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Combating Antibacterial Resistance through Novel Cationic Biocides

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M.S., Emory University, 2022

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An abstract of
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James T. Laney School of Graduate Studies of Emory University
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Abstract

Combating Antibacterial Resistance through Novel Cationic Biocides

By Christian A. Sanchez

Cationic biocides serve as the first level of defense against human pathogens in the effort towards infection prevention and control. Through poor management and overuse, bacterial pathogens have begun to develop resistance mechanisms to overcome cationic disinfectant treatment. The investigations contained herein focus on the development and study of novel cationic biocides as they relate to bacterial pathogens. Through our efforts, we have built upon previous structure-activity relationships for quaternary ammonium compounds (QACs) and quaternary phosphonium compounds (QPCs) as amphiphilic disinfectants. In the evaluation of nearly 200 novel cationic biocides, we have uncovered potent scaffolds that inhibit growth in gram-positive and gram-negative species. Narrowing in on a QPC from a previous campaign, P6P-10,10 displays a high degree of potency against cationic biocide resistant clinical isolates of *Acinetobacter baumannii* surpassing the efficacy of commercial and previously reported, experimental QACs. Similar studies in *Pseudomonas aeruginosa* revealed a similar efficacy. Mechanistic studies uncovered a distinct mode of action in *P. aeruginosa* and other gram-negative species, which is hypothesized to be based on specific structural features—namely the presence of delocalized lipophilic cations. Further investigations into whether the disinfectant mechanism of action affects the virulence phenotype of resistant strains revealed no substantial differences between the resistance phenotypes of commercial disinfectant benzalkonium chloride (BAC) and P6P-10,10. However, our results illustrate the significant consequences that cationic biocide resistance exerts on virulence-associated phenotypes of *P. aeruginosa*.

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Abbreviations*:*

AMR	antimicrobial resistance
CB	cationic biocide
QAC	quaternary ammonium compound
BAC	benzalkonium chloride
DDAC	didecyldimethylammonium chloride
CPC	cetylpyridinium chloride
BEC	benzethonium chloride
SAR	structure-activity relationship
TMEDA	tetramethylethylenediamine
TSC	trivalent sulfonium compound
QPC	quaternary phosphonium compound
MIC	minimum inhibitory concentration
MSSA	methicillin-susceptible *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
HA-	hospital acquired
CA-	community acquired
RBC	red blood cell
Me	methyl
Et	ethyl
Cy	cyclohexyl
Ph	phenyl
NMR	nuclear magnetic resonance
DST	dyanmic surface tension
NPN	N-phenyl-1-naphthylamine
LCMS	liquid chromatography-mass spectrometry
MRSN	Multidrug-Resistant Organism Repository and Sruveillance Network
CTAB	cetyltrimethylammonium bromide
CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
MDR	multidrug resistant
PA	*Pseudomonas aeruginosa*
$DiSC_3$-(5)	3,3'-dipropylthiadicarbocyanine iodide
GP	generalized polarization
OD	optical density

Spd	spermidine
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
OCT	octenidine
CHX	chlorhexidine
MFS	major facilitator superfamily
DLC	delocalized lipophilic cation
COVID-19	coronavirus disease 2019
-R	resistant
PYO	pyocyanin
PVD	pyoverdine
WT	wildtype

Chapter 1: Introduction

1.1 The Bacterial Problem

One in eight deaths worldwide are associated with bacterial infections.[1,2] In fact, deaths by infectious disease are projected to increase in the coming decades. This is partly due for the ability for bacteria to evolve and adapt to the pressure placed on them by antimicrobials in the environment and in the clinic. We refer to this phenomenon as antimicrobial-resistance (AMR). It is estimated that nearly 5 million deaths were associated with antimicrobial-resistant infections, and this is projected to increase to 10 million deaths per year by 2050.[3] As deaths from microbial infections begin to surpass deaths from cancer, our innovation pipeline is left in a dire situation.[4] Most large pharmaceutical companies expend little to no investment in antibiotic discovery, and the last broad-spectrum antibiotic class (fluoroquinolones) was brought to market in 1987.[5,6] As a result, bacteria continue to develop resistance to our currently available arsenal of antimicrobials as the pipeline remains fairly empty.[7] The danger then is not posed only for the sick, vulnerable, and immunocompromised. As bacterial pathogens develop resistance to multiple classes of antibiotics, routine medical procedures that require prophylaxis might end in bacterial infections as prophylactic treatments lose their efficacy.[8] A scrape on the sidewalk, a sinus infection, a urinary tract infection—though easily treatable now, these all have the potential to hold significantly more gravity in the future should the current trend continue. It is appropriate to declare that our world is in a public health crisis as we grapple with the dwindling power of our treatments against bacterial pathogens.

1.2 Addressing Bacterial Pathogens

1.2.1 Overview of Strategies

In the past, advanced microbial infections were incurable. In 1900, the leading causes of death were pneumonia, tuberculosis, and diarrhea and enteritis.[9] However, with the advent of modern sanitation and medicine, bacterial infections became not only treatable but also preventable. The three main ways that medicine addresses the bacterial problem are through the use of antibiotics, vaccines, and disinfectants.

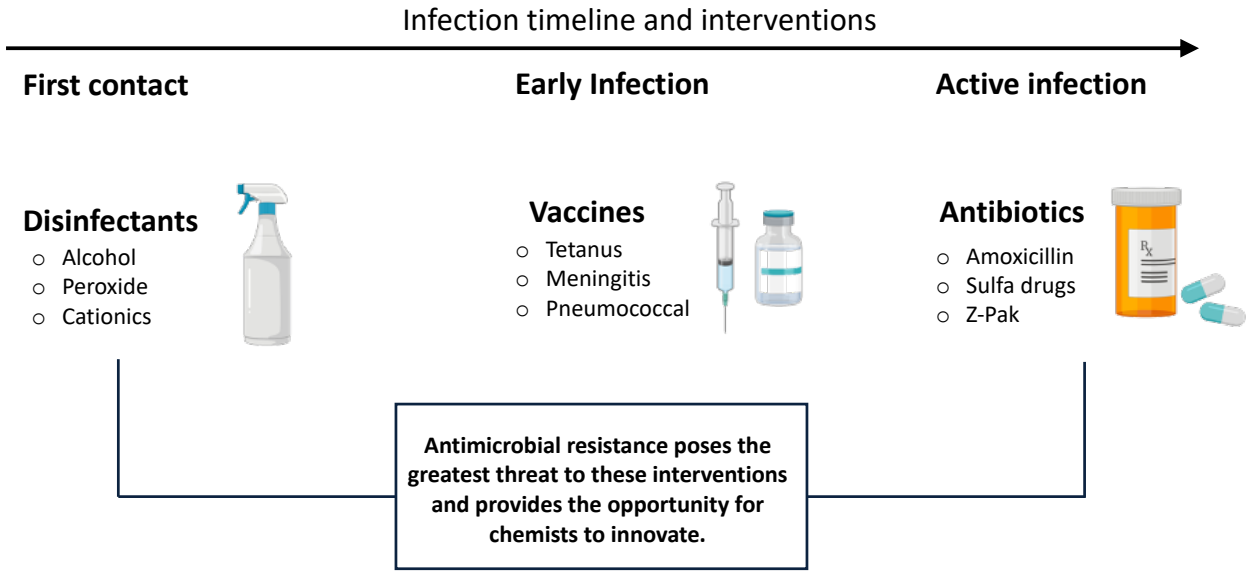

Figure 1.1 Infection timeline and interventions: disinfectants, vaccines, and antibiotics

1.2.2 Antibiotics

Antibiotics are one of the first interventions that come to mind when discussing bacterial infections. This is for good reason. When penicillin first came into the clinic in the 1940's, it was hailed as "yellow magic."[10] It truly transformed modern medicine with its power to save patients on the brink of death who were suffering from advanced infections. Antibiotics excel in the business of saving lives and staving off infections as prophylactics.[11] However, antibiotics are the last line of defense against bacterial pathogens. When antibiotics are needed, all preventative

measures along with the host's innate immune system have failed to prevent an infection from developing. Thus, when antibiotics begin to fail due to bacterial resistance, there are few places left to turn to. In an ideal world, foreign pathogens would never reach this point and would instead be halted earlier on in the process.

1.2.3 Vaccines

The modern era of immunization began nearly 140 years ago with Louis Pasteur's rabies virus vaccine.[12] However, several years earlier, he made significant contributions towards the creation of a live attenuated vaccine for the bacterium *Bacillus anthracis*, or anthrax.[13] This was one of the first pursuits of a method to prevent harmful bacteria from infecting the hosts upon contact. Fundamentally, the preventative concept behind vaccine is that the potential host should be able to preclude a bacterial infection from occurring upon sufficient exposure.[14] This rests one step behind antibiotic treatment and eliminates the need for antibiotics. As the timeframe for the intervention is moved back (i.e., from active infection to no infection), healthcare costs and time are saved. Furthermore, should immunizations fail, antibiotics function as a form of failsafe and protect patients from succumbing to their infections.

1.2.4 Disinfectants

Disinfectants stand at the front lines in the fight against bacterial infections. They beg the question: what if susceptible populations never came into contact with pathogenic bacteria to begin with? Effective infection prevention and control rests on the ability to eliminate human pathogens from high-contact surfaces and provide sterile surfaces in healthcare settings, though disinfectant use extends to a broader range of settings including agriculture, food industry, cosmetics, and domestic cleaning.[15,16] 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).[17] These cationic surfactants act upon bacteria by disturbing their cell envelopes, leading ultimately to membrane lysis and death.[18] By focusing on preventative measures like disinfectants, we can hopefully reduce burden further down the pipeline for interventions like antibiotics.

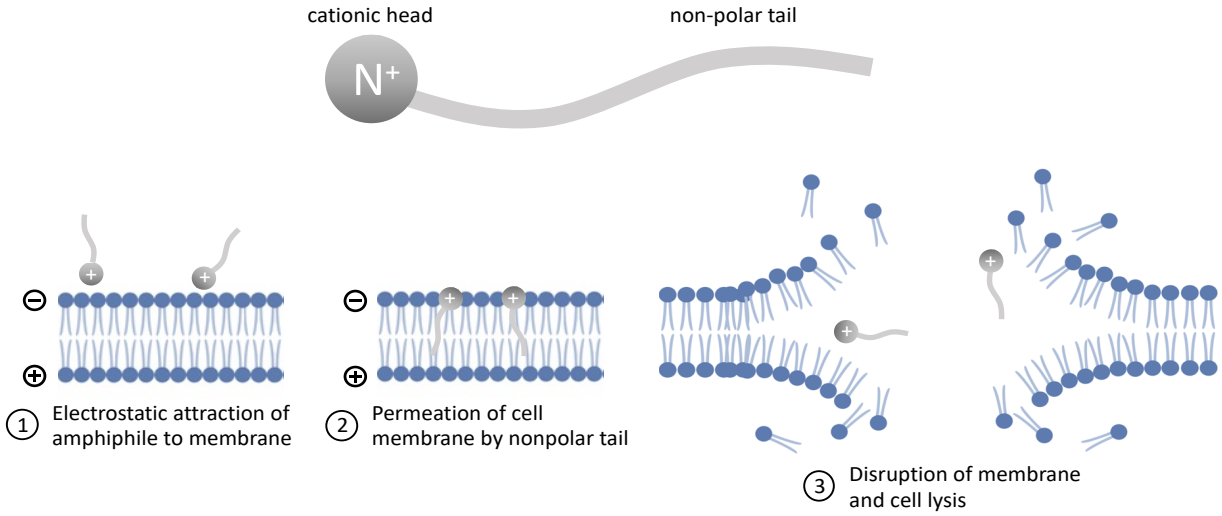

Figure 1.2 General mechanism of action for cationic biocides

1.3. Conclusion

This thesis will discuss projects that explored novel structures and scaffolds for cationic biocides, investigated the mechanism of actions of certain biocides, and assessed biological ramifications of cationic biocide resistance in *Pseudomonas aeruginosa*. Chapter 2 will present an overview of work performed towards the investigation of novel cationic biocides (quaternary ammonium compounds and quaternary phosphonium compounds). This chapter will demonstrate the success of highly collaborative efforts to identify the scaffolds and features of next-

generation cationic biocides that can overcome disinfectant resistance in the environment. We explore different scaffolds, the effect of multiple cationic centers, and—most interestingly—the effect of the heteroatom on biological activity. The chapter ends with promising results that display the efficacy of a novel quaternary phosphonium compound P6P-10,10 against a panel of *Acinetobacter baumannii* clinical isolates. The superior efficacy of P6P-10,10 compared to other QACs (commercial and experimental) led us to the investigation presented in chapter 3. The third chapter builds on P6P-10,10 as a potent antimicrobial against clinical isolates of the pathogen *Pseudomonas aeruginosa*. Similar to previous work, we observed the high potency of P6P-10,10 compared to commercial monocationic QACs and further investigated whether there were any mechanistic nuances between the disinfectants. From this work, we uncovered a crucial mechanistic difference in the activity of P6P-10,10 and two other commercial biocides that appears to be conserved across gram-negative species. The final chapter involves an investigation into whether this mechanistic difference between P6P-10,10 and BAC affects virulence and pathogenicity in resistant mutants. The aim for this thesis is to highlight the whole process of disinfectant discovery from identification in biological screens to mechanistic studies to resistance studies and show the promise of new cationic biocides.

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Chapter 2: Exploring Novel Classes of Cationic Biocides as Bacterial Disinfectants

2.1 Previous Research into Novel Classes of Cationic Biocides

Cationic biocides, namely QACs, have a rich history of use as disinfectants in the past 100 years. Following the report of Ehrlich leading to the discovery of salvarsan in the early 1900's, a renewed interest in using chemical methods to discovery novel therapeutic agents was generated.[1] Among the following successes was the discovery of the bactericidal action of hexamethylenetetramine salts. Reported by Walter A. Jacobs in 1916, these first reports of quaternary ammonium compounds began the launch into modern disinfection practice whose innovation arose from the work of synthetic organic chemists.[2,3] Some 20 years later in 1935,

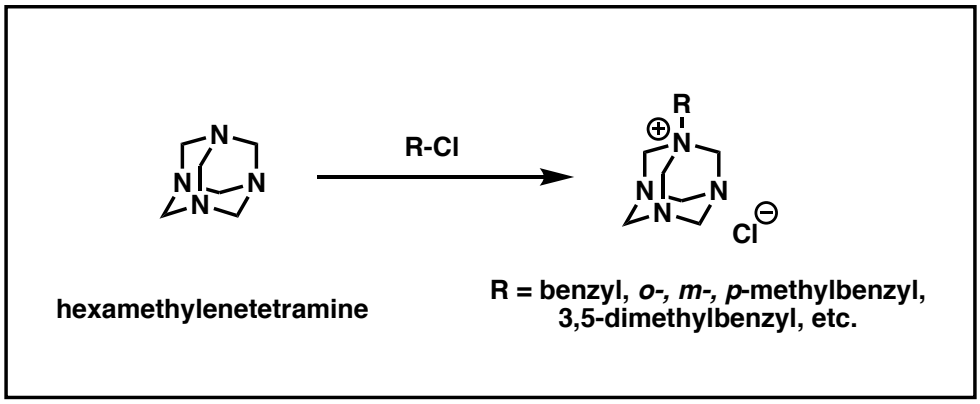


Figure 2.1 First report of antibacterial quaternary ammonium compounds

Gerhard Domagk, who would later win the Nobel Prize for his work on small molecule antibiotics, reported a new class of disinfectants that possessed a quaternized benzylamine core with various alkyl chain linkers, later to be named in English as benzalkonium chloride (BAC).[4] He discovered if at least one of the alkyl chains were long, then the compound possessed the ability to kill microorganisms. Indeed, BAC became the first QAC approved for disinfection use in the United States in 1947.[5] Currently, BAC is formulated as mixtures comprising 8-18 carbon chains originating from the dimethyl benzylammonium group. Since then, a multitude of novel QACs

and analogs have been developed and implemented for commercial use. Among these is the highly potent QAC dimethyldidecylammonium chloride, better known as DDAC.[6]

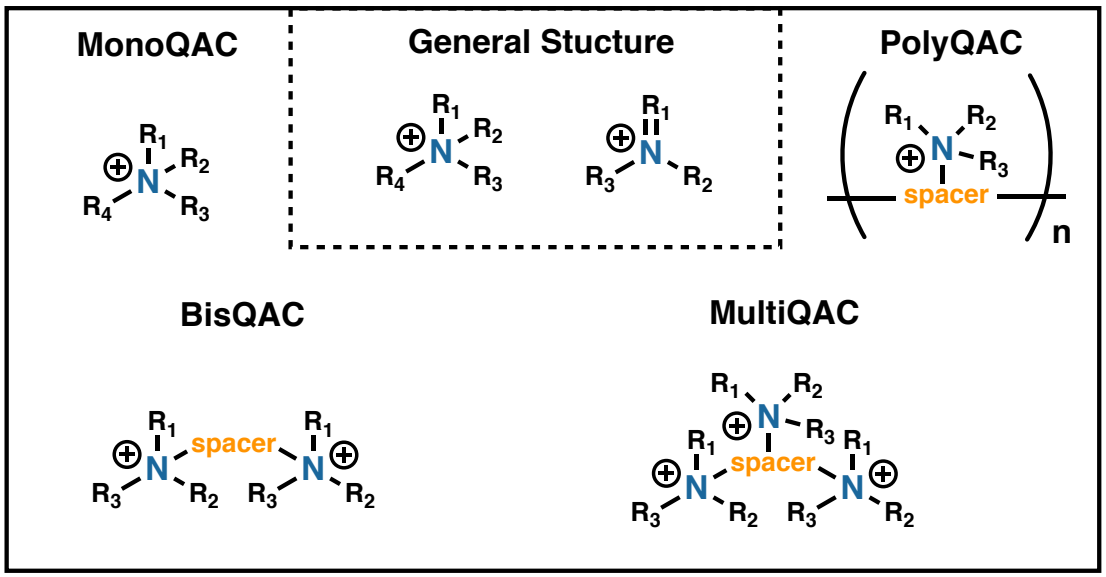


Figure 2.2 The general structure of QACs and the four major classes of QACs

QAC structures possess a positively charged nitrogen atom typically bound to four substituents (though heterocyclic QACs such as CPC are only bound to three). As a counterion, chlorides or bromides are typical, though experimental disinfectants or specially formulated QACs may possess a variety of anions. When classifying QACS, distinctions are made according to the number of positively charged nitrogen, or ammonium groups. Current classifications include monoQACs, bisQACs, multiQACs, and polyQACs. MonoQACs encompass the common commercially available QACs such as BAC, DDAC, BEC, and CPC.[7] Additionally, bisQACS include commercially QACs dequalinium and octenidine. Symmetry is a common feature of QACs with more than one cationic center, and these bisQACs are typically connected by an alkyl spacer. When bisQACs have an internal mirror plane, they can be further subclassified as "twin" or "gemini" QACs. MultiQACs containing three or more ammonium centers similarly contain the cation-spacer-cation moiety and possess branched molecular scaffolds. Additionally, polyQACs

such as polyquaternium-1 (also known as polidronium chloride) are polycationic, linear chains separated by spacers following the (cation-spacer-cation)$_n$ pattern.

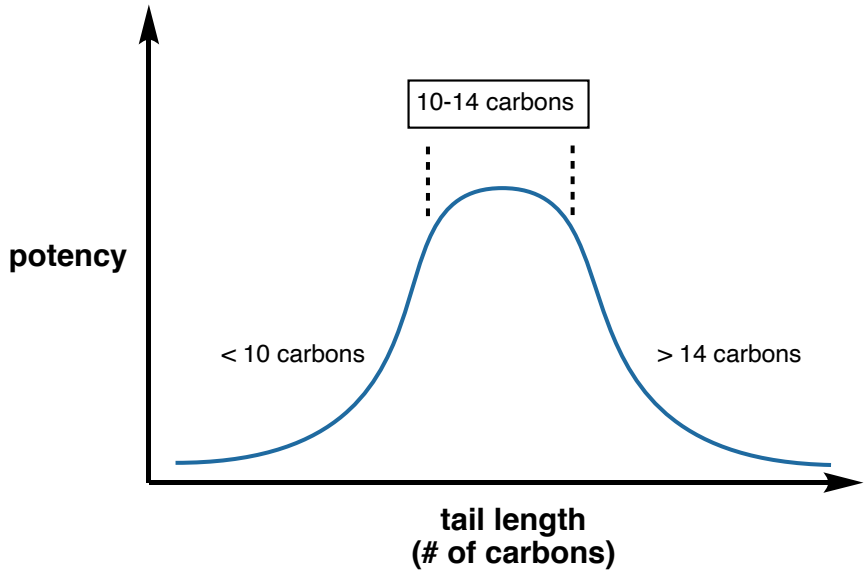


Figure 2.3 Parabolic relationship of tail length and biological activity

Defining the structure-activity relationships (SAR) for quaternary ammonium disinfectants has a long-going effort for near a century as groups have worked to understand what makes an effect biocide. One of the most apparent trends that occurs relates to tail length originating from the cationic center. When studying series of experimental QACs, one often observes the parabolic correlation of activity versus tail length.[8] Shorter tail length (<10 carbons long) display less potent activity bacterial species, especially more intrinsically resistant gram-negative species. Between 10 and 14 carbons length, the inhibitory potency of the QAC typically peaks, and subsequently longer tail lengths (>14 carbons long) begin to see a decrease in activity. For QACs to be effective, there needs to be a sufficient lipophilicity to favorably interact—and disrupt—the membrane of bacterial species. However, longer tail lengths can promote insolubility and the increased

flexibility of the tail as length increases may not promote the necessary interactions for effective cell membrane lysis.[9]

Approaches towards developing novel disinfectants have included increasing the number of cationic centers, varying the lipophilic groups, and changing the cationic heteroatom away from nitrogen. For this thesis, each relevant approach will be reviewed prior to the research performed in addition to relevant previous research from the Wuest and Minbiole group, between which there has been a decade's worth of collaborative efforts and greater than 850 compounds synthesized and tested.[10]

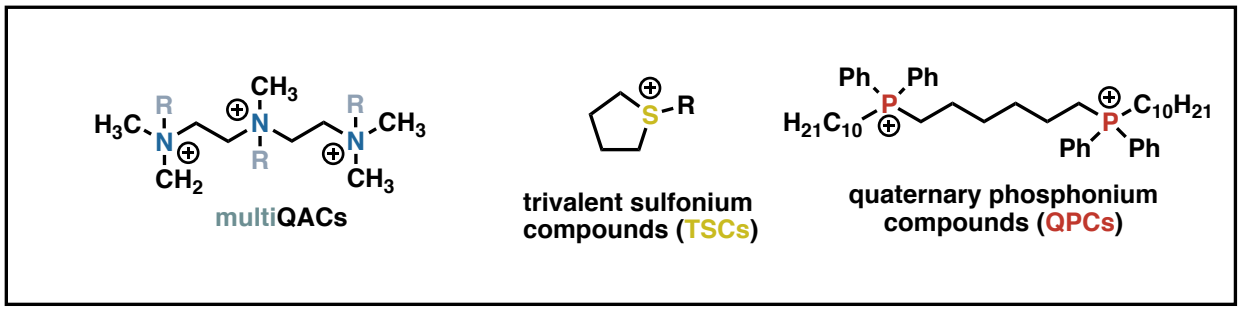


Figure 2.4 Representation of next-generation cationic biocides from the Wuest and Minbiole groups

Previous research has revealed two important concepts that have guided much of the research presented herein: (1) multiple cationic centers display more potent biological activity and (2) non-nitrogenous cationic biocides hold great promise as new scaffolds to overcome resistance. Beginning with the biscationic TMEDA-derived QACs spanning to the tetracationic tris(2-dimethylaminoethyl)amine derived "Super-T" multiQACs, the Wuest and Minbiole group have a decade-long history of demonstrating the power of multicationic disinfectants over monocationic species.[10,11] Because of this, most, if not all, current efforts involve developing cationic biocides with multiple cationic centers. Expanding beyond nitrogenous disinfectants, trivalent sulfonium compounds explored by the Minbiole and Wuest group were unimpressive in terms of biological

activity for the scaffolds tested.[12] However, branching into quaternary phosphonium compounds (QPCs) has resulted in the identification of highly potent phosphorous-based disinfectants.[13] Namely, a series of bis(diphenylphosphonium) disinfectants resulted in the identification of new class of QPCs with promising bioactivity. Among this class was P6P-10,10, a 1,6-bis(diphenylphosphonium)hexane QPC with a ten-carbon alkyl chain originating from the phosphorous, shown in Figure 2.4. P6P-10,10 displayed single digit micromolar MICs across a panel of clinically relevant bacteria.[13] Further investigations into the activity of P6P-10,10 is explored towards the end of this chapter with mechanistic studies and resistance development studies covered in chapters three and four, respectively.

