
Mes-4,4,4	>250	>250	>250	>250	>250	>250	>250	250
Mes-6,6,6	>250	>250	>250	>250	>250	>250	>250	250
Mes-4^,4^,4^	>250	>250	>250	>250	>250	>250	>250	250
Mes-6^,6^,6^	2	2	8	8	8	63	32	250
Mes-8^,8^,8^	8	16	125	63	63	16	125	4
Mes-10^,10^,10^	63	125	>250	250	>250	>250	>250	63
Mes-12^,12^,12^	63	250	>250	>250	>250	>250	>250	125
Mes-4*,4*,4*	250	250	>250	>250	>250	>250	>250	250
Mes-6*,6*,6*	1	1	4	4	4	4	8	16
Mes-8*,8*,8*	16	16	>250	>250	>250	250	250	63

other in all tests. Further, these three "double-chained" compounds all displayed a slight edge over the previously prepared "single arm" **pX-12,12** which showed some susceptibility to resistance from HA-MRSA (MIC = 16 μM). And in another surprise, **Mes-6*,6*,6*** was the only other compound that showed strictly single-digit micromolar inhibition against the entire bacterial panel, being the most potent of all the "triple-chained" compounds, in spite of the shorter six carbon chain lengths that usually correlate to weak antimicrobial performance. The structural variety in these highly potent compounds (**oX-8^,8^**, **mX-8^,8^**, **pX-8^,8^**, **Mes-6*,6*,6***), which differ in the disposition of the cationic residues in the solid state, suggests that potent and broad bioactivity can be identified in a variety of bushy-tailed architectures.

While our biscationic QACs have historically displayed little to no resistance from bacteria, for a surprising number of structures in this investigation, HA-MRSA showed distinctly higher MIC values than its MSSA counterpart. Apart from a few particularly potent compounds mentioned above, resistance was observed as a significant limitation. For example, the moderately active structure **mX-10^,10^** showed what we think to be the strongest resistance characteristics observed in our

laboratories, with an MIC of 2 μM versus MSSA but an MIC of >250 μM versus HA-MRSA; its isomers also showed significant resistance behavior. The appearance of bacterial resistance for select compounds within this set of structures is a target for further inquiry; further, the distinct association with the hospital environment might signal acquired resistance via repeated exposure in such settings.

From a wider perspective, this exploration of "bushy-tailed" QACs was based on a hypothesis that an improvement in therapeutic index would be observed for shorter-chained compounds, ideally resulting from a diminished eukaryotic toxicity (as determined by a RBC lysis proxy as measured by $lysis_{20}$ values). Unfortunately, this was not the case. All of the highly potent compounds described herein (**Bn-8*-Br**, **Bn-10^-Cl**, **oX-8^,8^**, **mX-8^,8^**, **pX-8^,8^**, **Mes-6*,6*,6***, as well as DDAC) display significant propensity for RBC lysis, measured as $lysis_{20}$ at 8–32 μM. Disappointingly, this tracks with our previous reports wherein strong antimicrobial activity aligned with toxicity. While we have no solid explanation for this observation, we can perhaps take insights from select compounds with startlingly poor therapeutic indices, such as **Bn-10*-Br** and **Mes-6^,6^,6^**, whose RBC lysis capacity far outstripped their antimicrobial activity.

In sum, we have determined that bushy-tailed QACs represent a promising architectural class for the development of novel disinfectants. Highly potent antimicrobial compounds from a wide variety of structural classes were observed and, in many cases, no resistance was observed. However, surprises in optimal chain lengths as well as an unsuccessful venture into improving therapeutic indices suggest that there is much more to learn in this structural class.

Acknowledgements

We thank Samantha Brayton for preliminary investigations into this synthetic work. This work was funded by the National Institutes of Health, specifically the National Institute of General Medical Sciences (R35 GM119426 to W.M.W.; T32 GM008602 to C.A.S.; DK126467 to G.G.V.C.), and the National Institute of Allergy and Infectious Diseases (R15 AI185860 to K. P. C. M.) as well as Major Research Instrumentation grants from the National Science Foundation (CHE-1827930 and CHE-2018399 to K.P.C.M.).