Our approach towards developing novel cationic biocides is a collaborative effort between the Minbiole group at Villanova University and the Wuest group at Emory University. All compounds are synthesized at Villanova and then sent down to Emory for biological evaluation. Our biological evaluation is restricted to bacterial pathogens and does not include relevant groups often targeted by disinfectants such as viruses, fungi, and algae. For our panel of bacteria, we generally focus on five bacterial species. Included in the panel are three strains of *Staphylococcus aureus* (methicillin-susceptible [MSSA SH1000], community-acquired methicillin resistant [CA-MRSA USA300-0114], and hospital-acquired methicillin resistant [HA-MRSA ATCC 33591]), *Enterococcus faecalis* [OG1RF], *Escherichia coli* [MC4100], *Acinetobacter baumannii* [ATCC 17978] and *Pseudomonas aeruginosa* [PAO1]). By selecting these strains, the panel includes both gram-positive (*S. aureus*, *E. faecalis*) and gram-negative (*E. coli*, *A. baumannii*, *P. aeruginosa*) strains. Gram-negative strains, with their additional outer membrane, are possess a degree of intrinsic resistance towards QACs and thus pose the greatest challenge to develop new chemical disinfectants that effectively neutralize them.[14] Evaluation of biological activity begins with

determining the minimum inhibitory concentrations (MICs) of each compound. This is the lowest concentration of compounds that fully inhibits the visual growth of a defined dilution of bacteria. In commercial application of disinfectants, complete neutralization of bacteria is the metric used for efficacy. However, time-kill assays are more laborious and lower throughput, which prohibits large screens of compounds across a panel of bacteria.[15] While the MIC assay measures biological activity that is not in line with its in-use functions of killing bacteria, it serves as a decent proxy for compound activity. Furthermore, due to the non-specific nature of QAC activity on membranes, assessing the mammalian cytotoxicity of compounds is included in our biological assessments.[16] As a proxy for cytotoxicity, the red blood cell (RBC) lysis assay is utilized to assess the effect that experimental QACs may unintendedly have on non-bacteria cells. From the RBC lysis assay, a $lysis_{20}$ value is obtained. This measures at what concentration of compound results in the lysis of 20% of RBCs. Through these two screening assays, we can readily evaluate novel disinfectant classes against a panel of clinically relevant bacteria and compare them to commercial standards. The following sections are a summary of the work that I performed towards this end, and they are concluded by some final remarks and conclusions.

2.2. Exploring Novel Classes of Cationic Biocides as Bacterial Disinfectants

2.2.1 BAC Derivatization Strategy

Benzalkonium chloride is a mainstay of current disinfection practices. Beyond gram-positive and -negative bacteria, BAC is also indicated for targeting pathogenic fungi, envelope and non-envelope viruses, mold/mildew, and algae. As shown in Table 2.1., BAC is found is all areas of life for microbe control.[17] In recent decades, concerns have arisen due to the use of BAC from

Use Category	Use Site
Industrial processes and water system	Industrial re-circulating water systems, pulp and paper facilities, cooling water towers, disposal water, oil field operations, and oilfield water flood or saltwater disposal.
Swimming Pools	Swimming pools, outside spas, whirlpools, and hot tubs
Aquatic Areas	Golf courses, recreational parks, amusement parks, universities, cemeteries, and greenhouse/nurseries
Wood Treatment	Pressure treatment, double vacuum, and dip/spray surface treatment
Agricultural Premise and Equipment	Hatcheries, swine/poultry/turkey farms, animal housing facilities, farrowing barns, dressing plants, mushroom farms, citrus farm, florist/flower shops/ and greenhouses/nurseries
Residential and Public Access Premises	Homes, mobile homes, cars, boats, playgrounds, boats, public facilities, campgrounds, campers, trailers, and trucks
Medical Premises and Equipment	Hospitals, health care facilities, medical/dental offices, nursing homes, autopsy rooms, finderal homes, mortuaries, medical research facilities, acute care instituions, alternatice care institutions, newborn nurseries, day-care facilities, and sick rooms.
Commerical, Institutional, and Industrial Premise and Equipment	Athletic/recreational facilities, exercise facilities, health clubs, dressing/locker rooms, schools, colleges, universities, transportation terminals, libraries, motels, hotels, barber and beauty salons, convenience stores, offices, commercial/ institutional laundry mats, emergency vehicles, factories, commercial florist, and correctional facilities
Food Handling/Storage Establishments Premises and Equipment	Restaurants, food service establishments, food processing/storage/handling plants and facilities, beverage processing plants, supermarkets, breweries, bars, cafeterias, fishery/citrus/wine/ice cream/potato processing plants, egg processing plants, dairies, institutional kitchens, fast food operations, rendering plants, school lunchrooms, and packing plants

Table 2.1 Benzalkonium chloride uses

its impact on human health and the increasing occurrence of bacterial resistance towards this disinfectant.[16] Regarding human health, BAC has been associated with adverse health effects such as a skin irritation, asthma, increased inflammatory cytokines, and altered serum lipid levels.[18] Concerns arise for the highly exposed populations that use QAC-containing products, such as those in domestic cleaning, maintenance, healthcare, and food-related industries. Furthermore, in addition to its impact on human health, bacterial resistance to BAC has been studied since at least 1968, yet BAC remains the mainstay of commercial disinfectants.[19] While this disinfectant has been very successful in the past, its relevant as a disinfectant is shrinking due to the emergence of higher levels of antimicrobial resistance.[20]

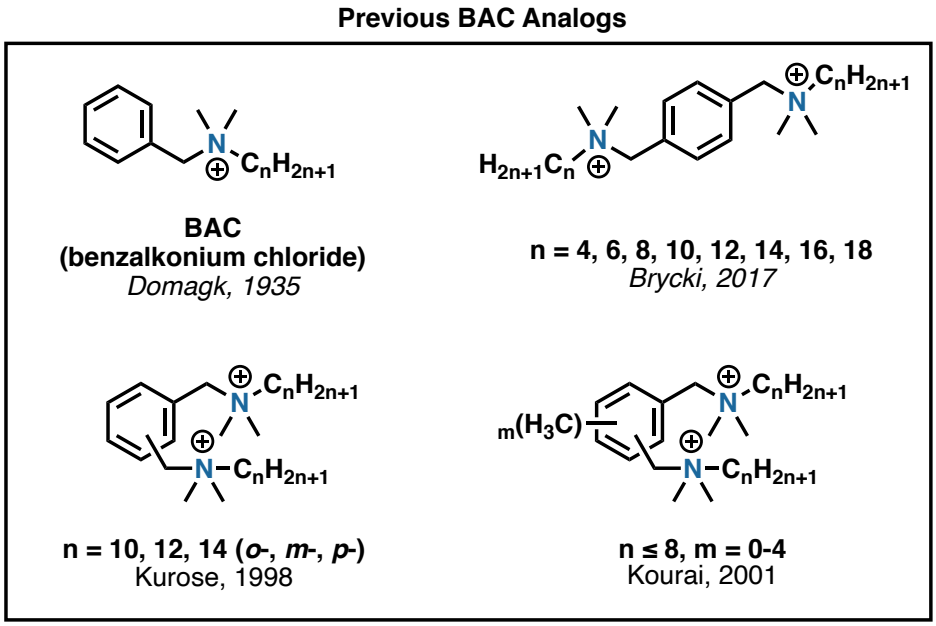
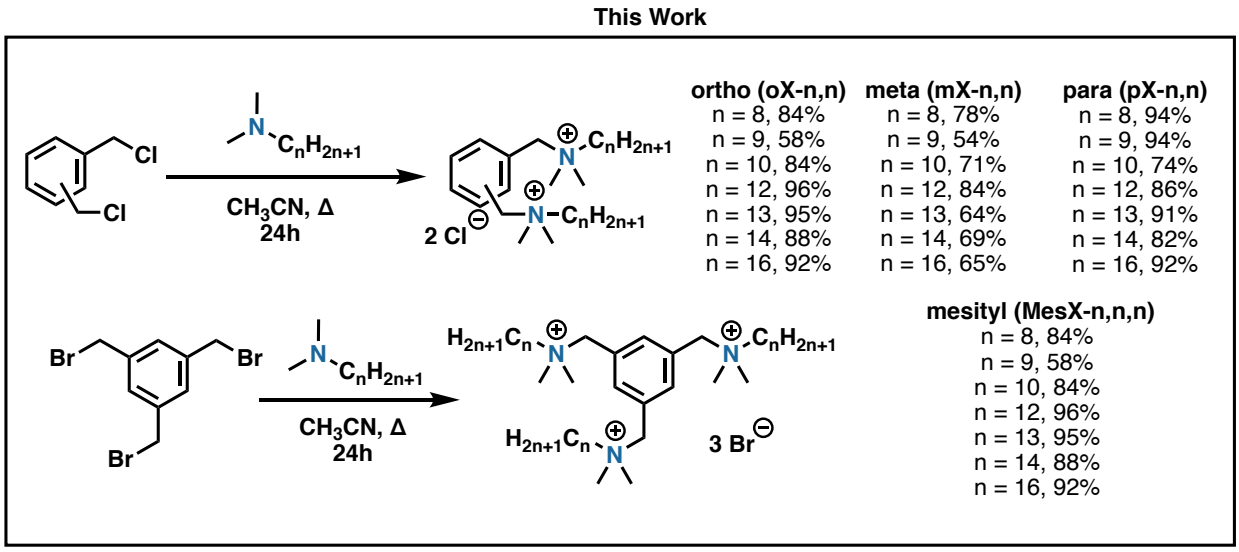


Figure 2.5 Previous benzalkonium chloride analogs

Due to the history of success with BAC, there has been interest in developing derivatives of the current structure to expand upon this effective core architecture. To accomplish this, modifications have focused on the further functionalization of the benzene ring. In 2017, Brycki and coworkers demonstrated the success of this strategy by synthesizing a series of eight

symmetric para-xylyl-disubstituted bisQACs.[21] Centered around a benzene ring and possessing the dimethyl benzylammonium moiety, these analogs displayed potent activity when a 10- or 12-carbon chain was attached to the ammonium. These analogs possessed low micromolar activity against bacterial pathogens and fungal pathogens alike, exhibiting promise for this strategy. Furthermore, earlier patent literature describes these longer chain lengths (10, 12, 14) on ortho- and meta-dimethyl benzylammonium moieties in addition to the para-substitutions reported by Brycki.[22] In addition, another patent reports similar bisQAC BAC analogs with methyl groups on the positions on the benzene core unsubstituted by benzylammoniums.[23]



Scheme 2.1 Synthesis of benzalkonium chloride analogs

Despite the literature precent previously described, there had been no systematic study of BAC analogs that examined the effect of chain lengths, number of benzylammoniums, and substitution patterns on antibacterial activity. Through a collaboration with the Minbiole group, we sought to examine the biological activity of the BAC analogs were synthesized. We directly compared their toxicity, biological activity, and ability to inhibit the growth of antibiotic resistant and susceptible strains of *Staphylococcus aureus*.

Compounds	MIC (μM)							Lysis20
	MSSA	CA-MRSA	HA-MRSA	*E. faecalis*	*E. coli*	*A. baumannii*	*P. aeruginosa*	
BAC	4	4	16	63	63	32	250	63
pX-8,8	125	125	>250	>250	>250	>250	>250	250
pX-9,9	16	8	63	125	125	>250	>250	250
pX-10,10	2	2	4	16	8	63	250	125
pX-12,12	1	1	1	2	2	4	8	8
pX-13,13	2	4	2	4	4	16	16	63
pX-14,14	4	4	4	16	32	>250	63	125
pX-16,16	8	16	8	>250	>250	>250	>250	250
mX-8,8	125	63	>250	>250	>250	>250	>250	250
mX-9,9	8	8	63	125	125	250	>250	250
mX-10,10	2	2	4	8	16	63	250	250
mX-12,12	2	1	1	2	2	2	8	8
mX-13,13	4	2	2	4	8	4	16	8
mX-14,14	8	2	2	16	32	16	32	8
mX-16,16	4	8	8	32	63	125	250	8
oX-8,8	>250	>250	>250	>250	>250	>250	>250	NT
oX-9,9	32	8	32	63	63	250	250	250
oX-10,10	4	1	2	16	8	32	32	125
oX-12,12	4	1	2	2	2	4	8	8
oX-13,13	4	2	8	16	4	2	16	8
oX-14,14	4	4	63	32	16	125	63	32
oX-16,16	16	8	8	250	125	250	250	250
Mes-8,8,8	4	2	32	63	32	>250	250	250
Mes-9,9,9	1	1	2	8	4	63	16	125
Mes-10,10,10	2	1	1	2	4	8	4	16
Mes-12,12,12	8	8	4	8	16	32	16	8
Mes-13,13,13	32	8	32	63	32	>250	125	63
Mes-14,14,14	63	125	125	63	125	>250	>250	63

Table 2.2 Biological activity of benzalkonium chloride analogs

In preparing the broad set of multicationic BAC analogs, a standardized alkylation strategy was used. Accordingly, ortho-, meta-, and para-bis(chloromethyl)benzene were exposed to the standard alkylation conditions (acetonitrile, [~ 1 M], reflux, ≤ 24 h) with a tertiary amine nucleophile (2 equiv) bearing two methyl groups and an aliphatic tail. Through this procedure, twenty-one biscationic quaternary ammonium compounds (bisQACs) were constructed. Yields ranged from 54–96% were named for their relative substitution (o-, m-, or p-) as well as the chain length of their amphiphilic alkyl tail. Additionally, a series of trisQACs were synthesized in high yields (84–99%) from 1,3,5-tris(bromomethyl)benzene to similar alkylation conditions and the resulting mesitylene- derived compounds were named Mes-n,n,n.

Four of the synthesized compounds displayed single digit micromolar activity against all seven bacterial strains tested. Interestingly, these compounds were distributed between the four classes generated: oX-12,12, mX-12,12, pX-12,12, and Mes-10,10,10. While confirming the that

these multi-cationic QACs based on the BAC structure are potent disinfectants, it indicated that chain length seemed to be the most important predictor of activity. Nevertheless, the multicationic amphiphiles displayed superior potency in contrast to monocationic BAC in both gram-positive and gram-negative bacteria, where BAC displayed MIC values between 32 and 250 μM. Comparing between methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA), we observe 4-fold reduction of BAC activity from MSSA to HA-MRSA, but our multicationic analogs retain their activity between antibiotic resistant and susceptible strains. Furthermore, our most potent multiQACs had near equipotency in gram-positive versus gram-negative species.

Chain length was the greatest predictor of bioactivity for the bis- and tris-QACs. For the bisQACs, activity generally increased with increasing chain length until it reached its maximum at 12 carbons. After 12 carbons, the activity began to decrease again. This is in accordance with previous reports by our group and others that suggest that between 10-14 carbon chain lengths attached cationic heteroatoms is optimal for antibacterial activity. While the 12-carbon tail compounds had the lowest inhibitory concentrations of the sets, this is shadowed by the red blood cell (RBC) hydrolysis results, which is used as a proxy for cytotoxicity. For the three dodecyl-substituted bisQACs, the therapeutic index—the ratio between efficacy and toxicity— for *Pseudomonas aeruginosa* is 1, which could potentially be problematic considering the potential human exposure to these compounds if commercialized. Some of 13-carbon tail compounds, while requiring slightly higher concentrations, have more promising therapeutic indices. These previously unreported compounds (oX-13,13, mX-13,13, pX-13,13) possess MIC values ≤ 16 μM across the entire bacterial panel. From this set, pX-13,13 has a promising therapeutic index of 4. Furthermore, there was little to no difference in the biological activity of our prepared biscationic

compounds according to molecular geometry. While this is consistent with the trends observed in the patent literature, [25,26] our study demonstrated that this trend also operates in a wider range of chain lengths—with the exception of mX-16,16 which displayed up to 8-fold improved activity over its ortho- and para- isomers.

Similar to the bisQACs, trisQAC activity initially increases with increasing chain length and then reaches its maximum at 10 carbons. In fact, trsiQACs may be even more sensitive to this phenomenon as both Mes-9,9,9 and Mes-12,12,12 marked a more noticeable change in performance (MIC 16–63 μM) against *P. aeruginosa* and *A. baumannii* than was observed in analogous changes in the bisQACs. The 10-carbon tail compound, Mes-10,10,10, displayed the best activity for the entire set of bisQACs and trisQACs. Mes-10,10,10 possesses MIC values ≤8 μM against the bacterial panel and has a maximum therapeutic index of 2 observed against *P. aeruginosa*. With these consideration, Mes-10,10,10 stands out as the best compound due to its potency and desirable therapeutic index.

In conclusion, this series exemplifies how multicationic species may delivery superior bioactivity as compared to the monocationic speies, using the BAC scaffold as an example. While an elevated MIC value (16 μM) was observed for BAC against HA-MRSA, multicationic BAC species show no such difference in performance. Interestingly, shorter non-polar chain length in the bis- and triscationic variants resulted in more desriable therapeutic indicies. The potent activity of multicationic variants, coupled with their efficient synthesis, provide support for the development of multicationic amphiphiles as the future of disinfection. Of course, this will vary depending on molecular scaffold, but the results presented herein were nonetheless promising.

2.2.2 Atom Economic Alkyl Phosphonium Biocides

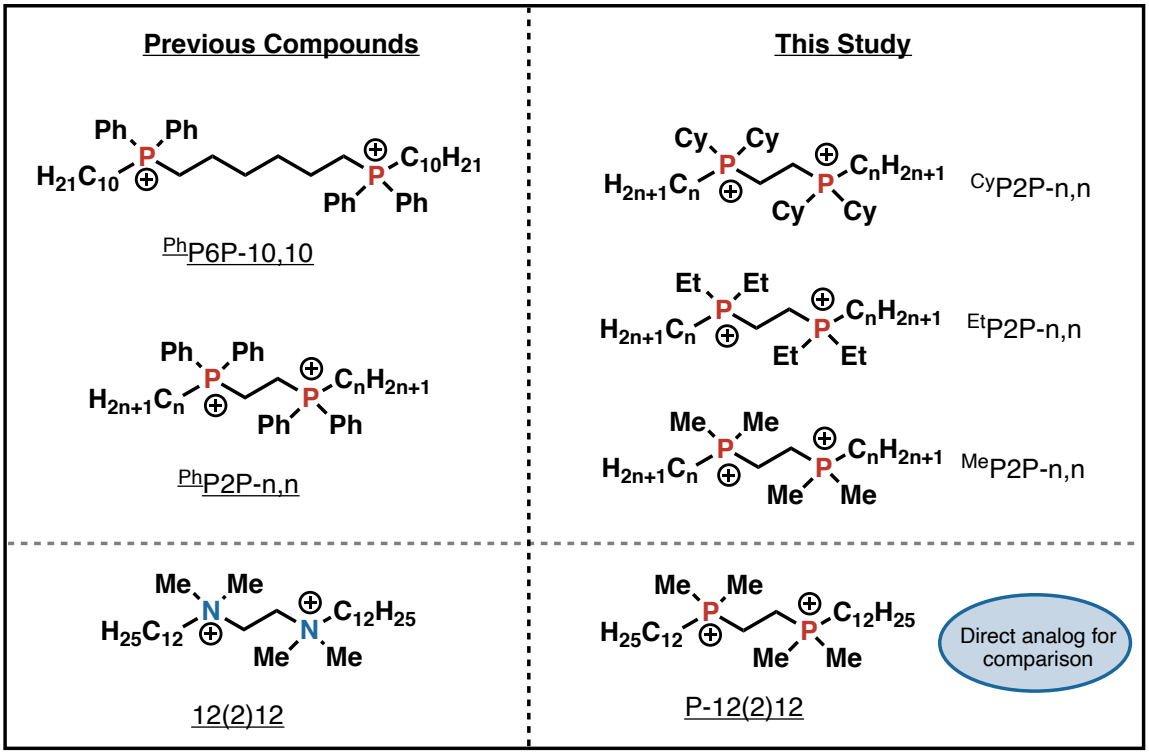
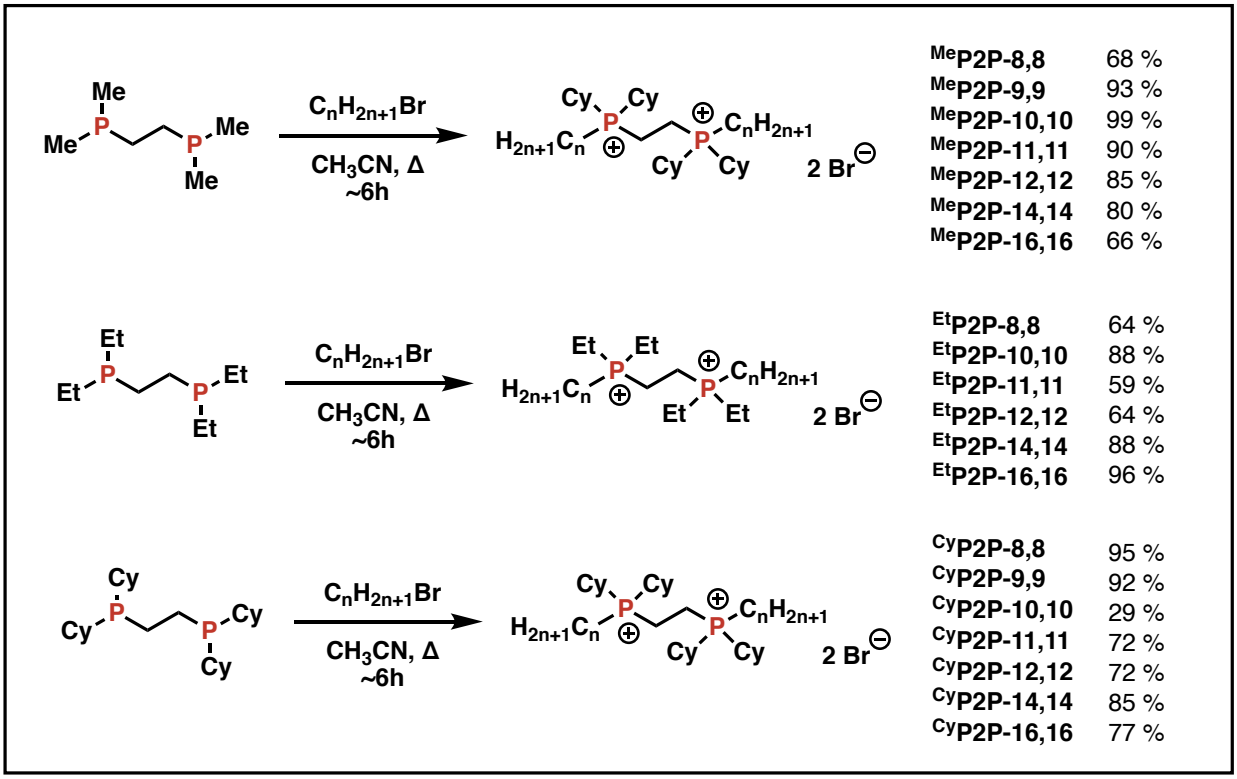


Figure 2.6 Atom-economical QPCs and direct nitrogen analogs

Based on the success of the early generation of bisQPC disinfectants (*vide supra*), new series of compounds were desired to expand this class of cationic amphiphiles. While the previous set proved to be potent against the bacterial panel, the molecular weights of the top performing compounds hover around 900 g/mol, while common commercially available QACs (BAC, DDAC, benzethonium chloride, and cetylpyridinium chloride) are all under 500 g/mol. These high molecular weights bring with them concerns about solubility, due to their high lipophilic character; ability to diffuse, due to their high molecular weight; and atom economy. In addition to expanding the current toolset of QPCs, a major question was still unanswered: is there any benefit to phosphorous over nitrogen as the cationic element? Through this work, two parallel goals were set: (1) determine if the bulky phenyl groups could be replaced with smaller alkyl groups, rendering



Scheme 2.2 Synthesis of atom economical QPCs

the disinfectant structures more atom economical, and (2) prepare exact nitrogen- and phosphorus-bearing analogs in amphiphilic disinfectants, thus scrutinizing the role of the cationic element in the antimicrobial action. Inspiration for the project came from tetramethylethylenediamine-derived bisQACs from previous work, specifically 12(2)12, and prior work in comparing analogous monoQAC and monoQPC compounds.[24,25] In order to prepare analogous structures, the phenyl rings on the ^{Ph}P2P-n,n structure were replaced with alkyl (methyl, ethyl, and cyclohexyl) substituents, leading to a series dubbed ^{Cy}P2P-n,n, ^{Et}P2P-n,n, and ^{Me}P2P-n,n, respectively. Cyclohexyl-substituted QPCs would serve to inform the transition from aromatic substituents in the first generation of bisQPCs to alkyl substituents, presumably providing steric protection from air oxidation for the phosphorus atom. Preparation of methyl- and ethyl-substituted bisQPC analogs would serve to maximize atom economy within this structural motif and identify minimum structural requirements for strong bioactivity. The methyl-substituted phosphonium compounds

allow for direct comparison of bisQPC to bisQAC activity since the only change in the molecules would be the pnictogen atom (nitrogen or phosphorus) of the cationic headgroup.

Preparation of the alkyl-substituted bisQPCs was achieved through the treatment of the corresponding bisphosphinoethane with excess alkyl bromide in acetonitrile at elevated temperatures. BisQPCs were isolated in 60–95% yields, with lower yields stemming from the difficulty of handling the waxy and glassy products. Reaction time required to reach maximum conversion correlated with steric hindrance of the phosphine ligand, with the less sterically hindered methyl- and ethyl- substituted phosphines finishing within 5–6 h, while cyclohexyl phosphines generally required heating for 24 h to ensure complete alkylation. It is notable that,

	MIC (μM)							
Compounds	MSSA	CA-MRSA	HA-MRSA	*E. faecalis*	*E. coli*	*A. baumannii*	*P. aeruginosa*	Lysis20
Control - BAC	4	4	16	63	63	32	250	63
Control - ^{Ph}P6P-10,10	1	1	1	2	2	4	2	8
Control - ^{Ph}P2P-10,10	1	0.5	1	8	4	2	8	16
Control - 12(2)12	**2**	**2**	**1**	**8**	**2**	**2**	**8**	**16**
^{Me}P2P-8,8	125	125	250	>250	>250	>250	>250	250
^{Me}P2P-9,9	8	8	16	125	125	250	>250	250
^{Me}P2P-10,10	2	2	2	16	16	63	32	63
^{Me}P2P-11,11	2	1	1	2	4	16	8	16
^{Me}P2P-12,12	**2**	**2**	**1**	**4**	**4**	**8**	**8**	**8**
^{Me}P2P-14,14	8	4	8	32	32	125	63	8
^{Me}P2P-16,16	32	32	63	250	250	>250	>250	125
^{Et}P2P-8,8	63	125	250	>250	>250	>250	>250	250
^{Et}P2P-10,10	2	1	8	16	16	32	32	63
^{Et}P2P-11,11	1	1	2	4	4	8	16	16
^{Et}P2P-12,12	1	1	1	16	2	2	8	8
^{Et}P2P-14,14	8	4	4	63	8	32	63	8
^{Et}P2P-16,16	16	16	32	63	125	260	125	16
^{Cy}P2P-8,8	2	2	8	63	63	125	250	125
^{Cy}P2P-9,9	1	1	2	16	16	16	32	125
^{Cy}P2P-10,10	1	1	1	8	8	8	8	16
^{Cy}P2P-11,11	1	1	1	8	4	4	8	8
^{Cy}P2P-12,12	2	2	2	16	8	4	16	16
^{Cy}P2P-13,13	2	2	1	32	32	16	63	8
^{Cy}P2P-14,14	4	4	2	32	63	63	63	8
^{Cy}P2P-16,16	8	2	2	125	125	125	63	32

Table 2.3 Biological activity of atom economic QPCs

while reactions with alkyl bromides were uniformly successful, the use of alkyl chloride electrophiles led to incomplete reactions and, ultimately, significant phosphorus oxidation in the Minbiole lab.

Beginning with the biological assessment, we began to examine the activity of the alkyl QPCs in comparison to phenyl-containing analogs. In line with expectations, the most potent antimicrobial activity was observed for the structures with alkyl chain lengths of 10–12 carbons. Performance progressively diminished as chain lengths either increased to 16 carbons or decreased to eight carbons. The amphiphiles with the lowest MICs within this series ^{Cy}P2P-11,11, ^{Et}P2P-12,12, and ^{Me}P2P-12,12; all display single-digit micromolar activity with only one exception in the EtP2P-12,12 panel. Compounds with chain lengths of 11 and 12 having the highest activity hardly came as a surprise. As previously reviewed, this seems to be the "sweet spot" for potent antibacterial activity. Among the three top-performing compounds, ^{Cy}P2P-11,11 has a slight overall advantage in broad-spectrum potency within this data set, with MIC values of 1–8 μM. While these compounds have lower MICs than commercial BAC, they do not reach the level of performance observed with our current best-in-class QPC, ^{Ph}P6P-10,10, which has MIC values of 1–4 μM across the panel. Nevertheless, the top-performing compounds possessed good efficacy against the Gram-negative pathogens in the panel, which are historically more different to inhibit due to their intrinsic resistance mechanisms, such as their protective outer membrane and their ability to express multidrug efflux pumps with cross-resistance to QACs. Finally, the hemolysis ($lysis_{20}$) assays for ^{R}P2P-n,n compounds demonstrate no observed impact corresponding to the substituting group (R). When considering toxicity, ^{Cy}P2P-9,9 is the top-performing compound, which displayed low MIC values for gram-positive strains and slightly higher for gram-negative strains. Coupled with and a modest $lysis_{20}$ value of 125 μM, the therapeutic index of 5 is promising

for further development. This represents an improvement in antimicrobial performance with diminished hemolysis over the commercial BAC control. This work supports that QPCs are a nascent, structurally diverse disinfectant class that possess sustained activity against antimicrobial resistant bacteria.

While the top performing compounds had virtually identical activity, it should be noted that there appeared to an important nuance added to our understanding of chain length optimization. For the Et and the Me substituents, the dodecyl chains are the optimal side chain (n=12). However, when the substituents are Cy or Ph, the optimal alkyl chains are decyl and undecyl (n=10 and 11). There potentially exists some additive effect on heteroatom substitution and chain length. As lipophilicity to the amphiphile as heteroatom-substituents, it is possible that less lipophilicity is needed on alkyl tails. However, it could be a more complex interaction related to compound diffusion through solution or micelle formation. Regardless, the exact physical phenomena related to diffusion, surface tension, and membrane interactions remain underexplored in our pursuits and might illuminate important factors that we have yet to consider. Nonetheless, preliminary physical characterization of analogous QPC and QAC structures presented in subsequent sections represent our first foray into this side of characterization.

Thinking further about the alkyl QPC series in contrast to previous series, we observe similar performance between ^{Ph}P2P-10,10 and ^{Cy}P2P-11,11, which suggest that electronic differences do not lead to a marked change in bioactivity with this particular linker and chain length, though it may not necessarily be generalized to QPCs at a whole based on this work alone. Alternatively, the retention of activity may not be a result of electronic agnosticism, but there may be steric effects in place that orient the amphiphile in favorable positions.

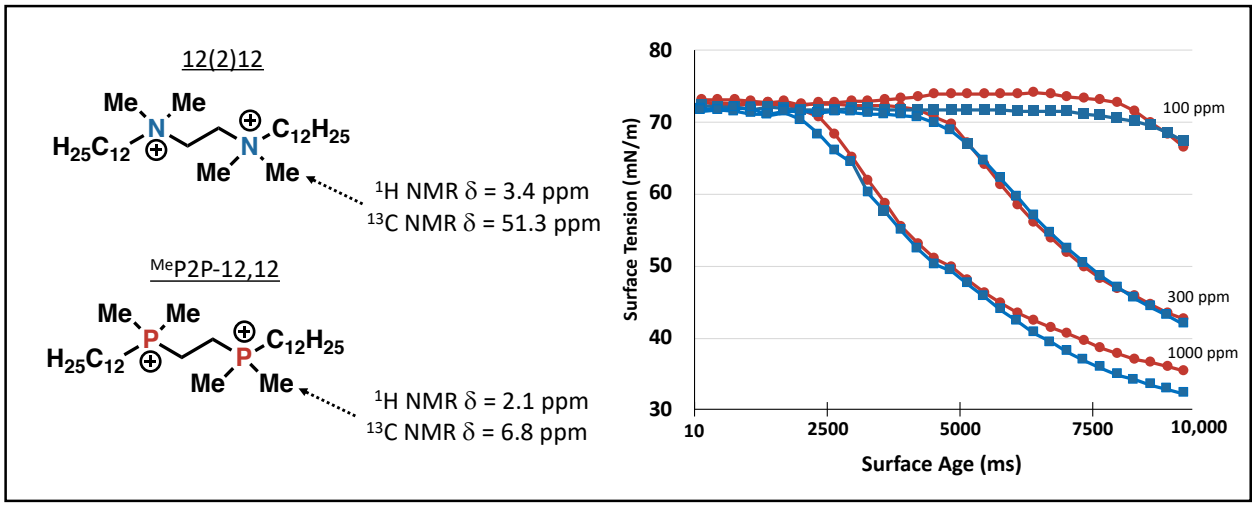


Figure 2.7 Physical comparison of quaternary nitrogen and phosphorus analogs

From this work, we can directly compare a structurally analogous bisQAC and bisQPC to examine the effect on heteroatom on physical properties and biological activity. Cationic biocides 12(2)12 and ^{Me}P2P-12,12 share the same carbon scaffold, only differing in the ammonium or phosphonium center. Two methods to characterize their physical properties were used: nuclear magnetic resonance (NMR) to understand cationic effects on nearby atoms and dynamic surface tension to understand solution dynamics. Comparing the NMR data of ^{Me}P2P-12,12 and 12(2)12, there were some appreciable differences in chemical shifts. Both ^{1}H NMR and ^{13}C NMR reveal upfield shift in the chemical shifts for the phosphonium methyl substituent compared to the ammonium methyl group. A lower chemical shift (upfield) indicates less deshielding occurring on the proton and carbon atoms in the QPC. Less deshielding in QPCs denotes a greater electron density on those carbon atoms compared to the methyl groups in the ammonium. Because phosphorous has a lower electronegativity than carbon, it retains its partial positive charge. This results in partially negative carbon atoms, which we observe with the lower chemical shifts. By contrast, nitrogen is more electronegative than carbon. Thus, nitrogen possesses a partial negative charge with partial positives on the adjacent α-carbons, which is reflected in the downfield chemical shifts for the QACs. Further, we sought to analyze the solution behavior of our

amphiphiles to compare between analogous QPCs and QACs. Interested in whether the heteroatom of the cationic species influenced dynamic surface tension (DST), we examined the nature of this amphiphiles in solution. The dynamic surface tension experiments were completed at three different concentrations of amphiphile (100, 300, and 1000 ppm). When comparing the dynamic surface tension (DST) of 12(2)12 and ^{Me}P2P-12,12, we observe virtually identical behavior despite the change of cationic center. This indicates that structural similarity may be the prevailing factor in physicochemical properties and antimicrobial behavior rather than the cationic element.

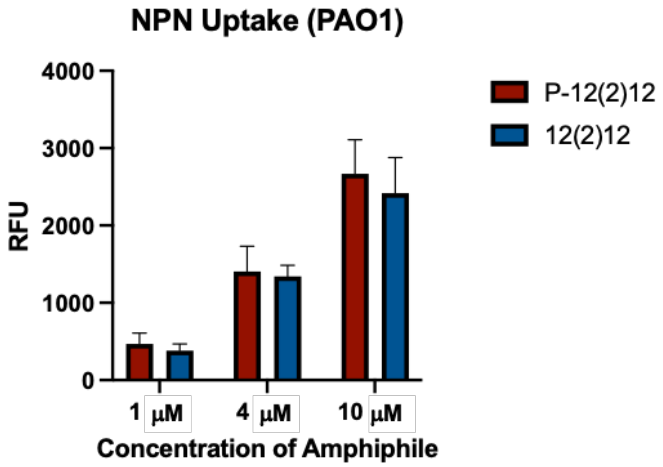


Figure 2.8 Outer membrane perturbation of quaternary nitrogen and phosphorus analogs

Though little difference was observed in MIC values and physical properties, we sought to examine whether there may be any nuance in membrane interactions. To accomplish this, we utilized the NPN uptake assay, which is used to study outer membrane disruption of gram-negative bacteria. Upon disruption of the bacterial outer membrane, NPN intercalates into the outer membrane which results in an increase of fluorescence. This assay is simple to perform, requires an inexpensive fluorophore, and rapidly generates valuable data. Upon testing 12(2)12 and its phosphorous analog (dubbed P-12(2)12) at increasing concentrations, we observe no significant

difference in outer membrane disruption. This revealed to us that there was no effect of heteroatom on amphiphile interactions with the outer membrane.

Revisiting the goals of this project, we were indeed able determine if the bulky phenyl groups could be replaced with smaller alkyl groups and (2) prepare exact nitrogen- and phosphorus-bearing analogs disinfectant for comparison. We were able to observe that smaller alkyl groups for QPC amphiphiles were indeed efficacious in inhibiting the bacteria in our panel. These lower molecular weight amphiphiles displayed comparable activity while decreasing the molecular weight of the active components by over 200 g/mol compared to previous series of QPCs. Furthermore, these compounds had promising MBECs, which demonstrate potential in being translated to disinfectant surfaces that have already been colonized by bacteria. When comparing analogous QPCs and QACs, there were no significant differences observed in biological activity, dynamic surface tension measurements, and amphiphile-membrane interactions. Despite some charge localization differences, the choice of heteroatom has no real impact on performance. What then is the advantage of continuing to study QPCs over the traditional QACs? I propose that structural diversity is the key motivation to continue studying QPCs. Commercial availability of starting material varies widely from phosphorous (III) substrates compared to nitrogen. While there are certainly more commercially availability amines that serve as potential precursors for disinfectants, the available phosphines differ significantly enough from the amines to make their study worthwhile. In this series, analogous phosphines and amines were used, but previous studies such as the initial phenyl-substituted series contained scaffolds or substituents (i.e., the diphenyl pattern) that are less readily accessible or inaccessible by amine chemistry.

2.3.4 Rigidity Effects on Phosphorous Scaffolds

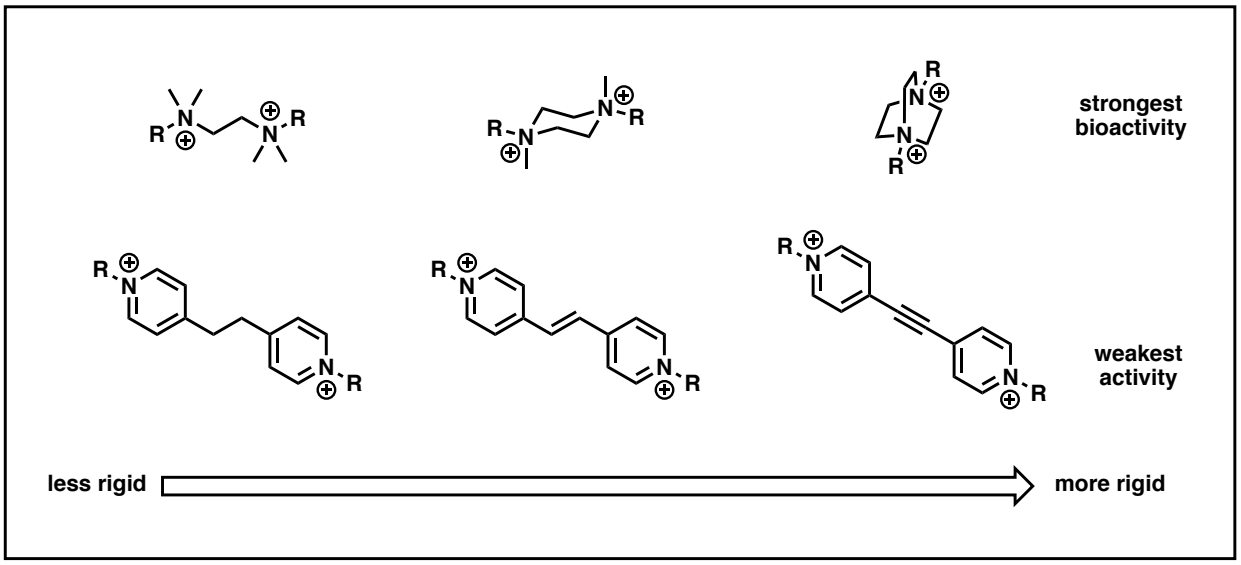
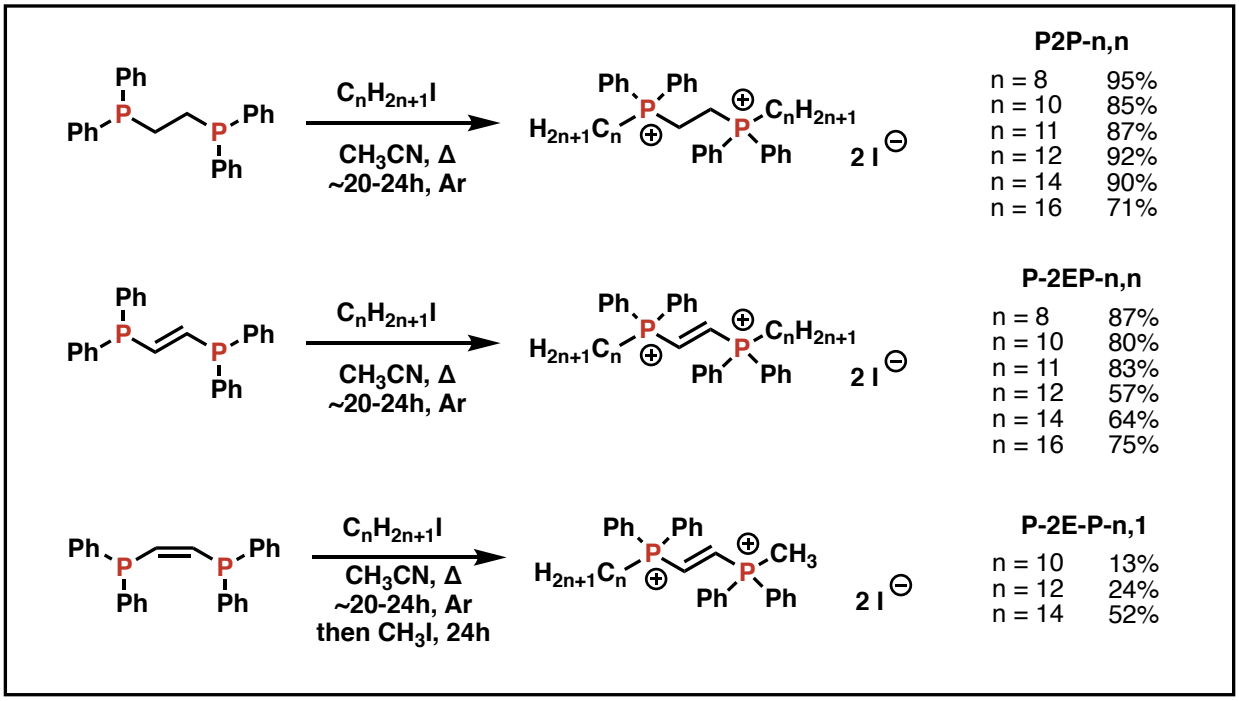


Figure 2.9 Rigidity effects of QACs from previous studies

Because QPCs have been underexplored in the past, there is little data on their structure-activity relationships (SAR).[24,26] Due to our previous success with multicationic amphiphiles, we sought to further dive into bisQPCs to understand how different structural motifs affect their inhibitory activity against the panel of bacteria. In order to develop greater SAR knowledge of bisQPCs, we aimed to mimic previous reports from the Wuest and Minbiole laboratories that focused on the rigidity of the linkers between the cationic centers.[27,28] Results from the alkyl amine report revealed that more rigid structures improved activity, while pyridine derived compounds showed the opposite effect. Examining closer, the dissonance between the two conclusions might be rectified by examining the relative placement of the cations. As the cations are brought closer together (or at least have the conformation flexibility to be near one another), activity increases. In this study, we evaluated a series di(bisphenyl)phosphonium amphiphiles with 2-carbon linkers. These linkers had varying degrees of rigidity (alkyl, *E*-alkene, and *Z*-alkene). Hypothesizing that

these would display differential antimicrobial activity based on rigidity, this set of amphiphiles were synthesized in the Minbiole laboratory and delivered to us for biological evaluation.