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

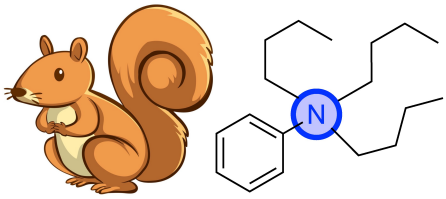
The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Amphiphiles · Benzalkonium chloride · DDAC · Disinfectants · QAC

[1] G. Domagk, *Dtsch. Med. Wochenschr.* **1935**, *61*, 829–832, https://doi.org/10.1055/s-0028-1129654.
[2] O. Rahn, W. P. V. Eseltine, *Ann. Rev. Microbiol.* **1947**, *1*, 173–192, https://doi.org/10.1146/annurev.mi.01.100147.001133.
[3] B. Merchel Piovesan Pereira, I. Tagkopoulos, *Appl. Environ. Microbiol.* **2019**, *85* (13), e00377–19, https://doi.org/10.1128/AEM.00377–19.
[4] G. Kampf, *Antiseptic Stewardship.* **2018**, Cham.: Springer. 371–394, https://doi.org/10.1007%2F978-3-319-98785-9 11.
[5] P. Gilbert, L. E. Moore, *J. Appl. Microbiol.* **2005**, *99* (4), 703–715, https://doi.org/10.1111/j.1365-2672.2005.02664-x.
[6] J. M. Boyce, *Antimicrob. Resist. Infect. Control.* **2023**, *12*(32), 1–14, https://doi.org/10.1186/s13756-023-01241-z.
[7] U. Tezel, S. G. Pavlostathis, *Curr. Opin. Biotechnol.* **2015**, *33*, 296–304, https://doi.org/10.1016/j.copbio.2015.03.018.
[8] Y. Morita, T. Murata, T. Mima, S. Shiota, T. Kuroda, T. Mizushima, N. Gotoh, T. Nishino, T. Tsuchiya, *J. Antimicrob. Chemother.* **2003**, *51* (4), 991–994, https://doi.org/10.1093/jac/dkg173.
[9] M. E. Wand, J. M. Sutton, *Microbiology* **2022**, *168* (11), 001263, https://doi.org/10.1099/mic.0.001263.
[10] A. A. Huet, J. L. Raygada, K. Mendiratta, S. M. Seo, G. M. Kaatz, *Microbiology* **2008**, *154*, 3144–3153, https://doi.org/10.1099/mic.0.2008/021188–0.
[11] S. Oh, M. Tandukar, S. G. Pavlostathis, P. S. G. Chain, K. T. Konstantinidis, *Environ. Microbiol.* **2013**, *15* (10), 2850–2864, https://doi.org/10.1111/1462-2920.12154.
[12] S. Oh, Z. Kurt, D. Tsementzi, M. R. Weigand, M. Kim, J. K. Hatt, M. Tandukar, S. G. Pavlostathis, J. C. Spain, K. T. Konstantinidis, *Appl. Environ. Microbiol.* **2014**, *80* (19), 5892–5900, https://doi.org/10.1128/AEM.01255–14.
[13] S. Buffet-Bataillon, A. Le Jeune, S. Le Gall-David, M. Bonnaure-Mallet, A. Jolivet-Gougeon, *J. Antimicrob. Chemother.* **2012**, *67* (12), 2837–2842, https://doi.org/10.1093/jac/dks321.
[14] M. S. Sidhu, E. Heir, T. Leegaard, K. Wiger, A. Holck, *Antimicrob. Agents Chemother.* **2002**, *46 (9*), 2797–2803, https://doi.org/10.1128/aac.46.9.2797-2803.2002.