Scheme 2.3 Synthesis of QPC rigidity series

Initially, the synthesis was attempted using alkyl bromides as carbon chain as typically done in previous sets of amphiphiles. However, the reactions were sluggish and resulted in poor conversion when attempted on the alkene-linked phosphines. To promote the reaction, the more electrophilic alkyl iodides carbon chains were used and resulted in good-to-moderate conversion for the alkyl P2P-n,n series and the P-EP-n,n series. However, when the bisalkylation was performed on the Z-alkene, the reactions were unsuccessful. This is believed arise from steric hindrances from the first large alkyl group in such close proximity to the mirroring phosphine. The lack of conformational flexibility arising from the alkene then renders the second nucleophilic phosphorous unable to react with a second large electrophile, in contrast to the alkyl P2P-n,n series. However, a set of three asymmetric bisQPCs were synthesized with a 10, 12, and 14 carbon chain

on one end using the Z-alkene as a precursor. Subsequently, a methyl iodide was used as a single carbon source to quaternize the free phosphine resulting in the P-2E-P-n,1 series. Upon inspection, it was revealed that the Z-alkene isomerized to the E-alkene, thus rather unambiguously communicating that E-alkenes are not a viable option in this bisQPC analog series using typical alkylation conditions. Finally, in an attempt to access rigid alkyne bisQACs, the alkyne linked (bis)phenylphosphine was exposed to 10 equivalents of alkyl iodide chain in dimethylformamide at reflux, followed by column chromatography purification. Rather than providing the bisQPC of interest, an interesting side reaction first reported by Stang et al. was observed.[29] The alkyne was reduced to the E-alkene and the iodide counterion was transformed to the triiodide ion.

		Minimum Inhibitory Concentration (μM)						$Lysis_{20}$ (μM)
	Compound	MSSA	CA-MRSA	HA-MRSA	*E. facaelis*	*E. coli*	*A. baumannii*	
QACs	BAC	4	8	8	32	63	32	16
	CPC	2	2	2	32	16	16	16
	DDAC	1	2	2	8	8	8	8
Alkyl QPCs	P2P-8,8 Br[a]	1	0.5	8	250	>250	ND	125
	P2P-10,10 Br[a]	1	1	2	8	16		16
	P2P-11,11 Br[a]	1	1	2	8	32		32
	P2P-12,12 Br[a]	1	2	8	32	63		16
	P2P-8,8 I	4	4	63	>250	250	>250	125
	P2P-10,10 I	8	8	1	8	8	8	16
	P2P-11,11 I	4	4	2	16	16	8	32
	P2P-12,12 I	8	16	2	63	63	32	16
	P2P-14,14 I	4	8	4	63	250	32	16
	P2P-16,16 I	16	16	32	250	>250	>250	16
E-alkene QPCs	P-2E-P-8,8 I	2	4	16	63	32	32	125
	P-2E-P-10,10 I	2	2	4	16	16	16	63
	P-2E-P-11,11 I	1	1	2	32	32	32	16
	P-2E-P-12,12 I	1	2	2	16	63	32	16
	P-2E-P-14,14 I	2	2	2	32	250	32	16
	P-2E-P-16,16 I	16	4	4	125	>250	>250	16
	P-2E-P-10,1 Br I	125	125	16	8	63	63	250
	P-2E-P-12,1 Br I	4	2	4	8	32	16	125
	P-2E-P-14,1 Br I	4	4	4	2	32	32	32
	P-2E-P-8,8 I_3	63	125	125	>250	>250	>250	125
	P-2E-P-10,10 I_3	16	16	32	125	250	125	125
	P-2E-P-11,11 I_3	125	125	16	>250	>250	>250	125
	P-2E-P-12,12 I_3	125	125	125	>250	>250	>250	125
	P-2E-P-14,14 I_3	250	>250	32	>250	>250	>250	250
	P-2E-P-16,16 I_3	125	125	63	>250	>250	>250	125

Table 2.4 Biological activity of QPC rigidity series

Though the initial series of increasing rigid bisQPCs with varying conformational flexibility was elusive, a sufficient series of analogs had been prepared for biological investigation. Beginning with the most flexible bisQPCs, we observe positive results in bacterial inhibition and low red blood cell lysis, with the top performing compound being P2P-10,10 I^-. Compared the previously synthesized analog P2P-10,10 I^-, we observe little variation in activity for this scaffold when exchanging the counterion. Comparing the 8-, 11-, and 12-carbon chained amphiphiles for the P2P-n,n series, we again see little variation in MICs, though the activity is more modest. Moving on to the more rigid *E*-alkenes, it is clear that for this scaffold, 10-carbon length chains yield the compounds with the lowest MICs. While P2P-10,10 I^- possessed lower MIC values than P-2EP-10,10 I^-, the unsaturated linker seems to increase the $lysis_{20}$ value suggesting a decrease in mammalian toxicity. Though this decrease in toxicity only occurs at this chain length, suggesting that this may not be a general trend to follow when designing subsequent analog series. Aside from this nuance, there were no general trends observed from the rigidification of the phosphonium linker. Transitioning from the unsaturated iodide compounds to the intriguing triiodide species, the latter class were wholly disappointing as a class. Compared to the analogous iodide species, the *E*-alkenes with the I_3^- was over 100 times less active. This is believed to be due to a combination of the instability and insolubility caused by the more lipophilic anion. Paired with the low activity was consistently high $lysis_{20}$ values. Since these compounds had little activity against bacterial cell membranes, their low lytic potential is not surprising. Finally, the 12- and 14-carbon chain asymmetric compounds had excellent inhibitory activity against MSSA, CA-MRSA, HA-MRSA, and *E. faecalis*, but activity substantially increased when tested against gram-negative *E. coli* and *A. baumannii*. These MIC values, however, were comparable to BAC against when tested against these gram-negative pathogens.

Overall, the incorporation of rigidity into these scaffolds did not substantially affect the activity of the bisQPCs. Counterion swapping from iodide to bromide only seemed to diminish activity, with activity even more affected by the transition from iodide to triiodide. We believe that this is due primarily to solubility issues with the iodide and triiodide species.

2.3.5 "Soft" QPC Amphiphiles

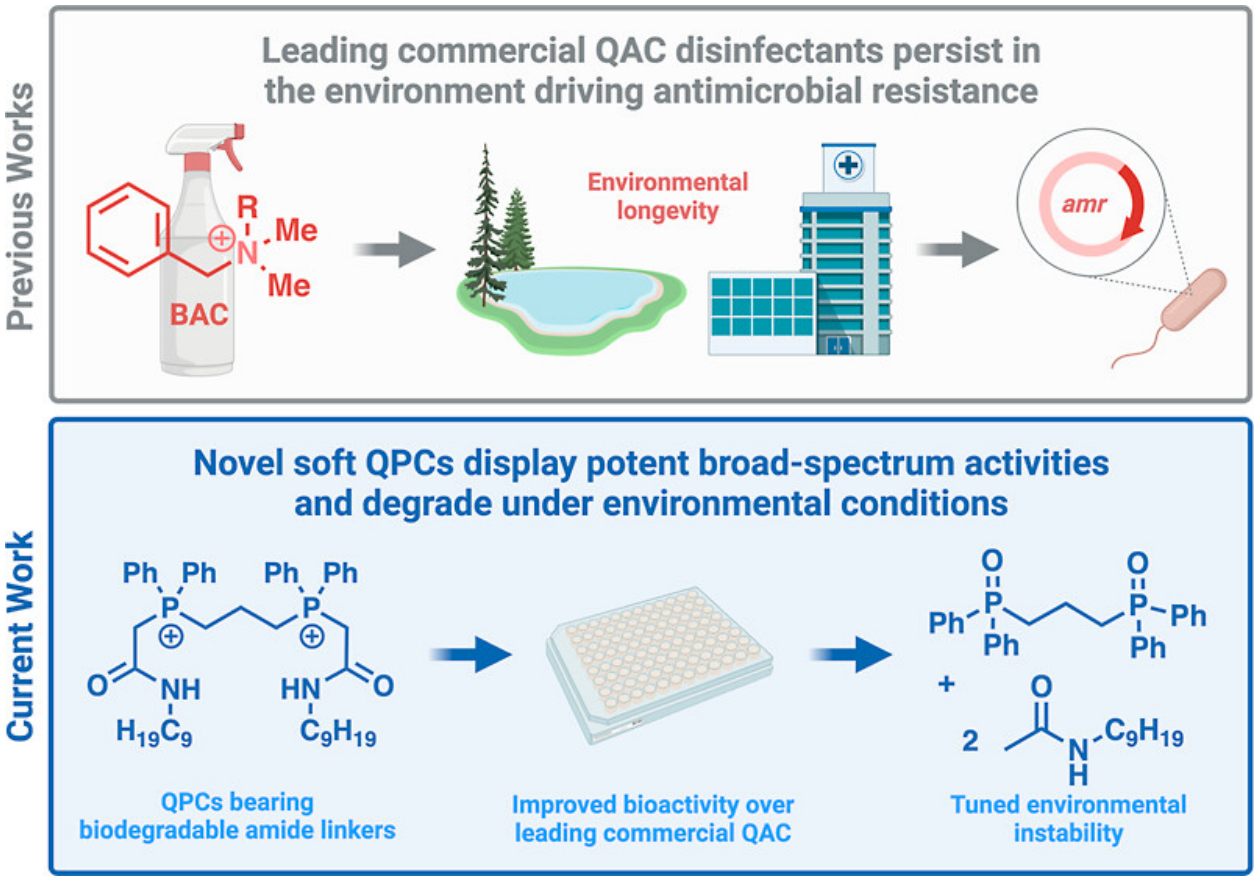


Figure 2.10 "Soft" amphiphiles to delay resistance in the environment

Roughly 75% of QACs once used are released into wastewater treatment plants with the remaining 25% directly entering the environment.[30] In most cases, treatment plants are effective in removing QACs via absorption onto activated sludge.[6] Despite treatment, residual QAC concentrations of 20–300 μg/L have been found in surface water even after processing and even higher concentrations can be found when downstream from municipal/industrial wastewater

treatment plants and hospitals.[31] Long-term environmental exposure to QACs has shown growth inhibition and lethal effects in most aquatic organisms.[32] BAC in particular possesses an environmental half-life of 9 months due to its chemical stability and slow biodegradation.[33] From their longevity, increased antimicrobial resistance has emerged as these compounds persist in the environment.[34] Currently, there exists a need for novel cationic disinfectant that can balance potency and environmental considerations, such as slow biodegradation. This is challenging considering stability of active compounds is paramount for effective commercialization. Though, we were led into this area in order to innovate to produce a series of compounds that is stable in most environmental conditions unless treated in a specific manner.

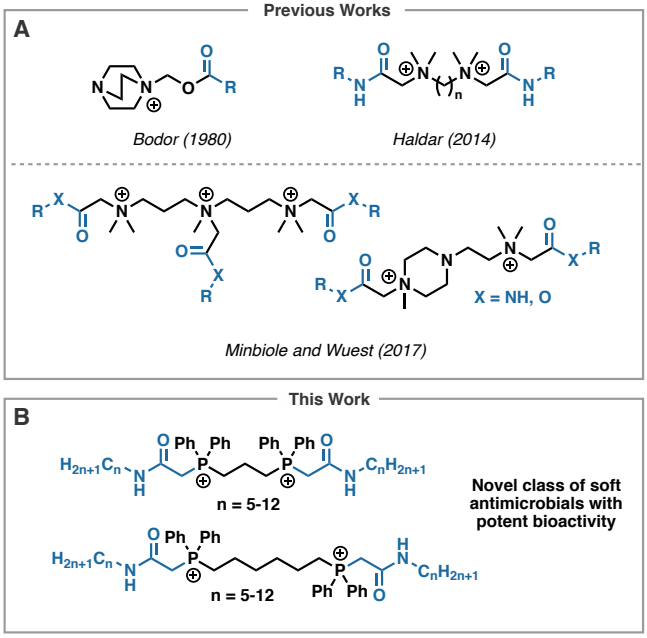
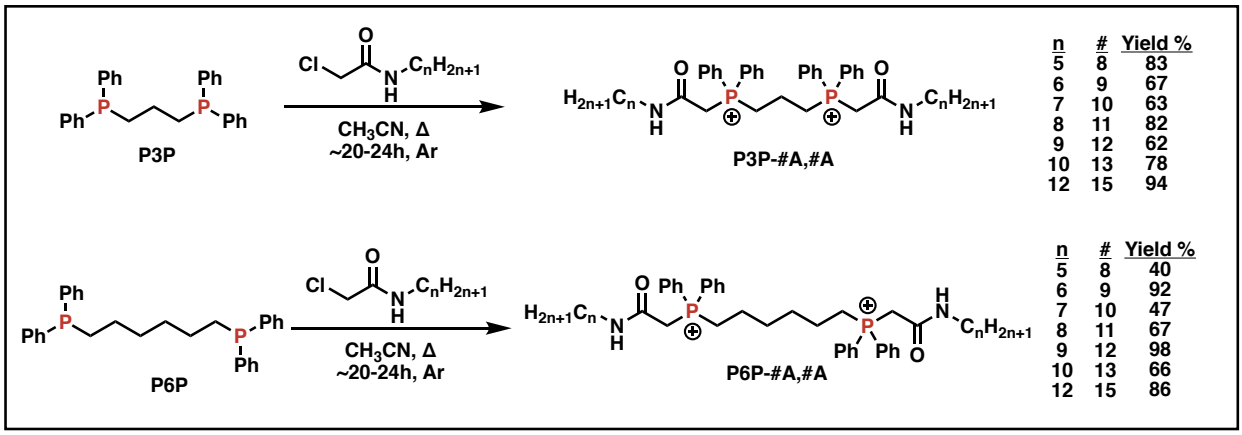


Figure 2.11 Previous work in nitrogenous "soft" amphiphiles

Over the past decade, efforts between the Wuest and Minbiole group have led to the preparation and biological assessment of over 850 novel QACs. Previously, investigations towards development of novel hydrolyzable as antimicrobial agents with tuned instability were performed. This was ultimately aimed at preparing amphiphiles whose environmental impacts could be minimized by decomposing into non-lytic components once in the environment. Focusing on

multicationic QAC architectures, the Wuest and Minbiole group reported the synthesis of 40 QACs with either ester or amide linkers in 2017.[35] This initial foray provided encouraging antimicrobial and stability data. While the ester-containing QACs were generally short-lived in aqueous solution, the stability of the amide-containing QACs displayed a direct correlation with the overall pH of the sample. Under acidic conditions, the amides immediately decomposed, but the stability of over 24 h was apparent in deionized water and buffered solutions of pH ≥ 7. Importantly, these amide-based QACs displayed potent antimicrobial activity, with minimum inhibitory concentration (MIC) values at single-digit micromolar values. Our work added to a growing literature of QACs with designed instability—dubbed "soft QACs"—joining the efforts of Bodor, who coined the term "soft antimicrobials," as well as Ahlstrom, Haldar, and others.[36–39]



Scheme 2.4 Synthesis of softQPCs

As the cationic biocide research program began to delve into QPCs, it was time to revisit the soft antimicrobial concept with the current lead scaffold. Because QPCs had been shown to be effective at inhibiting the growth of various bacterial pathogens (*vide supra*), there was great promise in building upon current QPC knowledge by evaluating whether QPC could be designed as soft antimicrobials.

The soft QPCs were synthesized with the same general synthetic strategy as other QPCs and QACs, though in 2 steps rather than the usual single step syntheses. The quaternarization step involved commercially available bisphosphines as nucleophiles. Two series of soft QPCs were produced—one using 1,3-bis(diphenylphosphino)propane and the second using 1,6-bis(diphenylphosphino)hexane. These served as the core scaffold prior to alkylation with N-alkyl-2-chloroacetamide building blocks. These acetamides were synthesized with 5, 6, 7, 8, 9, 10, 12 carbon alkyl chains tethered to the amide. These were constructed by exposing the corresponding alkyl amines to chloroacetyl chloride. Altogether, this strategy afforded 14 novel soft QPCs.

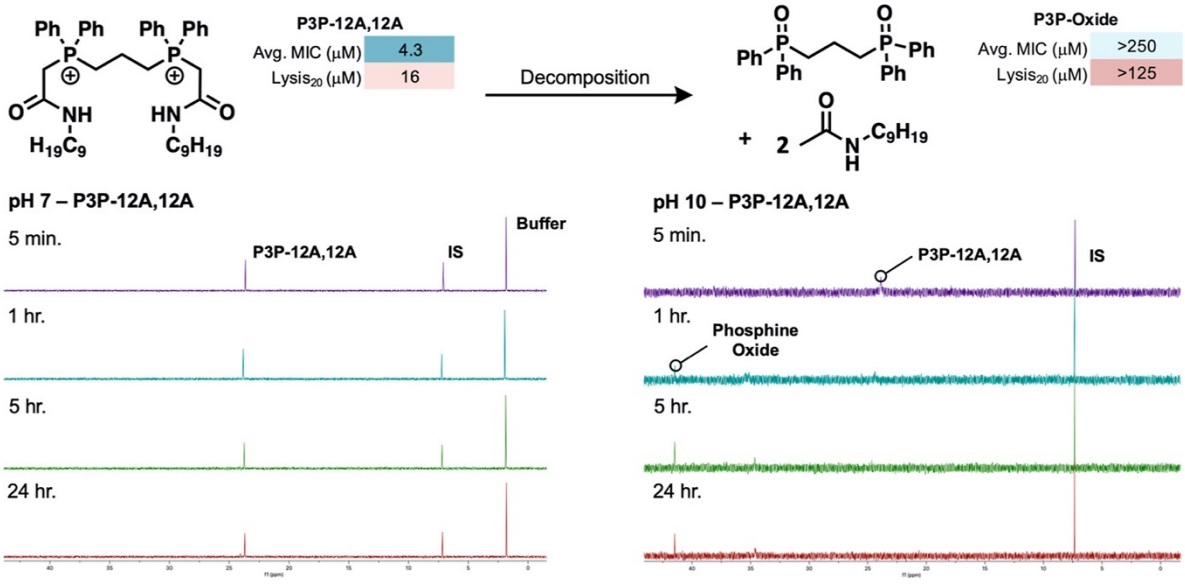


Figure 2.12 P3P-12A,12A decomposition studies in neutral and basic conditions

The stability of these soft QPCs was assessed using P3P-9A,9A and P6P-9A,9A as model substrates for each class. The relative stability was assessed by exposing the substrates buffered solutions of pH 4, 6, 7, and 10 as well as deionized water. Solutions (10mg/mL) were prepared with sodium hypophosphate pentahydrate ($NaH_2PO_2 \cdot 5H_2O$) as a nonreactive internal standard. While previous soft QACs readily hydrolyzed under acidic conditions, P3P-9A,9A and P6P-

9A,9A immediately precipitated at pH 4, though the two QPCs remained stable after 24 hours in milder conditions (pH 6 and pH 7). Under basic conditions (pH 10), P3P-9A,9A decomposed to the oxidized (diphenylphosphino)propane. Likewise, P6P-9A,9A decomposed to the oxidized (diphenylphosphino)hexane albeit at a longer timescale whereby it displayed stability over 24 hours and decomposed over the course of a week. These studies were repeated in the Minbiole group for the P3P-12A,12A and P6P-12A,12A under neutral (pH 7) and basic (pH 10) conditions. As with previous results, these compounds were both stable over 24 hours but decomposed under basic conditions within 24 hours. Interestingly, these compounds underwent hydrolysis at the phosphonium center rather than at the amide, as confirmed by ^{31}P NMR, and LCMS analysis of the test solutions.

In assessing the bioactivities of the soft QPCs, we measured bacterial MICs, biofilm eradication, hemolysis activity, and mitochondrial toxicity. The latter assay was included to assess whether the aryl phosphoniums species possessed any mitochondria targeting activity as suggested

		Minimum Inhibitory Concentration (μM)							
		Gram-Positive Pathogens				Gram-Negative Pathogens			$Lysis_{20}$
		MSSA	CA-MRSA	HA-MRSA	*E. faecalis*	*E. coli*	*A. baumannii*	*P. aeruginosa*	(μM)
QACs	BAC	4	4	8	32	16	16	125	63
	DDAC	1	1	4	8	4	4	32	32
	P6P-10,10	1	1	2	2	2	4	2	8
P3P-based QPCs	P3P-8A,8A	63	63	>250	>250	>250	>250	>250	>125
	P3P-9A,9A	8	16	63	250	250	>250	>250	>125
	P3P-10A,10A	2	2	8	16	8	32	63	>125
	P3P-11A,11A	1	1	8	8	4	4	32	32
	P3P-12A,12A	2	4	4	4	4	4	8	16
	P3P-13A,13A	4	2	4	8	8	8	63	16
	P3P-15A,15A	1	2	2	63	32	63	250	16
P6P-based QPCs	P6P-8A,8A	16	16	125	250	125	>250	>250	>125
	P6P-9A,9A	2	4	8	32	16	250	250	>125
	P6P-10A,10A	1	1	2	4	4	32	63	125
	P6P-11A,11A	1	1	2	2	2	4	16	16
	P6P-12A,12A	2	2	2	2	2	4	8	8
	P6P-13A,13A	1	2	2	8	8	8	16	8
	P6P-15A,15A	2	2	4	32	32	125	63	16
Br⁻ QPCs	P3P-13A,13A(Br-)	1	4	8	8	16	8	32	16
	P6P-8A,8A(Br-)	1	2	1	2	2	4	16	16
	P6P-11A,11A(Br-)	1	2	1	4	2	8	16	16
	P6P-15A,15A(Br-)	2	32	32	125	250	125	125	63
Ox.	P3P-Oxide	>250	>250	>250	>250	>250	>250	>250	>125
	P6P-Oxide	>250	>250	>250	>250	>250	>250	>250	>125

Table 2.5 Biological activity of SoftQPC series

by previous reports. The soft QPCs were broadly potent against gram-positive bacteria. While the parabolic relationship of activity relating to tail length was still observed, it was much less prominent in gram-positive species than in previous screens (*vide supra*). Little variation was observed between the P3P and the P6P classes, and the strongest determinant of potent inhibition was the length of the nonpolar alkyl chain. Considering inhibition of both gram-positive and gram-negative bacteria, P3P-12A,12A and P6P-12A,12A proved to be the most effective amphiphiles tested with average MICs against the whole panel at 4.29 μM and 3.14 μM, respectively. With these promising MIC results, we tested the ability for P3P-12A,12A and P6P-12A,12A to eradicate established biofilm. We were pleased to report that the soft QPCs displayed single-digit micromolar biofilm eradication activity comparable to DDAC, when tested against HA-MRSA. However, when measured against gram-negative pathogen *P. aeruginosa*, no biofilm eradication was detectable when tested below 250 μM. Though this was unsurprising since best-in-class QAC DDAC is unable to eradicate biofilm below 250 mM against *P. aeruginosa*.

	MBEC (μM)	
compounds	HA-MRSA	*P. aeruginosa*
P3P-12A,12A	4	>250
P6P-12A,12A	4	>250
^{Me}P2P-12,12	4	>250
DDAC	8	250

Table 2.6 Biofilm eradication activity of SoftQPC series

To evaluate the toxicity profiles of the best-in-class soft QPCs, we used the standard hemolysis assay along with a mitochondrial toxicity assay. When measuring hemolytic potential of the compounds, P3P-12A,12A had a slight advantage over P6P-12A,12A whereby the therapeutic indices were 2 and 1, respectively, against *P. aeruginosa*. Despite this, the compounds overall had favorable $lysis_{20}$ values, with higher therapeutic indices than commercial disinfectants

BAC and DDAC against hard-to-treat *P. aeruginosa*. To evaluate any potential mitochondrial toxicity, we sought the guidance of the Quave laboratory to aid our efforts in branching into mammalian cell culture, specifically with the assistance of Dr. Caitlin Risener.

Using a Promega Mitochondrial ToxGlo kit for the analysis, we cultured human hepatocellular carcinoma cells (HepG2) as the cell line for our tests. Since the experiment requires serum-free media, we initially cultured the cells in serum free RPMI-1640 at 37ºC and 5% CO_2. While the cells proliferated under these conditions, they were not able to reach an appropriate confluency for additional culture splitting. After several weeks of efforts towards promoting growth under these conditions, we were able to detect any further cell proliferation or viable cells. We began the culture again from freezer stocks this time with 10% fetal bovine serum (FBS) with the intention of washing the cells and resuspending them in FBS-free media prior to experimentation. As controls, BAC was used for a comparison to other cationic biocides while CCCP was used as a positive control due to its ability to stall mitochondrial function. We tested the active QPCs alongside their corresponding PxP-oxides that form upon decomposition. Pleasingly, no mitochondrial toxicity was observed for the QPCs tested.

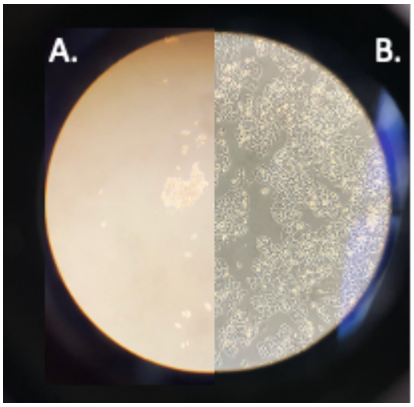


Figure 2.13 HepG2 cell culture in RPMI01640 A) without serum B) with serum

In summary, two highly potent QPCs with reduced environment impact were identified from a series of 14 novel "soft" amphiphiles with bisquaternary phosphonium centers. While multiple compounds displayed favorable activity, P3P-12A,12A and P6P-12A,12A displayed the most potent combination of inhibitory activity and low hemolytic potential. Furthermore, these compounds, when exposed to basic conditions, degrade into non-bioactive compounds. By

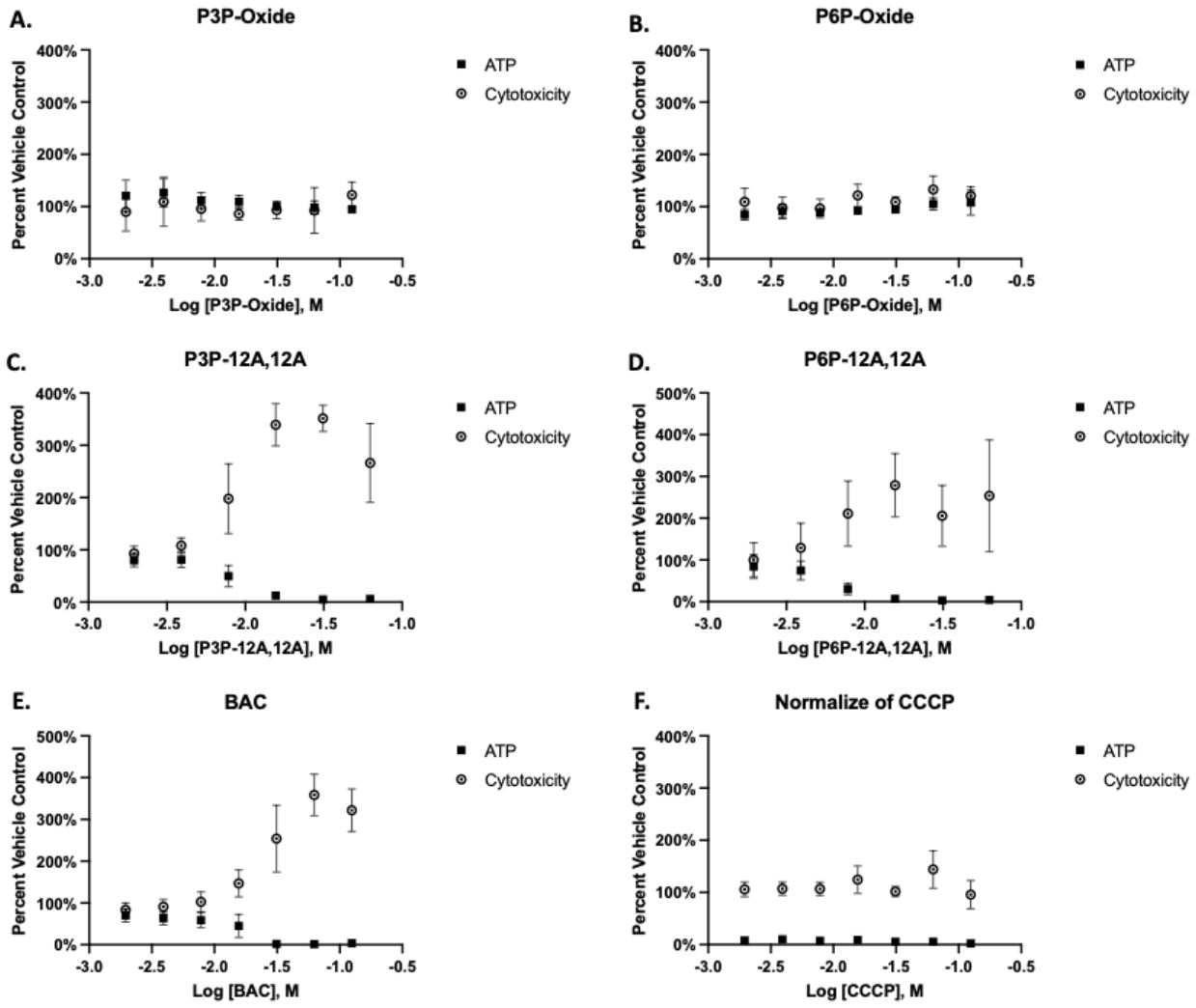


Figure 2.14 Mitochondrial toxicity of compounds with the Mitochondrial ToxGlo™ Assay. Panel A. and B. show P3P-Oxide and P6P-Oxide, respectively, with no changes in ATP or membrane integrity (MI). Panel C., D., and E. show P3P-12A,12A, P6P-12A,12A, and control benzalkonium chloride (BAC), respectively, with a reduction in ATP with commensurate MI changes indicating the occurrence of primary necrosis. Panel E. shows the protonophore CCCP reducing ATP concentration with no changes in MI, indicating mitochondrial toxicity.

designing these types of "soft" cationic biocides, we hopefully can prevent the development of antimicrobial resistance to these compounds by reducing their environmental half-life. While certain quaternary phosphonium compounds have shown toxicity against mammalian cells, we demonstrate that neither our best-in-class compounds or their decomposition products display any mitochondrial-specific inhibition or disruption.

2.3.5 Evaluation of Cationic Biocides Against a Panel of *Acinetobacter baumannii* Clinical Isolates

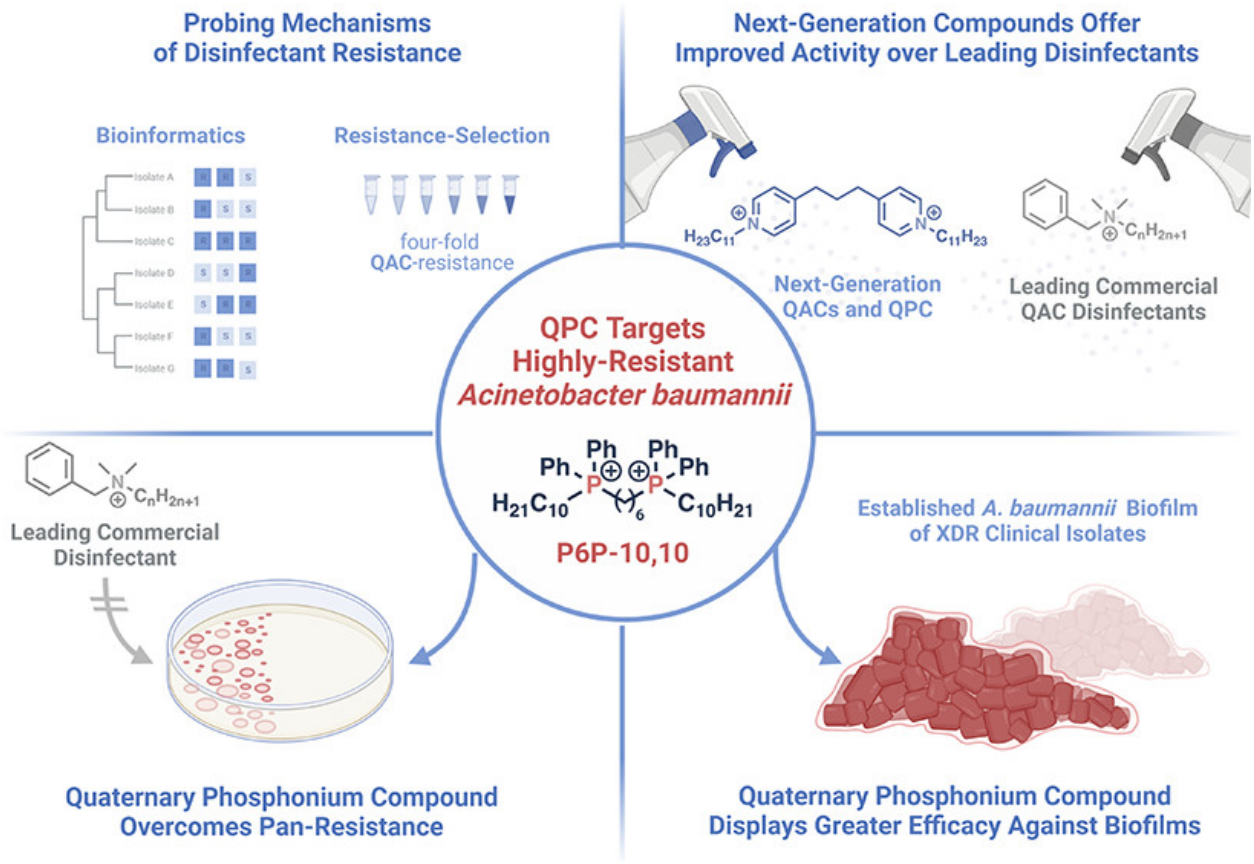


Figure 2.15 Overview of disinfectant studies on Acinetobacter baumanii

Acinetobacter baumannii represents one of the deadliest antimicrobial resistant pathogens.[40] One of the main contributors to the success of *A. baumannii* as a pathogen is in its ability to withstand desiccation and form robust biofilms on a variety of surfaces.[41] The growing burden of *A. baumannii* in nosocomial infections underscores the need for effective disinfectants to prevent the spread of bacterial threats. However, little work has been performed in understanding the degree of resistance to disinfectants in gram-negative species. For effective infection prevention and control, it is crucial to understand the relative efficacy of commercial cationic disinfectants against pathogenic strains beyond current laboratory strains commonly used. In 2020, Galac et al. reported a curated panel of *A. baumannii* clinical isolates for use in antimicrobial development.[42] As part of the Multidrug-resistant Organism Repository and Surveillance Network, these isolates came from blood, respiratory swabs, surveillance swabs, and would samples. Furthermore, these samples were isolated across four continents between 2003 and 2017, providing geographic and temporal diversity. Overall, the 100-strain panel encompasses

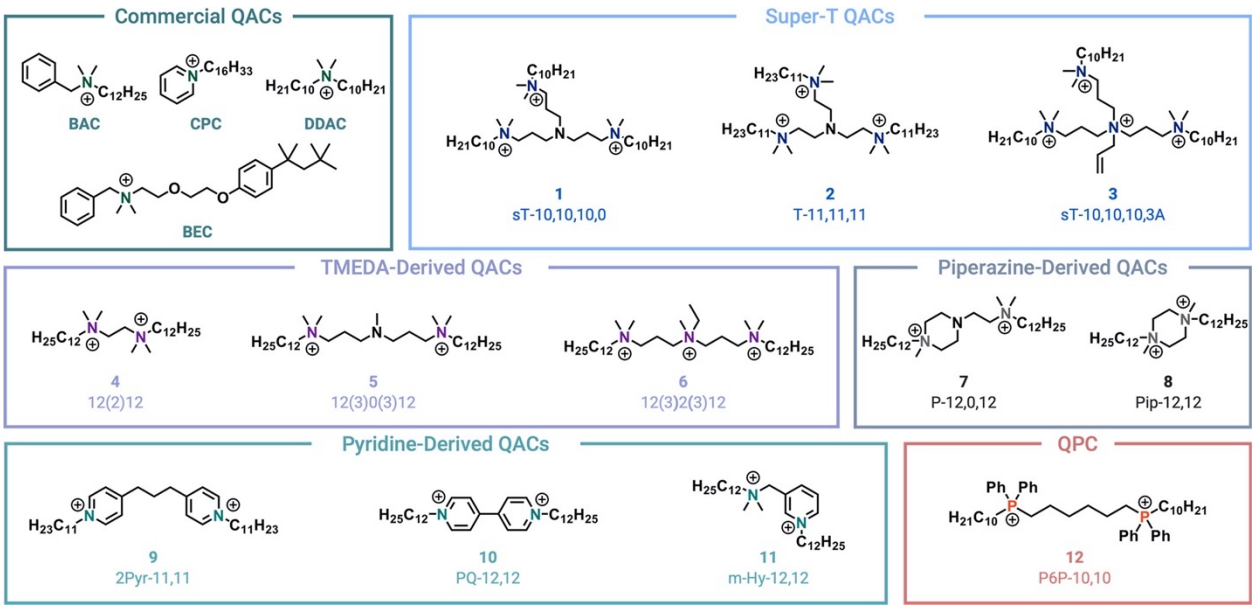
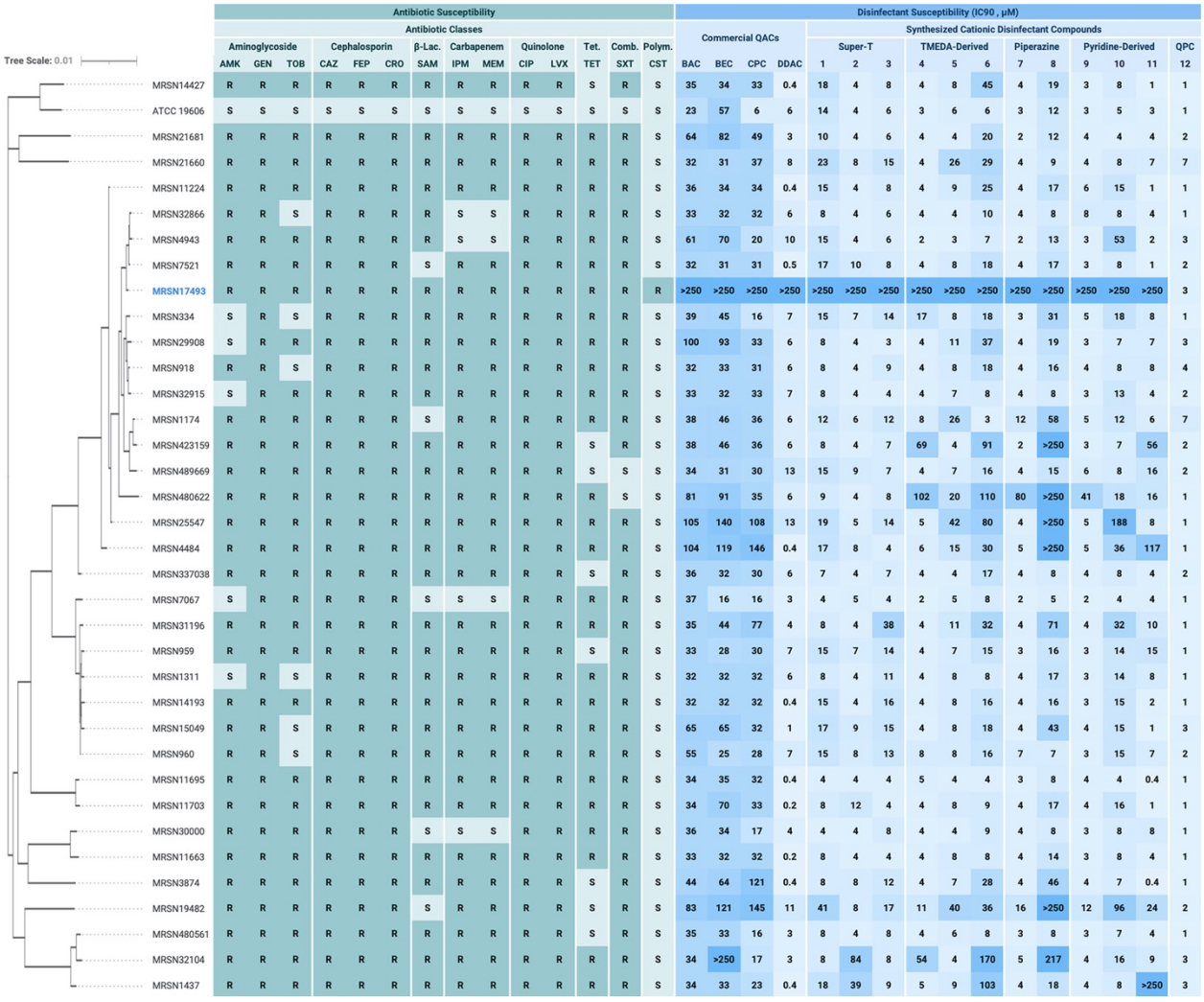


Figure 2.16 List of disinfectant used in current study

the genetic diversity of the species, both antibiotic-susceptible and -resistant isolates, and pandemic, epidemic, and sporadic clones.

Using an abbreviated form of this panel (35 strains), our group sought to evaluate the extent of disinfectant resistance within *A. baumannii*. Additionally, based on the efficacy displayed by previous reports of novel QACs and QPCs from the Minbiole and Wuest groups against gram-negative pathogens, we hypothesized that our best-in-class compounds would display high efficacy against clinical isolates of *A. baumannii*.[11,13,43–45] This project involved multiple generations of group members over the span of several years from the initial disinfectant screening



	Antibiotic Susceptibility														Disinfectant Susceptibility (IC90 , µM)												
	Antibiotic Classes														Commercial QACs				Synthesized Cationic Disinfectant Compounds											
	Aminoglycoside			Cephalosporin			β-Lac.	Carbapenem		Quinolone		Tet.	Comb.	Polym.					Super-T			TMEDA-Derived			Piperazine		Pyridine-Derived			QPC
	AMK	GEN	TOB	CAZ	FEP	CRO	SAM	IPM	MEM	CIP	LVX	TET	SXT	CST	BAC	BEC	CPC	DDAC	1	2	3	4	5	6	7	8	9	10	11	12
MRSN14427	R	R	R	R	R	R	R	R	R	R	R	S	R	S	35	34	33	0.4	18	4	8	4	8	45	4	19	3	8	1	1
ATCC 19606	S	S	S	S	S	S	S	S	S	S	S	S	S	S	23	57	6	6	14	4	6	3	6	6	3	12	3	5	3	1
MRSN21681	R	R	R	R	R	R	R	R	R	R	R	R	R	S	64	82	49	3	10	4	6	4	4	20	2	12	4	4	4	2
MRSN21660	R	R	R	R	R	R	R	R	R	R	R	R	R	S	32	31	37	8	23	8	15	4	26	29	4	9	4	8	7	7
MRSN11224	R	R	R	R	R	R	R	R	R	R	R	R	R	S	36	34	34	0.4	15	4	8	4	9	25	4	17	6	15	1	1
MRSN32866	R	R	S	R	R	R	R	S	S	R	R	R	R	S	33	32	32	6	8	4	6	4	4	10	4	8	8	8	4	1
MRSN4943	R	R	R	R	R	R	R	S	S	R	R	R	R	S	61	70	20	10	15	4	6	2	3	7	2	13	3	53	2	3
MRSN7521	R	R	R	R	R	R	S	R	R	R	R	R	R	S	32	31	31	0.5	17	10	8	4	8	18	4	17	3	8	1	2
MRSN17493	R	R	R	R	R	R	R	R	R	R	R	R	R	R	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	3
MRSN334	S	R	S	R	R	R	R	R	R	R	R	R	R	S	39	45	16	7	15	7	14	17	8	18	3	31	5	18	8	1
MRSN29908	S	R	R	R	R	R	R	R	R	R	R	R	R	S	100	93	33	6	8	4	3	4	11	37	4	19	3	7	7	3
MRSN918	R	R	S	R	R	R	R	R	R	R	R	R	R	S	32	33	31	6	8	4	9	4	8	18	4	16	4	8	8	4
MRSN32915	S	R	R	R	R	R	R	R	R	R	R	R	R	S	33	32	33	7	8	4	4	4	7	8	4	8	3	13	4	2
MRSN1174	R	R	R	R	R	R	S	R	R	R	R	R	R	S	38	46	36	6	12	6	12	8	26	3	12	58	5	12	6	7
MRSN423159	R	R	R	R	R	R	R	R	R	R	R	S	R	S	38	46	36	6	8	4	7	69	4	91	2	>250	3	7	56	2
MRSN489669	R	R	R	R	R	R	R	R	R	R	R	S	S	S	34	31	30	13	15	9	7	4	7	16	4	15	6	8	16	2
MRSN480622	R	R	R	R	R	R	R	R	R	R	R	R	S	S	81	91	35	6	9	4	8	102	20	110	80	>250	41	18	16	1
MRSN25547	R	R	R	R	R	R	R	R	R	R	R	R	R	S	105	140	108	13	19	5	14	5	42	80	4	>250	5	188	8	1
MRSN4484	R	R	R	R	R	R	R	R	R	R	R	R	R	S	104	119	146	0.4	17	8	4	6	15	30	5	>250	5	36	117	1
MRSN337038	R	R	R	R	R	R	R	R	R	R	R	S	R	S	36	32	30	6	7	4	7	4	4	17	4	8	4	8	4	2
MRSN7067	S	R	R	R	R	R	S	S	S	R	R	R	R	S	37	16	16	3	4	5	4	2	5	8	2	5	2	4	4	1
MRSN31196	R	R	R	R	R	R	R	R	R	R	R	R	R	S	35	44	77	4	8	4	38	4	11	32	4	71	4	32	10	1
MRSN959	R	R	R	R	R	R	R	R	R	R	R	S	R	S	33	28	30	7	15	7	14	4	7	15	3	16	3	14	15	1
MRSN1311	S	R	S	R	R	R	R	R	R	R	R	R	R	S	32	32	32	6	8	4	11	4	8	8	4	17	3	14	8	1
MRSN14193	R	R	R	R	R	R	R	R	R	R	R	R	R	S	32	32	32	0.4	15	4	16	4	8	16	4	16	3	15	2	1
MRSN15049	R	R	S	R	R	R	R	R	R	R	R	R	R	S	65	65	32	1	17	9	15	4	8	18	4	43	4	15	1	3
MRSN960	R	R	S	R	R	R	R	R	R	R	R	R	R	S	55	25	28	7	15	8	13	8	8	16	7	7	3	15	7	2
MRSN11695	R	R	R	R	R	R	R	R	R	R	R	R	R	S	34	35	32	0.4	4	4	4	5	4	4	3	8	4	4	0.4	1
MRSN11703	R	R	R	R	R	R	R	R	R	R	R	R	R	S	34	70	33	0.2	8	12	4	4	8	9	4	17	4	16	1	1
MRSN30000	R	R	R	R	R	R	S	S	S	R	R	R	R	S	36	34	17	4	4	4	8	4	4	9	4	8	3	8	8	1
MRSN11663	R	R	R	R	R	R	R	R	R	R	R	R	R	S	33	32	32	0.2	8	4	4	4	8	8	4	14	3	8	4	1
MRSN3874	R	R	R	R	R	R	R	R	R	R	R	S	R	S	44	64	121	0.4	8	8	12	4	7	28	4	46	4	7	0.4	1
MRSN19482	R	R	R	R	R	R	S	R	R	R	R	S	R	S	83	121	145	11	41	8	17	11	40	36	16	>250	12	96	24	2
MRSN480561	R	R	R	R	R	R	R	R	R	R	R	S	R	S	35	33	16	3	8	4	8	4	6	8	3	8	3	7	4	1
MRSN32104	R	R	R	R	R	R	R	R	R	R	R	R	R	S	34	>250	17	3	8	84	8	54	4	170	5	217	4	16	9	3
MRSN1437	R	R	R	R	R	R	R	R	R	R	R	R	R	S	34	33	23	0.4	18	39	9	5	9	103	4	18	4	8	>250	3

Table 2.7 Antibiotic resistance and disinfectant susceptibility profiles of A. baumannii clinical isolate panel

to the resistance development and the bioinformatic analyses. This thesis will only focus on the MIC and MBEC results.