[15] J. E. LaDow, D. C. Warnock, K. M. Hamill, K. L. Simmons, R. W. Davis, C. R. Schwantes, D. C. Flaherty, J. A. L. Willcox, K. Wilson-Henjum, K. L. Caran, K. P. C. Minbiole, K. Seifert, *Eur. J. Med. Chem.* **2011**, *46*, 4219–4226, https://doi.org/10.1016/j.ejmech.2011.06.026.
[16] J. Hoque, P. Akkapeddi, V. Yarlagadda, D. S. S. M. Uppu, P. Kumar, J. Haldar, *Langmuir* **2012**, *28*, 12225–12234, https://doi.org/10.1021/la302303d.
[17] M. A. Mitchell, A. A. Iannetta, M. C. Jennings, M. H. Fletcher, W. M. Wuest, K. P. Minbiole, *ChemBioChem* **2015**, *16* (16), 2299–2303. https://doi.org/10.1002/cbic.201500381.
[18] B. Thomas, R. E. Duval, S. Fontanay, M. Varbanov, M. Boisbrun, *ChemMedChem* **2019**, *14*, 1232–1237, https://doi.org/10.1002/cmdc.201900151.
[19] N. V. Shtyrlin, M. V. Pugachev, S. V. Sapozhnikov, M. R. Garipov, R. M. Vafina, D. Y. Grishaev, R. S. Pavelyev, R. R. Kazakova, M. N. Agafonova, A. G. Iksanova, S. A. Lisovskaya, M. I. Zeldi, E. S. Krylova, E. V. Nikitina, A. E. Sabirova, A. R. Kayumov, Y. G. Shtyrlin, *Molecules* **2020**, *25*, 4341, https://doi.org/10.3390/molecules25184341.
[20] J. N. Marafino, T. M. Gallagher, J. Barragan, B. L. Volkers, J. E. LaDow, K. Bonifer, G. Fitzgerald, J. L. Floyd, K. McKenna, N. T. Minahan, B. Walsh, K. Seifert, K. L. Caran, *Bioorg. Med. Chem.* **2015**, *23*, 3566–3573, https://doi.org/10.1016/j.bmc.2015.04.020.
[21] A. N. Vereshchagin, N. A. Frolov, A. P. Minaeva, E. V. Detusheva, Y. V. Derkach, M. P. Egorov, *Mendeleev Commun.* **2021**, *31* (3), Pages 368–369, https://doi.org/10.1016/j.mencom.2021.04.028.
[22] A. N. Vereshchagin, N. A. Frolov, K. S. Egorova, M. M. Seitkalieva, V. P. Ananikov, *Int. J. Mol. Sci.* **2021**, *22* (13), 6793, https://doi.org/10.3390/ijms22136793.
[23] M. C. Jennings, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2015**, *1*, 288–303, https://doi.org/10.1021/acsinfecdis.5b00047.
[24] M. Tischer, G. Pradel, K. Ohlsen, U. Holzgrabe, *ChemMedChem* **2012**, *7*, 22–31, https://doi.org/10.1002/cmdc.201100404.
[25] F. Bureš, *Top. Curr. Chem.* **2019**, *377*, 14, https://doi.org/10.1007/s41061-019-0239-2.
[26] K. R. Morrison, R. A. Allen, K. P. C. Minbiole, W. M. Wuest, *Tetrahedron Lett.* **2019**, *60*, 150935, https://doi.org/10.1016/j.tetlet.2019.07.026.

[27] E. A. Saverina, N. A. Frolov, O. A. Kamanina, V. A. Arlyapov, A. N. Vereshchagin, V. P. Ananikov, *ACS Infect. Dis.* **2023**, *9* (3), 394–422, https://doi.org/10.1021/acsinfecdis.2c00469.
[28] M. S. Leatherbury, L. M. Thierer, C. A. Sanchez, G. G. Vargas-Cuebas, A. A. Petersen, L. E. Amoo, E. L. Bezold, K. C. Washington, M. B. Mistrot, M. J. Zdilla, W. M. Wuest, K. P. C. Minbiole, *ChemMedChem* **2024**, *19*, e202300718, https://doi.org/10.1002/cmdc.202300718.
[29] A. Kanazawa, T. Ikeda, T. Endo, *J. Polym. Sci. Part A.* **1993**, *31* (6), 1467–1472, https://doi.org/10.1002/pola.1993.080310615.
[30] Toagosei Co., Ltd. *Preparation of quaternary ammonium salts as bactericides and fungicides. European Patent Organization*, EP1156030 A1, November 21, **2001**.
[31] Toa Gosei Chemical Industry Co., Ltd. *Preparation of quaternary ammonium salts having antibacterial and antifungal activity. Japan*, JP2003137706 A, May 14, **2003**.
[32] C. K. L. Ng, D. Obando, F. Widmer, L. C. Wright, T. C. Sorrell, K. A. Jolliffe, *J. Med. Chem.* **2006**, *49*, 811–816, https://doi.org/10.1021/jm0508843.
[33] F. Brunela, C. Lautardb, F. Garzinoa, J.-M. Raimundoa, J.-M. Bollab, M. Camplo, *Bioorg. Med. Chem. Lett.* **2020**, *30*, 127389, https://doi.org/10.1016/j.bmcl.2020.127389.
[34] S. R. Yasa, S. S. Kaki, Y. Poornachandra, C. G. Kumar, V. Penumarthy, *Bioorg. Med. Chem.* **2016**, *26* (18), 1978–1982, https://doi.org/10.1016/j.bmcl.2016.03.002.
[35] R. Dey, K. De, R. Mukherjee, S. Ghosha, J. Haldar, *MedChemComm* **2019**, *10*, 1907, https://doi.org/10.1039/C9MD00329 K.
[36] A. Markova, M. Hympanova, M. Matula, L. Prchal, R. Sleha, M. Benkova, L. Pulkrabkova, O. Soukup, Z. Krocova, D. Jun, J. Marek, *Toxics* **2021**, *9*, 222, https://doi.org/10.3390/ toxics9090222.
[37] S. Ide, A. Ohki, M. Takagi, *Anal. Sci.* **1985**, *1* (4), 349–354, https://doi.org/10.2116/analsci.1.349.
[38] H.-M. Yang, Y.-H. Hung, C.-Y. Tu, *J. Taiwan Inst. Chem. Eng* **2014**, *45* (4), 1421–1427, https://doi.org/10.1016/j.jtice.2013.11.004.
[39] H.-M. Yang, D.-W. Lin, *Catal. Commun.* **2011**, *14*, 101–106.
[40] M. Benkova, O. Soukup, L. Prchal, R. Sleha, T. Eleršek, M. Novak, K. Sepčić, N. Gunde-Cimerman, R. Dolezal, V. Bostik, P. Bostik, J. Marek, *ChemistrySelect* **2019**, *4*, 12076–12084, https://doi.org/10.1002/slct.201902357.
[41] Z. E. A. Toles, A. Wu, C. A. Sanchez, M. E. Michaud, L. M. Thierer, W. M. Wuest, K. P. C. Minbiole, *ChemMedChem.* **2023**, *18*, e202300018, https://doi.org/10.1002/cmdc.202300018.

Manuscript received: April 26, 2024
Revised manuscript received: June 12, 2024
Accepted manuscript online: June 14, 2024
Version of record online: ■■, ■■



Bushy-tailed QAC disinfectants: Will multiple short chains improve therapeutic indices?

A series of nearly four dozen amphiphilic disinfectants is presented by the Minbiole/Wuest collaboration, in hopes of developing "bushy tailed" QAC architectures that will minimize eukaryotic toxicity while displaying strong antimicrobial activity against a wide variety of bacterial strains. These multiQACs feature ammonium groups bearing single, double, and triple substitutions of mid-length alkyl chains.

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