Initially, MIC values were attempted to be collected against the strain panel using four commercial disinfectants and 12 of the most potent cationic biocides developed by the Wuest and Minbiole groups as of 2022. However, when visually evaluating the microbroth dilution assays, small subpopulations of bacteria survived above the apparent MIC breakpoints, indicative of the presence of bacterial heteroresistance phenotype.[46] To address the ambiguity of the "true" MIC, IC_{90} calculations were performed to standardize measurements across all compounds and strains and eliminate the potential for bias when evaluating unclear MICs.

Overall, there was a moderate degree of reduced susceptibility to cationic biocide observed in the clinical isolate panel compared to the *A. baumannii* lab strain ATCC 19606. Pleasingly, the next generation compounds generally demonstrated improved activity compared to commercial QACs BAC, BEC, and CPC. Novel QPC P6P-10,10 displayed comparable or comparable activity to highly potent commercial QAC DDAC against most or all strains. From these results, we additionally observed that several strains displayed lower levels of susceptibility to cationic disinfectants than other strains. To probe whether there existed a genetic determinant of cationic disinfectant resistant or tolerance, a genomic analysis was performed to understand if there was a phylogenetic connection between the strains. However, the dendrograms generated from multi-sequence alignment illustrated no correlation. Among the strains with reduced susceptibility, we observed complete biocide tolerance in MRSN 17493 up to 250 μM for all QACs tested. Excitingly, P6P-10,10 possessed single-digit micromolar activity against MRSN 17493, suggesting that it is able to overcome traditional QAC resistance mechanisms. MRSN 17943 also possesses resistance against colistin, a cationic, membrane-targeting antibiotic. The resistance

determinant identified in MRSN 17943 was the *pmrCAB* operon, which affects structural modification of lipid A in outer membrane of the cell.[47,48] This mutation may be responsible for conferring such broad QAC resistance, but further experimentation would be required to validate this hypothesis.

To further interrogate the efficacy of P6P-10,10 against highly resistant *A. baumannii* isolates, we next evaluated the biofilm eradication properties of the compound. As mentioned above, Chng and coworkers highlighted the ability of pathogens, such as *A. baumannii*, to evade eradication via the formation of stable biofilms across hospital environments, underscoring the need for disinfectants with potent broad-spectrum efficacies against both planktonic cells and biofilms.[41] To this end, we determined the minimum biofilm eradication concentration (MBEC) for P6P-10,10 compared to our best-in-class QAC (2Pyr-11,11) and the two leading commercial QACs, BAC and DDAC. A combination of BAC to DDAC in a 94:100 ratio was also included, resembling the ratio present in Virex 256, a leading QAC-based commercial disinfectant utilized in an array of healthcare and industrial settings. Excitingly, P6P-10,10 exhibited greater potency against both wild-type *A. baumannii* strain ATCC 19606 and XDR clinical isolates MRSN 4484 and MRSN 29908 compared to the other QAC disinfectants. We were not able to test biofilm eradication efficacy of P6P-10,10 against PDR clinical isolate MRSN 17493 due to its inability to form robust mature biofilms in our hands. These results further support the viability of QPCs, such

	MBEC (μM)		
samples	ATCC 19606	MRSN 4484	MRSN 29908
BAC	500	500	500
DDAC	125	63	125
BAC/DDAC	125	125	250
2Pyr-11,11	250	250	500
P6P-10,10	63	32	63

Table 2.8 Minimum biofilm eradication concentrations of select disinfectants against select A. baumannii strains

as P6P-10,10, as a potentially valuable next-generation disinfectant compounds for combatting the spread of resistant pathogens, such as *A. baumannii*.

2.3 Conclusion and Future Work

The preceding work greatly emphasizes what is already known about QACs and QPCs: additional cationic centers aid in overcoming bacterial resistance mechanisms and medium length chains (10-14 carbons) originating from the cation center possess the strongest inhibitory activity. However, QPCs remain a nascent class of amphiphilic disinfectants with much to be explored. While we demonstrated that there was very little difference between analogous phosphorous and nitrogen structures in physical properties or biological activity, this was limited to the 12(2)12 bicationic scaffold. Are there scaffolds that show a difference in performance between analogous phosphorus and nitrogen structures? Exploring this question remains challenging due to the difference in phosphorus and nitrogen reactivity and electronic properties that preclude analogous structures with bisphenyl groups, as an example. Perhaps the biscationic structures masks difference that do exist and analogous monocationic species might be more telling of a difference between the use of phosphorous and nitrogen in cationic biocides.

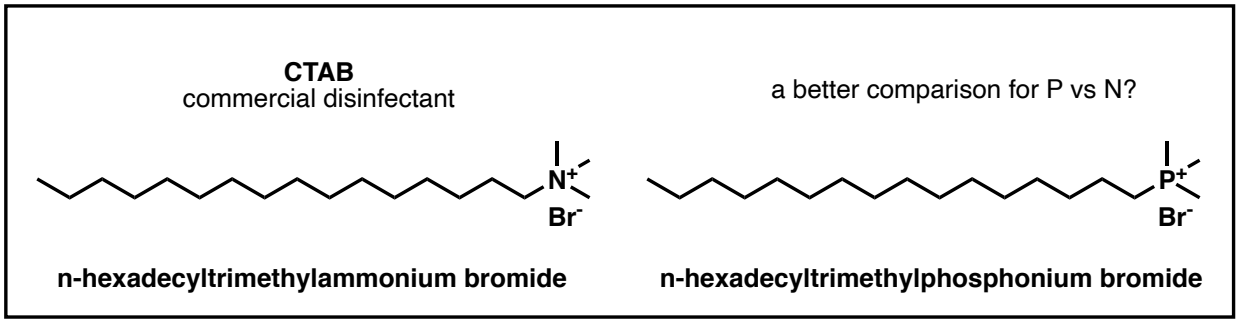


Figure 2.17 Proposed QPC and QACs for comparison studies

Biscationic QPCs such as P6P-10,10, P3P-12A.12A, and P6P-12A,12A displayed highly potent and promising inhibitory and cytotoxicity data. The ability for P6P-10,10 to overcome disinfectant resistant in *A. baumannii* MRSN 17493 leads to some interesting questions. How does P6P-10,10 overcome disinfectant resistance in *A. baumannii*? Is there a mechanistic difference, or does P6P-10,10 simply have a greater affinity for the negatively charged cell membrane?

In conclusion, our efforts towards screening experimental QAC and QPC biocides have definitively shown that there is great potential for novel classes of cationic disinfectants to be explored. While disinfectant resistance grows, it becomes ever more important to develop new classes that overcome these resistance mechanisms with whatever time remains. While not all our disinfectants might outperform compounds like BAC against laboratory strains, these strains are not representative of the reality and pervasiveness of disinfectant resistance. This makes investigations like our *A. baumannii* even more important as we assess the disinfectant resistance landscape.

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Chapter 3: Highly effective biocides against *Pseudomonas aeruginosa* reveal new mechanistic insights across gram-negative bacteria.

3.1 Introduction to the problem

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 commercially available quaternary ammonium compounds (QACs) and our best-in-class cationic biocides against a panel of *Pseudomonas aeruginosa* clinical isolates. Ranked in the Serious Threat category by the CDC and a pathogen of Critical Priority by the WHO, *P. aeruginosa* is an opportunistic gram-negative pathogen that is responsible for over 500,000 deaths annually.[1] Recently, Stribling *et al.* showed 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.[2] There is a paucity of innovation in this field, and the lack of mechanistic nuance in the biocide mode of action has led to a dire situation.

Cationic biocides generally act upon the bacterial cells by binding to and subsequently disrupting the phospholipid cell membrane.[3] With two cellular membranes, gram-negative bacteria possess an added barrier to the uptake of disinfectants and other antimicrobials.[4] Resistance to well-studied QAC disinfectants in gram-negative bacteria includes the expression of efflux pumps, upregulation of polyamines like spermidine, membrane lipid changes, and even biodegradation.[5] 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.[6] These findings suggest the presence of underlying mechanistic

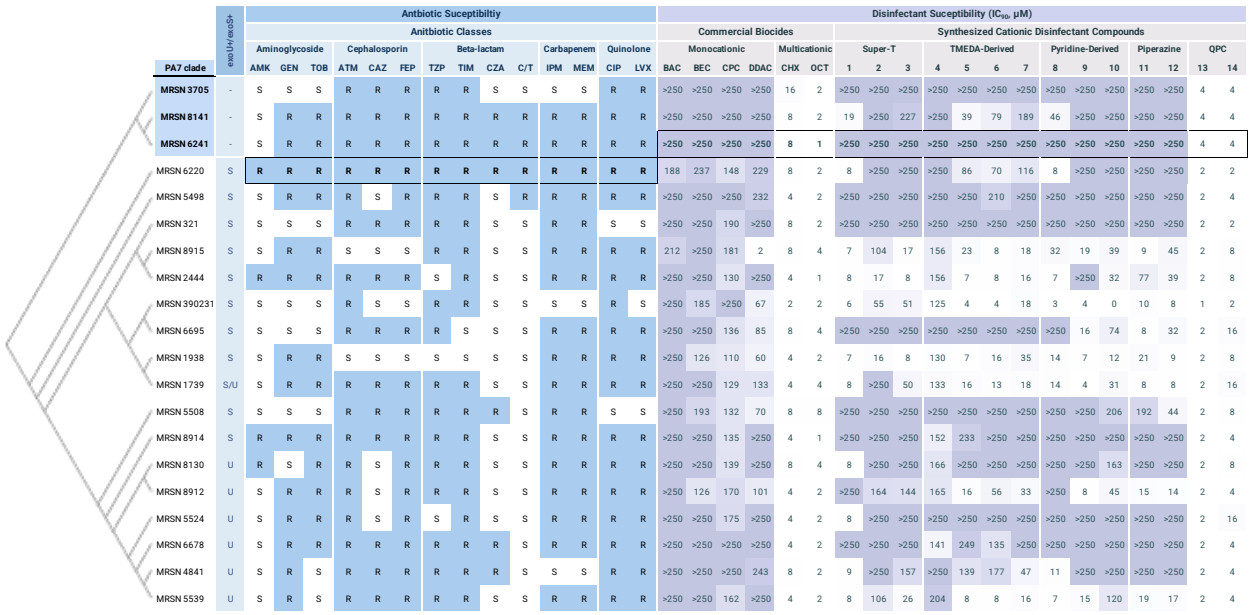
subtleties and underscore the importance of understanding how gram-negative bacteria are able to develop resistance to these biocides.

Through our screen, we observed broad resistance to QAC biocides among *P. aeruginosa* clinical isolates and the superior efficacy of our novel quaternary phosphonium compounds (QPCs). While exploring the bactericidal mechanism and resistance mechanisms of QPC P6P-10,10, we were led into uncovering distinct, structurally predictable mechanisms of actions 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 how this selects for predictable resistance mechanisms. These findings provide insight for rational design of cationic biocides against gram-negative bacteria.

3.2 Uncovering Novel Mechanistic Insights of Cationic Biocides

3.2.1 *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).[7] 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 20 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 attempted to determine MICs for each cationic biocide but were met with trailing growth for certain QACs in different isolate strains,



PA7 clade	exoU+/-/exoS+	Antibiotic Susceptibiltiy														Disinfectant Suceptibility (IC_{90}, 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	
		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

Figure 3.1. Susceptibility of the *P. aeruginosa* clinical isolates to a panel of 14 antibiotics, 6 commercial QACs, and 14 of our previously reported cationic disinfectant compounds. 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.

corresponding with a heteroresistance phenotype similar to our previous observations in *A. baumannii*.[6,8,9] Due the presence of resistant subpopulations above the MIC, we used IC_{90} values as a proxy for disinfectant efficacy. Across the panel, we observed a high degree of cationic biocide resistance in the clinical isolate panel in both commercially available QACs and our next-generation QACs. We observed no apparent correlation between antibiotic resistance and disinfectant resistance, but we found a strong correlation between disinfectant resistance and PA7-related species. The PA 7 clade is a taxonomic outlier that possess an extended resistance spectrum and typically possesses an increased biofilm forming character.[10] This represents the first work to our knowledge on a connection between disinfectant resistance and the PA7 clade.

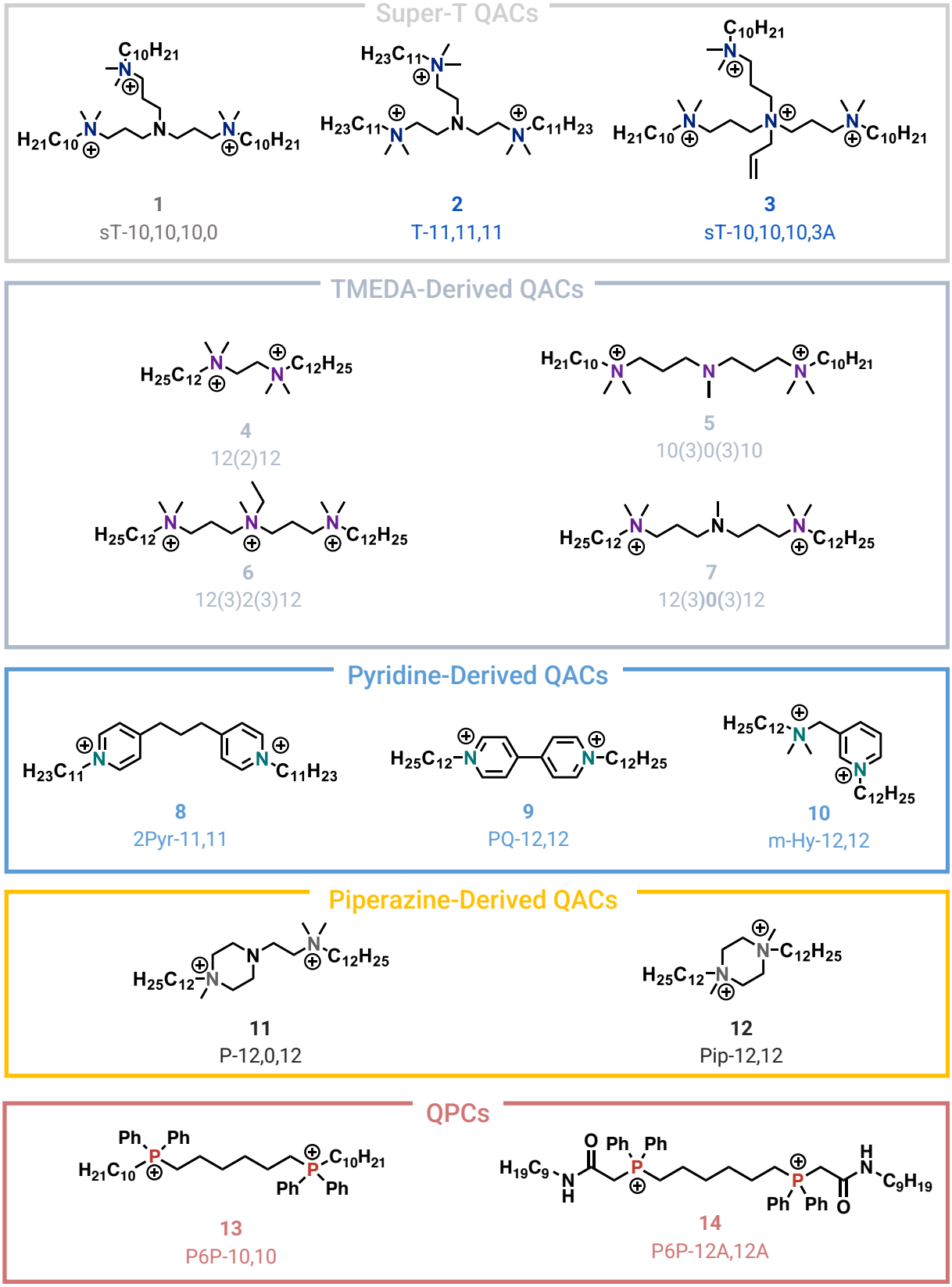


Figure 3.2. *Continued on next page*.

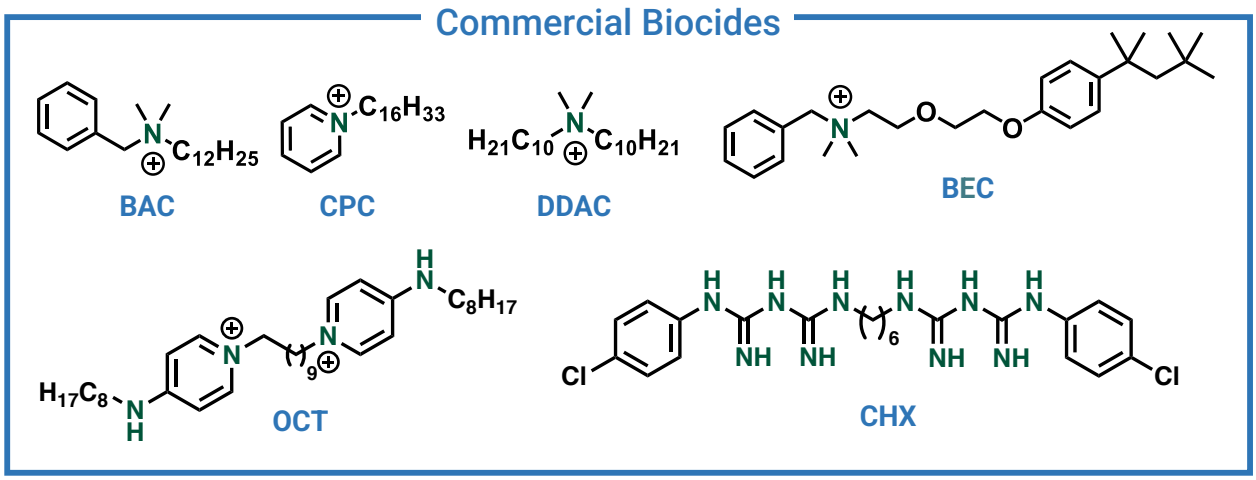


Figure 3.2. Cationic biocides used in screen against *P. aeruginosa* clinical isolates

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

Additionally, we observed the superior inhibitory efficacy of QPCs P6P-10,10 and P6P-12A,12A (**13** and **14** in **Fig 3.1**, 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.

3.2.3 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. 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) (**Fig 3.3A**). Upon dosing P6P-10,10 at sequentially higher

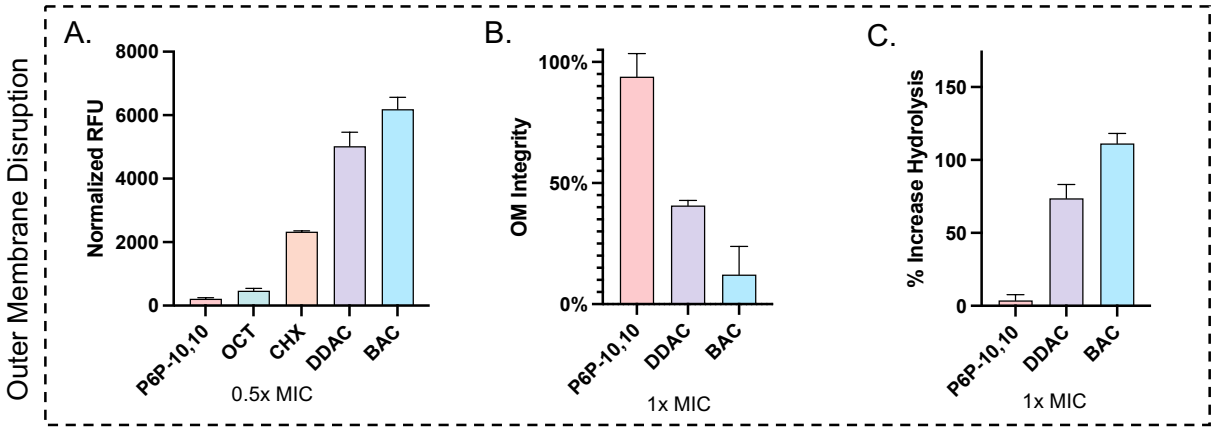


Figure 3.3. A) NPN uptake was assessed fluorometrically to measure outer membrane perturbation. B) Lysozyme permeability assay to assess outer membrane integrity through OD_{600} measurements upon lysozyme and disinfectant treatment. C) Effect of disinfectant treatment on nitrocefin hydrolysis mediated by outer membrane disruption.

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 3.4A**).

Testing at a lower cell density (OD_{600} = 0.05), we observed similar results (**Fig 3.4A**) To further investigate the of the effect of P6P-10,10 on the outer membrane, we used a lysozyme permeability assay. 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

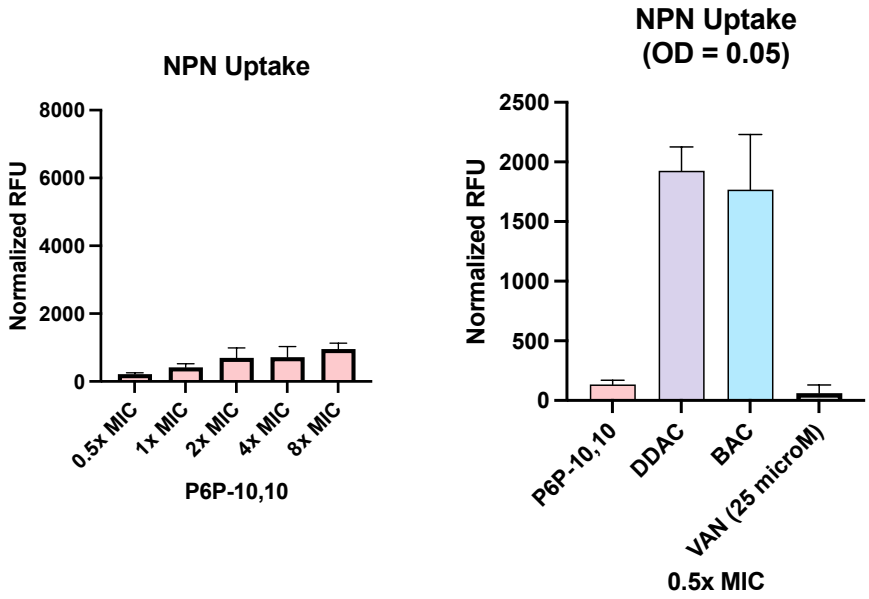


Figure 3.4. Outer membrane control assays **A)** P6P-10,10 dosed at sequentially higher concentrations in PAO1 B) NPN assay at lower cell density

membrane permeabilizing effects (**Fig 3.3B**), 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* (**Fig 3.3C**).

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

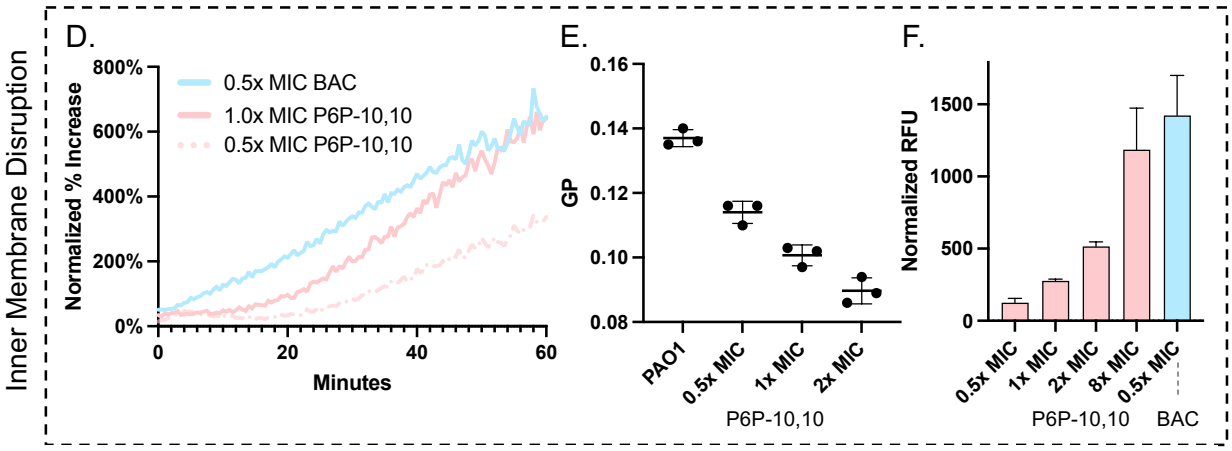


Figure 3.5. Inner membrane mechanistic assays D) Inner membrane depolarization measured by $DiSC_3$-(5) upon QAC and QPC treatment. E) Laurdan generalized polarization (GP) to assess inner membrane fluidity, where lower values indicate increases in fluidity. F) Propidium iodide cytoplasmic entry as a measure of inner membrane disruption.

Desiring 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). P6P-10,10 showed appreciable depolarization at 0.5x and 1x MIC comparable to BAC (**Fig 3.5D**). Furthermore, by using a Laurdan generalized polarization (GP) assay, we observe similar dose-dependent responses. A dose-dependent decrease in GP upon P6P-10,10 treatment is observed, which correlates to an increase in membrane fluidity consistent with an inner membrane disruption mechanism (**Fig 3.5E**). Additionally, P6P-10,10 dose-dependently induces membrane pore formation, as determined by increase in fluorescence of the dye propidium iodide (**Fig 3.5F**). 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 is observed when there is an increased in the number of organisms inoculated (**Fig S4**). 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.

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

To further explore the inner membrane specificity of P6P-10,10, we explored two different antagonism assays that would support the mechanism. Spermidine (Spd) is a cationic polyamine lipopolysaccharide of gram-negative bacteria. In *P. aeruginosa*, spermidine has been shown to protect the outer membrane from antibiotic treatment.[11,12] In addition, Kwon et al. show that addition of exogenous spermidine antagonizes activity of the cationic membrane disrupter polymyxin B.[13] Furthermore, an increase in spermidine production is a known transcriptional response to treatment with BAC, presumably because it masks the negative potential displayed on the exterior of the cell.[5] 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 hypothesize that if BAC and DDAC target the outer membrane but P6P-10,10 does not, then we should 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 (**Table 3.1**). However, the MIC of P6P-10,10 remained unchanged, suggesting 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.[14,15] Previous results while exploring P6P-10,10 in *P. aeruginosa* have shown that CCCP is antagonistic to QPC treatment.[16] We replicated this previous experiment and demonstrated that CCCP is antagonistic to P6P-10,10, while having no profound effect on BAC and DDAC treatment under the experimental conditions tested (**Table 3.1**) Taken together, these results further support the inner membrane specificity of P6P-10,10 activity.

MIC, µM (± 50 5mM Spd)						MIC, µM (± 50 µM CCCP)					
BAC		DDAC		P6P-10,10		BAC		DDAC		P6P-10,10	
- Spd	+ Spd	- Spd	+ Spd	- Spd	+ Spd	- CCCP	+ CCCP	- CCCP	+ CCCP	- CCCP	+ CCCP
100	300	32	50	2	2	125	125	32	63	2	40

Table 3.1. Spermidine (Spd) and CCCP antagonism assays

3.2.6 Distinct mechanisms of resistance to cationic biocides.

We hypothesized that since these cationic biocides show distinct mode of action, resistance to these compounds would be achieved through distinct mechanisms. 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 BAC and P6P-10,10 over a period of 15 days, and isolated mutants with stable increases in MIC for both cationic biocides. 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, correlating with these biocides having a distinct mode of action (**Fig 3.6A**). 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 3.6B**). 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, virulence, among others (**Fig 3.6B**). In contrast, loss of function mutations in *smvR*, the negative regulator of SmvA, were identified in all P6P-resistant mutants, indicating this efflux system is involved in resistance to this QPC. These mutants also showed weak cross-resistance to octenidine (OCT) and chlorhexidine (CHX), suggesting that SmvA is a major cationic biocide resistance determinant in *P. aeruginosa*. Adaptations to cationic biocides with distinct mechanism of action result in completely different resistance profiles in *P. aeruginosa*.

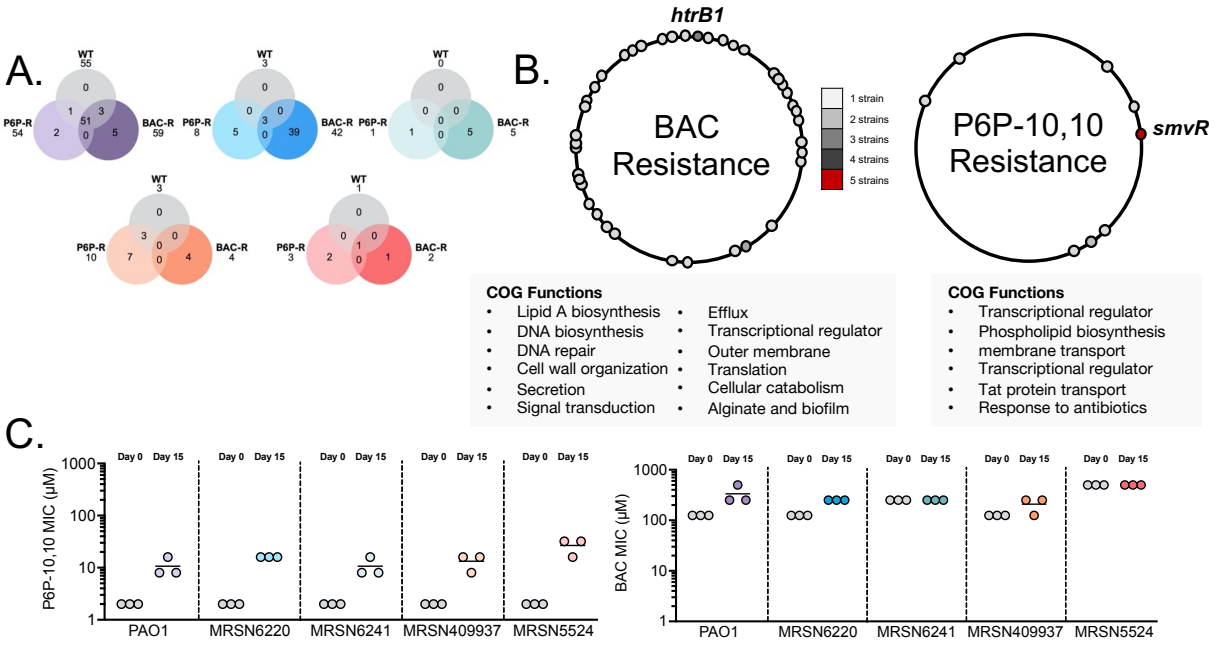
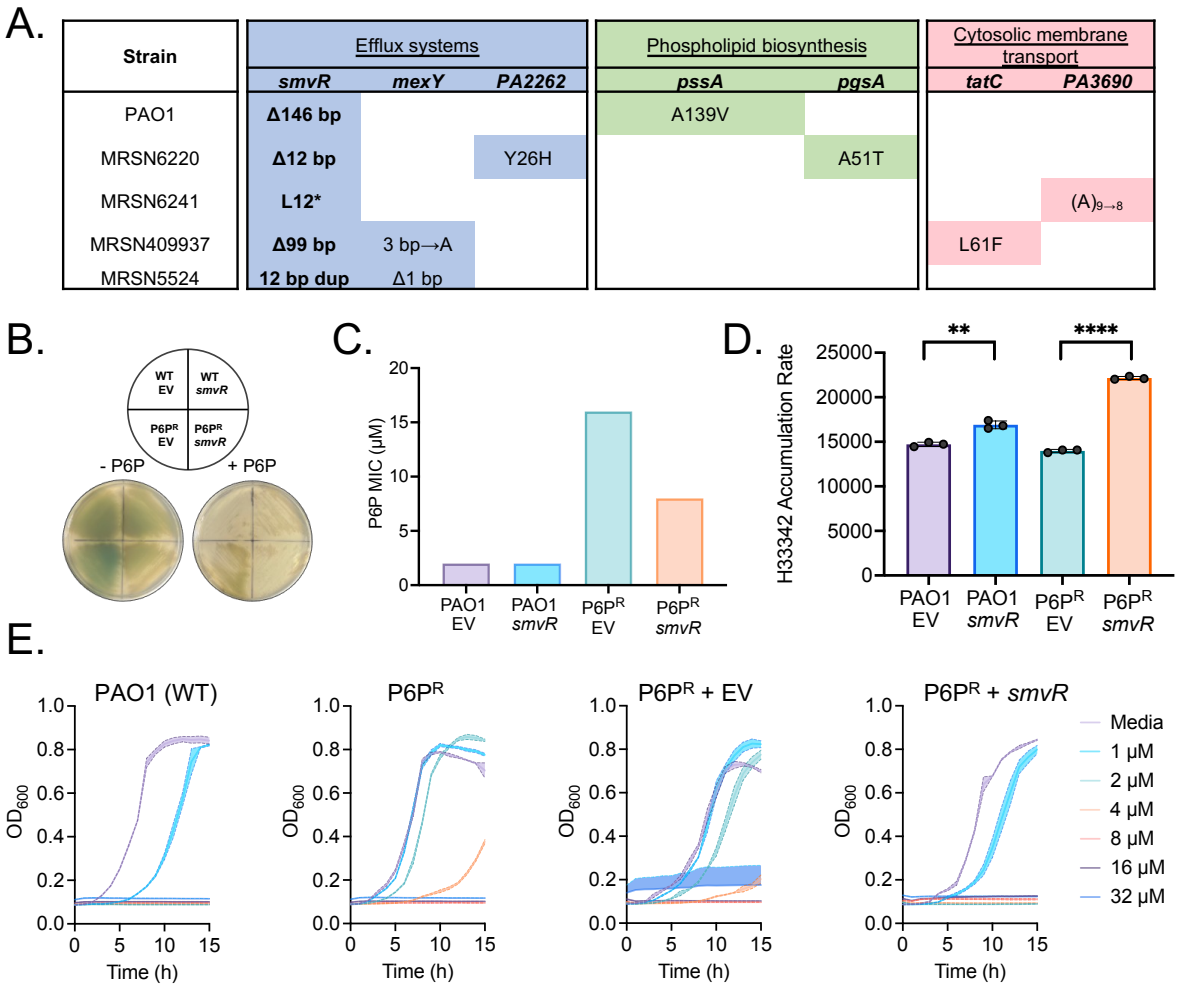


Figure 3.6. Distinct cationic biocide resistance profiles in response to P6P-10,10 and BAC. A) Venn diagrams of genetic variants identified in P6P- and BAC- resistant mutants of 5 *P. aeruginosa* PAO1, MRSN6220, MRSN6241, MRSN409937 and MRSN5524 strains. B) Schematic of genes with identified genetic variations 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. C) MIC values for P6P- (left) and BAC-resistant (right) before (Day 0) and completion of resistance selection assay (Day 15). Three independent biological replicates were performed.

3.2.7 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. Sequence homology analysis show that SmvA shows the highest homology (~35 %) to the known QAC efflux pump (QacA) of all the efflux pumps present in *P. aeruginosa*. Interestingly, a loss of function mutation in *smvR* was identified in strains resistant to P6P in all genetic backgrounds (**Fig 3.7A**). To explore 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 3.7B**). Restoration of SmvR function partially restored susceptibility to P6P-10,10 as measured by MIC assays (**Fig 3.7C**). This also correlated with restoration of relative expression levels of SmvA and efflux activity, as measured by Hoechst 33342 accumulation (**Fig 3.7D**). To further corroborate the involvement of SmvRA in P6P-10.10 resistance, we expose the wildtype and P6P-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 3.7E**). 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, this data shows different mutations associated

Figure 3.7. The efflux system SmvRA is a major resistance determinant of P6P-10,10. A) Mutations identified associated with P6P resistance in the 5 *P. aeruginosa* genetic backgrounds. B) *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). C) MIC values of PAO1 (WT) and P6P-resistant (P6P^R) strains with either empty vector (pUCP30T) or vector containing a functional copy of *smvR*. D) Hoechst 33342 accumulation assay in strains with either empty vector (pUCP30T) or vector containing a functional copy of *smvR*. E) 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.

with distinct mode of action of cationic biocides.

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

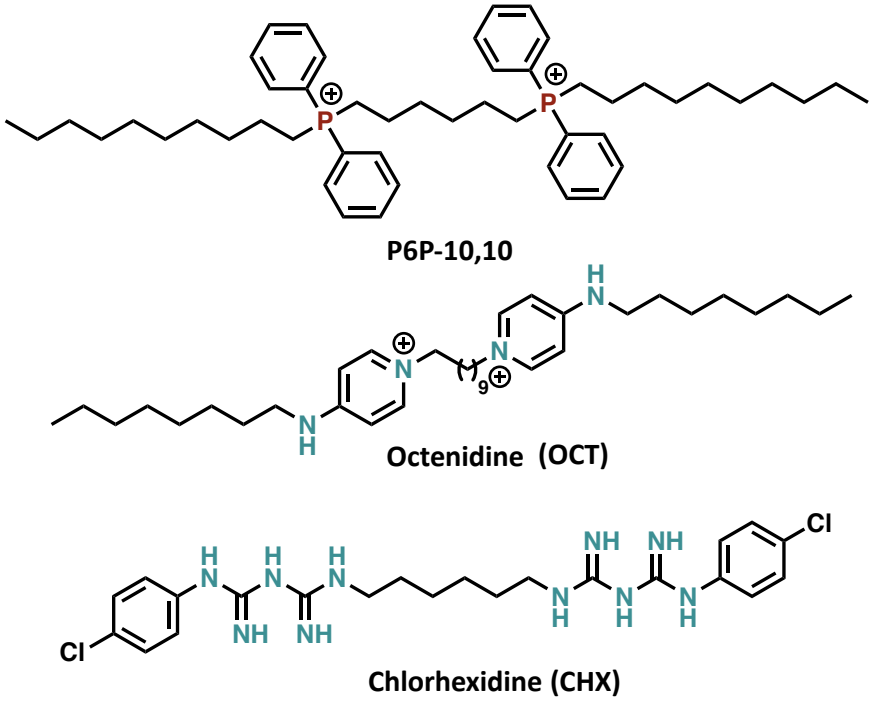


Figure 3.8. Multicationic biocides with delocalized lipophilic cations that display inner membrane selectivity.

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*.[17–19] 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, similar to 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 explored 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 3.9**). This

demonstrates that cationic biocides act via different mechanisms in gram-negative bacteria and uncovers a mechanistic difference that was previously unknown.

Through examining the molecular structures between the P6P-10,10, OCT, and CHX compared to QACs BAC and DDAC, we observed the presence of delocalized, lipophilic cations (DLCs) in the former three. By having the cationic atom in conjugation with a π system the cationic character then becomes diffused throughout the region. P6P-10,10 with its diphenylphosphonium moieties, OCT with its 4-aminopyridinium, and CHX with its dual biguanides all possess this type of resonance-stabilized cations.[20,21] It appears that when possessing DLC moieties separated by a linker, biocides selectively disrupt the inner membrane of gram-negative bacteria without producing a strong effect on the outer membrane.

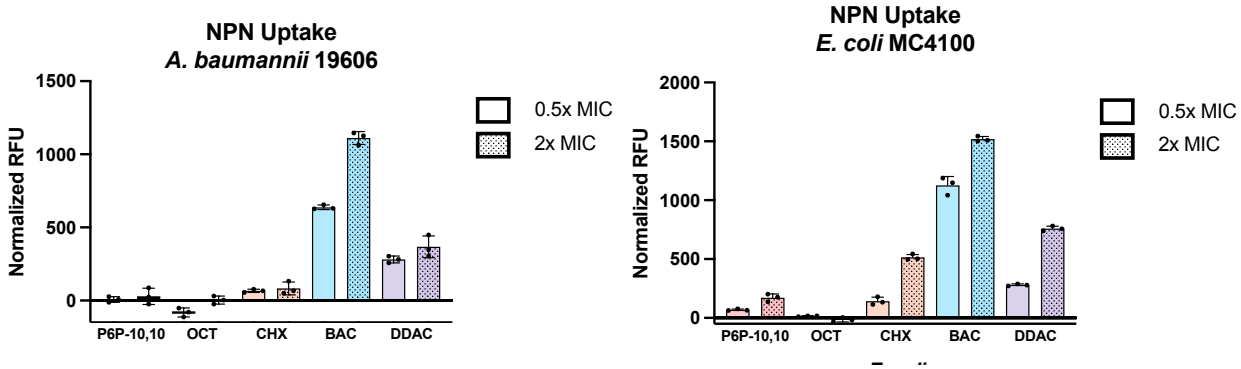


Figure 3.9. NPN assays in additional gram-negative species. NPN assays for P6P-10,10, octenidine (OCT), chlorhexidine (CHX), benzalkonium chloride (BAC), and didecyldimethylammonium chloride on gram-negative species *Acinetobacter*

3.3 A Mechanistic Distinction in Cationic Biocides Revealed

3.3.1 Discussion

P. aeruginosa is a troublesome opportunistic pathogen with a range of complicated clinical manifestations, especially in patients with cystic fibrosis.[1,22] 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 showing resistance to at least

one antimicrobial treatment group.[23,24] Recently, McGann *et al*. reported how a wounded soldier was co-infected with six bacterial strains, 3 of which were extremely drug-resistant *P. aeruginosa* strains, highlighting this pathogen threat during bellicose conflicts.[25] In addition, *P. aeruginosa* is a notoriously difficult pathogen to eradicate through biocide treatments due to its intrinsic mechanism of resistance to disinfectants.[26]

Cationic biocides, such as QACs, are an important part of disinfection protocols in healthcare and food industry and a barrier for pathogen spread. The widespread use of biocides in multiple settings has led to increase resistance, as consequence.[27] As alternative compounds to the overused QACs, our group has developed a series of QPCs that show good activity against a diverse group of pathogenic bacteria.[16,28–30] One of such compounds, P6P-10,10, showed potent antimicrobial activity against a panel of *A. baumannii* clinical isolates with high levels of antibiotic and biocide resistance.[6] The effectiveness of this compound against strains displaying high levels of resistance to diverse cationic biocides suggested that P6P-10,10 might possess a distinct mode of action compared to the other compounds.

In this work, we sought to explore the mechanistic nuances of cationic biocides antimicrobial activity by comparing the QPC P6P-10,10 and commercially available QACs. We tested our compound against *P. aeruginosa*, a gram-negative opportunistic pathogen notorious for its intrinsic resistance to disinfectants. To no surprise, a panel of highly antibiotic resistant *P. aeruginosa* clinical isolates also frequently displayed high levels of resistance to a wide range of biocides. Strikingly, the QPCs showed strong antimicrobial activity against all the isolates, suggesting that these compounds are effective regardless of preexisting mechanisms of antibiotic and biocide resistance. Biocides are believed to have multiple cellular targets, with membrane disruption being the main mechanism of action.[31] While BAC and DDAC showed potent outer

membrane disruption, P6P-10,10 show minimal disruption of the outer membrane. In contrast, when the integrity of the inner membrane was assessed, P6P-10,10 showed strong and dose-dependent disruption, suggesting that P6P-10,10 preferentially targets the inner membrane in *P. aeruginosa*. Our current hypothesis is that the delocalized lipophilic cationic nature of P6P-10,10, and similar compounds, preferentially target the inner membrane.

We have reported that *P. aeruginosa* strains resistant to BAC and P6P-10,10 are phenotypically different and differentially affect virulence (under review). We subjected these strains to whole genome sequencing to determine what resistance determinants define each phenotype, expecting to see significant overlap between these. To our surprise, there was no shared resistance determinant between BAC and P6P-10,10 resistant strains. While P6P-10,10 genetic determinants located to limited genetic loci, genetic variants associated with BAC resistance localized throughout the genome and with numerous cellular functions. In general, BAC resistance was associated with lipid A biosynthesis, as 3 out of the 5 strains showed mutations in lysophospholipid acyltransferase. These results are in accordance with a previous reports showing modifications of the outer membrane as a response to BAC, likely reducing the negative charge of the outer membrane. [32,33]

Additionally, we discovered that increase expression of the MFS efflux pump SmvA is the main resistance mechanism to the QPC, P6P-10,10, with all strains displaying a loss of function mutation in its negative regulator SmvR. Genetic complementation studies indicate that, while SmvA is the predominant resistance determinant, mutations in other genes (e.g. *pssA*) are also required for full complementation by MIC assay. Importantly, SmvA is also associated with resistance to octenidine and chlorhexidine, two compounds that also share a preference to disrupt

the inner membrane, suggesting that inner membrane-disrupting agents have might have a predictable resistance mechanism.

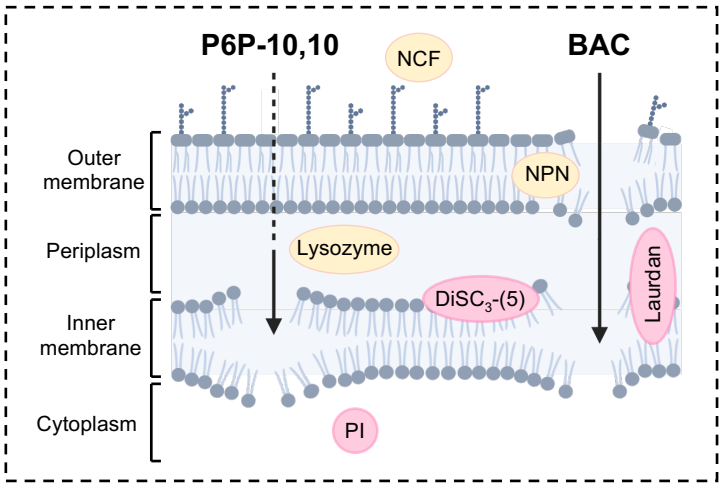


Figure 3.10. Mechanistic proposal for P6P-10,10 and BAC. Outer membrane assays represented in yellow. Inner membrane assays represented in pink.

3.4 Conclusion

In summary, this study starts to uncover the 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. The superior ability for multicationic biocides P6P-10,10, chlorhexidine, and octenidine to overcome disinfectant resistance in *P. aeruginosa* delivers much needed promise to the field of disinfectants. By understanding that cationic biocides may have distinct mechanisms of action based on structure and that this mechanism of action overcomes resistance mechanisms, we may hopefully be able to

rationally design novel cationic biocides that share the inner membrane specificity that we observed in this study.

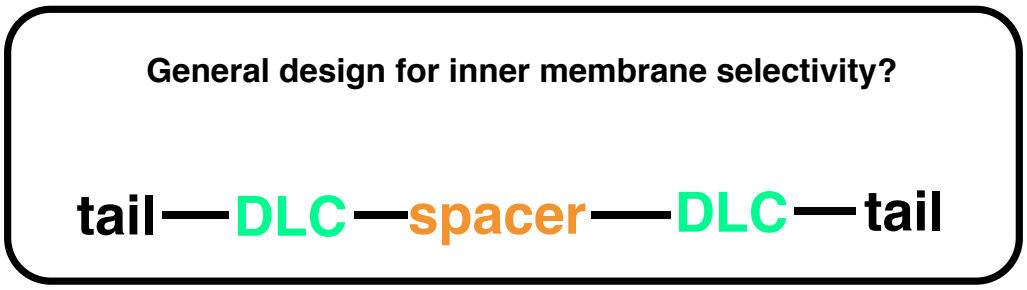


Figure 3.11. Proposed structure of inner membrane targeting cationic biocides.

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Chapter 4: Investigating the influence of cationic biocide resistance on virulence and pathogenicity in *Pseudomonas aeruginosa*

4.1 Cationic Biocide Resistance Phenotype Introduction

4.1.1 Overview of virulence and pathogenicity

Trillions of bacteria inhabit the human body protecting and providing benefit to the human host.[1] However robust this symbiosis may be, foreign bacterial invasion and dysbiosis can disrupt the complex relationship between humans and their indwelling bacterial company.[2] While previous chapters have focused on the eradication of bacteria and the elucidation of cationic biocide mechanism of action, little ink has been dedicated towards what this work is ultimately trying to prevent—namely, bacterial infections. To properly think about the bacteria that cause infection, it becomes important to possess appropriate terminology.[3]

When a bacterium is capable of causing host damage or disease, it can be labeled as a pathogen.[4] Disease may be defined as a disruption of normal homeostatic processes.[5] In turn, this disruption impairs the normal functioning of the body and its processes. Damage may be caused either by the host or the pathogen. Immune status plays a large role in this differentiation. In a scenario where the host possesses a weakened immune system, the pathogen is primarily responsible for the damage. However, as in the case of other infectious diseases like the 1918 'Spanish Flu,' a stronger host immune system can be detrimental as host-mediated damage can prove fatal.[6] In bacteria, an example of this is tuberculoid leprosy.[7] When describing the capacity of a microbe to cause damage, the term pathogenicity is used. This allows for a division between pathogenic and non-pathogenic strains and species. Furthermore, when discussing the degree of pathogenicity of a strain or species of bacteria, the term virulence is used.[8] Thus, a pathogenic bacterial strain that causes severe infections might be classified as a "highly virulent" strain.

Components of a pathogen that damage the host are termed virulence factors.[9] These vary significantly between bacterial genera but can generally be separated into two categories: factors that promote host cell colonization and factors that are involved in damaging host cells.[10,11] Alternatively, using more warfare-related language, virulence factors can be categorized as either offensive or defensive, as has been done since at least 1913.[12]

From this understanding, it is important to note that pathogenicity and virulence are not intrinsic characteristics; rather, they can be influenced by environmental factors or genetic changes within a species. We then sought to understand how subinhibitory exposure to cationic biocides affected bacterial pathogenicity and virulence in the bacterium *Pseudomonas aeruginosa*. Should the reduction of bioburden through cationic biocide treatment fail and instead the generation of cationic biocide resistant bacteria emerge, how does this ultimately affect the severity and occurrence of bacterial infections?

4.1.2 Disinfectants and Virulence-Associated Phenotypes

Recent years, resistance to disinfectants such as CBs has been exacerbated by COVID-19 pandemic, which resulted in a significant increase in disinfectant usage thereby inducing pressure toward disinfectant resistance.[13] 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.[14,15]

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.[16] Through a combination of defense strategies and virulence factors, *P.*

aeruginosa can establish deadly infections in immunocompromised hosts, especially in cystic fibrosis patients.[17] 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.[18] Furthermore, the pathogenicity of *P. aeruginosa* lies in what can be regarded as its "offense" strategies.[19,20] 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 4.1. Concerningly, *P. aeruginosa* can be transmitted from one person to another through contaminated equipment or surfaces in healthcare settings.[21] 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.[22] Previous reports have demonstrated that long term exposure to BAC can promote antibiotic resistance in bacteria including *P. aeruginosa*. 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.[14] McCay 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.[23] Loughlin et al. also reported the development of serially passaged *P. aeruginosa* strains in sublethal amounts of BAC to explore antibiotic cross-resistance.[24] 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.

4.1.3 Aim of Present Investigation into Disinfectant Resistance

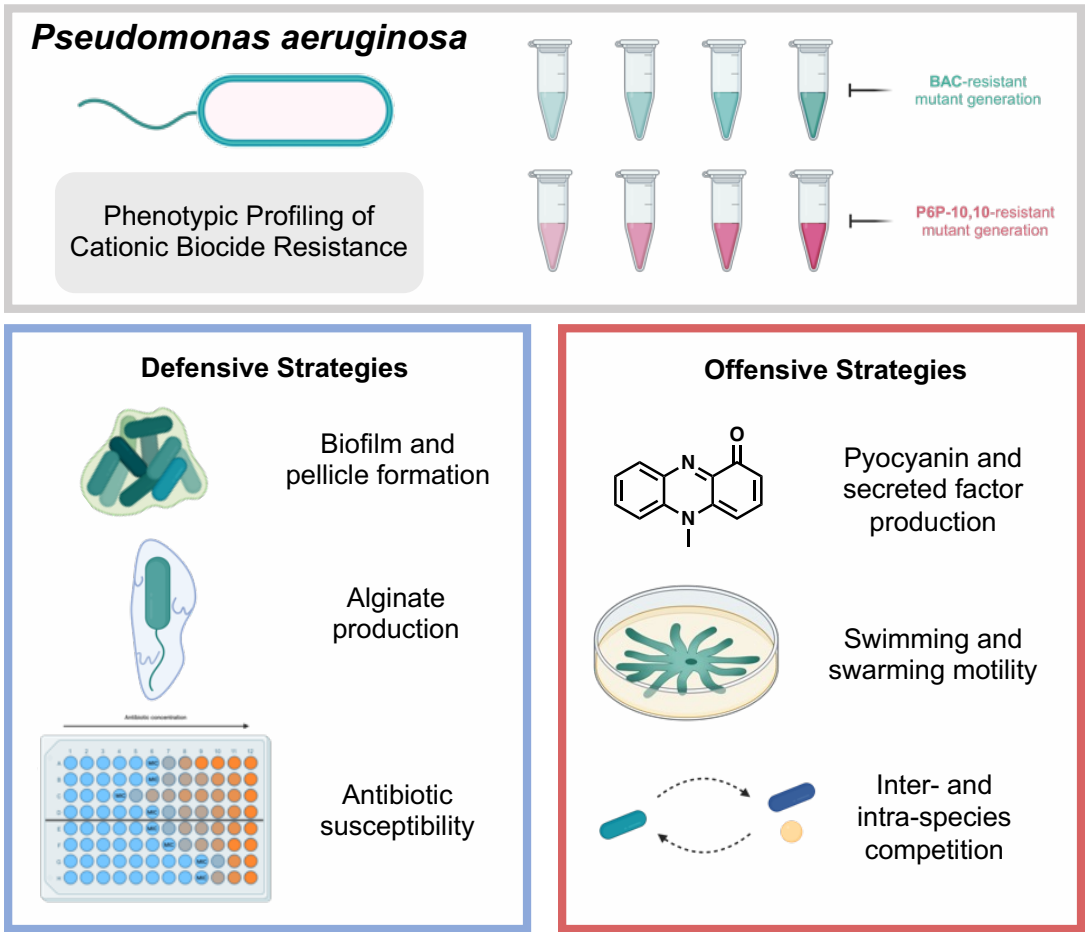

Figure 4.1. Schematic overview of *P. aeruginosa* virulence factors assessed after adaptation to cationic biocides (CBs). *P. aeruginosa* strains were exposed to increasing concentration of CBs over 15 days, and virulence-associated phenotypes were subsequently evaluated in these CB-resistant strains. 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.

In this study, we explored the effects of sublethal CB exposure utilizing *P. aeruginosa* strains from the recently developed panel of diverse clinical isolates from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN) discussed in Chapter 3.[25] By using isolates from this panel, we can 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,[26] as well as the novel quaternary phosphonium compound P6P-10,10, previously reported by our research groups.[27] By comparing these two dramatically different cationic biocides, we hoped to assess whether structural and mechanistic diversity in CBs influences the adaptation phenotypes *in P. aeruginosa* after long-term exposure.

We hypothesized that prolonged exposure of *P. aeruginosa* to a pair of disparate CB structures would result in an increase in bacterial defense mechanisms and offense strategies based on these concerning precedents. In turn, this resistant phenotype would be overall more pathogenic to hosts. To study this, we utilized the resistant-strains from the previous mechanism of action study with P6P-10,10. Alongside those strains, we additionally selected for resistance to BAC. The following results detail our findings on the implications of CB-resistance on the virulence and pathogenicity phenotype of *P. aeruginosa*.

4.2 Cationic Biocides Resistance and its Effect on Defensive Strategies

4.2.1 Cationic Biocide Resistance in *P. aeruginosa* Influences Antibiotic Susceptibility

The following experimentation was led and designed by C.A.S. with the invaluable technical support of E.L.B.

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

Antibiotic abbreviations: amikacin (AMK), gentamycin (GEN), tobramycin (TOB), aztreonam (ATM), ceftazidime (CAZ), cefepime (FEB), imipenem (IPM), meropenem (MEM), ciprofloxacin (CIP), levofloxacin (LVX), colistin sulfate (CST), polymyxin B (PMB)

Table 4.1. Minimum inhibitory concentrations (MIC) of parental (bold) and CB-resistant (R) strains of *P. aeruginosa*.

In our study, 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. Nevertheless, we did observe small increases in collateral sensitivity (Table 4.2). 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.[28] 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 gentamycin 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. 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. Though no cross-resistance was observed, our results suggest that some resistance development to CBs may increase susceptibility to certain antibiotic classes in *P. aeruginosa*.

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

Antibiotic abbreviations: amikacin (AMK), gentamycin (GEN), tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LVX)

Table 4.2. Minimum inhibitory concentrations (MIC) of parental (bold) and CB-resistant (R) strains of *P. aeruginosa*. Notable MIC differences are highlighted in red.

4.2.2 Cationic Biocide Resistance Increases Alginate Production in *P. aeruginosa*

The following experimentation was led and designed by C.A.S. with the invaluable technical support of G.M.W.

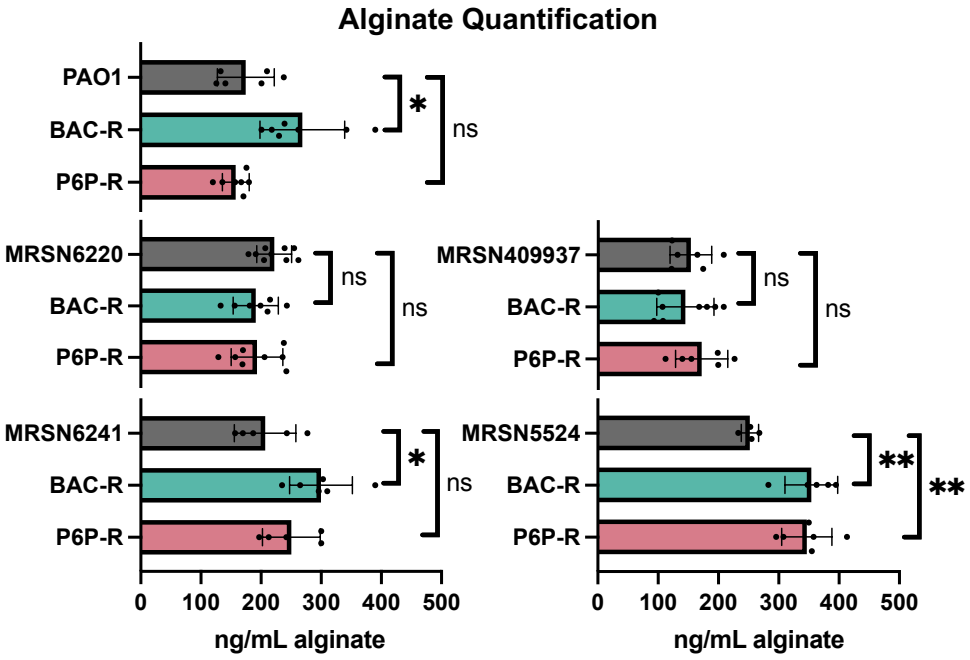

Figure 4.2. Alginate production. Alginate production was quantified in a mixture of boric acid/sulfuric acid/carbazole using a spectrophotometer (A_{550}) after alginate precipitation with 2% cetylpyridium and collection with isopropanol. The amounts of alginate produced were compared between the CB-resistant *P. aeruginosa* strains and their respective parental (WT) strain.

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.[29–31] 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.[32] It has been previously discussed that alginate production can be stimulated through varied factors such as nitrogen limitation or high oxygen environments. 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.[31] 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. 4.2A). 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.

4.2.3 Quantification of biofilm in CB-resistant strains of *P. aeruginosa*.

The following experimentation was led and designed by C.A.S. with the invaluable technical support of E.L.B.

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.[33] Biofilms are composed of a matrix of extracellular polymeric substances produced by bacteria that include nucleic acids, lipids, secreted proteins, polysaccharides, and water.[34,35] 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.[36] In *P. aeruginosa*, the main components of biofilms are the polysaccharides Pel (positively charged), Psl (charge-neutral), and alginate (negatively charged).[37] 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.[38,39] 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.

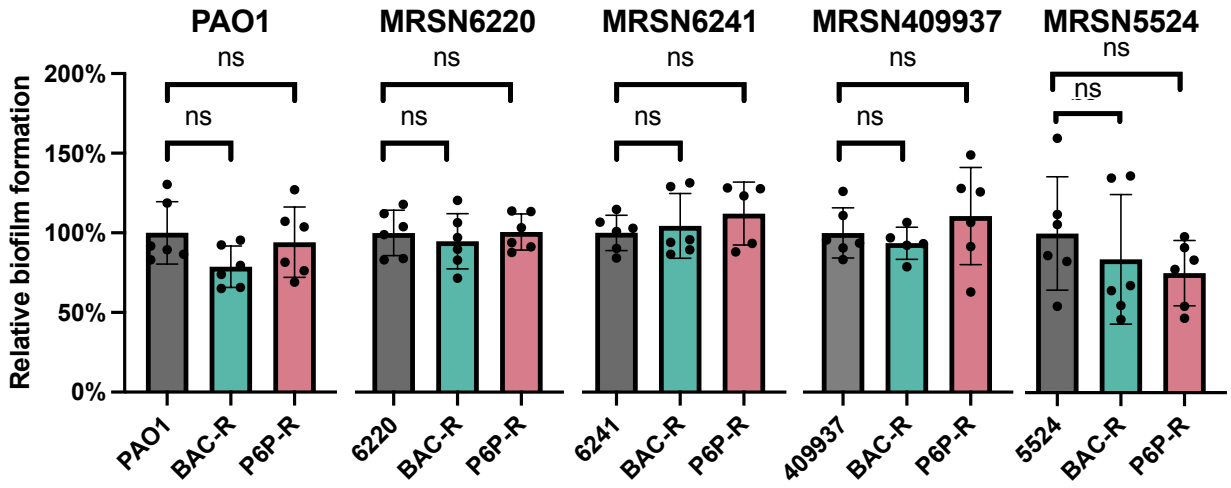

Figure 4.3. Biofilm quantification and comparison. Relative biofilm formation by CB-resistant and parental strains was evaluated in M63 media and quantified using crystal violet measuring absorbance at a wavelength of 570 nm

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. 4.2B). 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*.

4.2.4 Pellicle Formation Is Increased in the BAC-Resistant but not in the P6P-Resistant Strain Derived from PAO1.

The following experimentation was led and designed by G.V.C. and is included to provide context for the larger findings of the study.

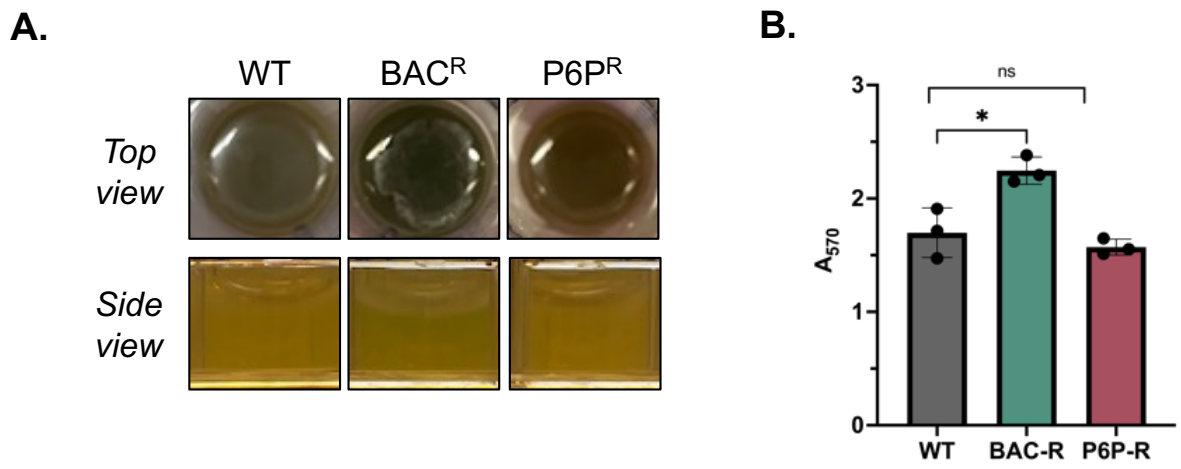

Figure 4.4. Pellicle formation. A) Pellicle formation of CB-resistant and parental PAO1 strains after overnight incubation. Differences in pellicle formation were clearly observed after overnight incubation with a thicker white film formed in the air-liquid interface (top and side view) in the BAC-resistant strain compared to the P6P-resistant or PAO1 parental strains. B) Pellicle formation was quantified using crystal violet and measuring absorbance at a wavelength of 570 nm (A570).

In addition to surface-liquid biofilms, bacteria can also form biofilms at the air-liquid interface known as pellicles.[40] In *P. aeruginosa*, the pellicle matrix is composed of the Pel and Psl exopolysaccharides.[40] An advantage of formation a pellicle at the air-liquid interface is the ability for bacteria to be able to acquire oxygen from the air while still accessing nutrients from the media.[41] In *Bacillus subtilis*, exposure to sub-lethal concentrations of chlorine dioxide bleach promotes the formation of pellicle biofilm.[42] Testing the pellicle formation of the CB-resistant strains, we observed an evident increase in pellicle formation for BAC-resistant PAO1 when

grown in LB (Fig. 4.2C). Biofilm peg microtiter plates were used to quantify the difference in pellicle formation, as previously described.[43] A significant increase in pellicle formation in the BAC-resistant strain when compared to the PAO1 was observed (Fig. 2D). Additionally, no significant difference in pellicle formation was observed in the P6P-10,10-resistant strain. While CB resistance seems to influence pellicle formation in BAC-resistant PAO1, it appears to have no effect on P6P-10,10 resistance.

4.3 Cationic Biocide Resistance and its Effect on Offensive Strategies

4.3.1 BAC adaptation reduces swimming behavior in clinical isolates.

The following experimentation was led and designed by C.A.S. with the invaluable technical support of E.L.B.

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

Table 4.3. 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.

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.[44] 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.[45] 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.[46] 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.[47] Similar observations were found for gram-positive *Listeria monocytogenes* as BAC-adapted mutants exhibited reduced swarming motility.[48]

Wanting to build upon this knowledge, we interrogated the relationship between CB resistance of *P. aeruginosa* clinical isolates and motility. As shown in Table 4.2, 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.

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

The following experimentation was led and designed by C.A.S. with the invaluable technical support of S.S.

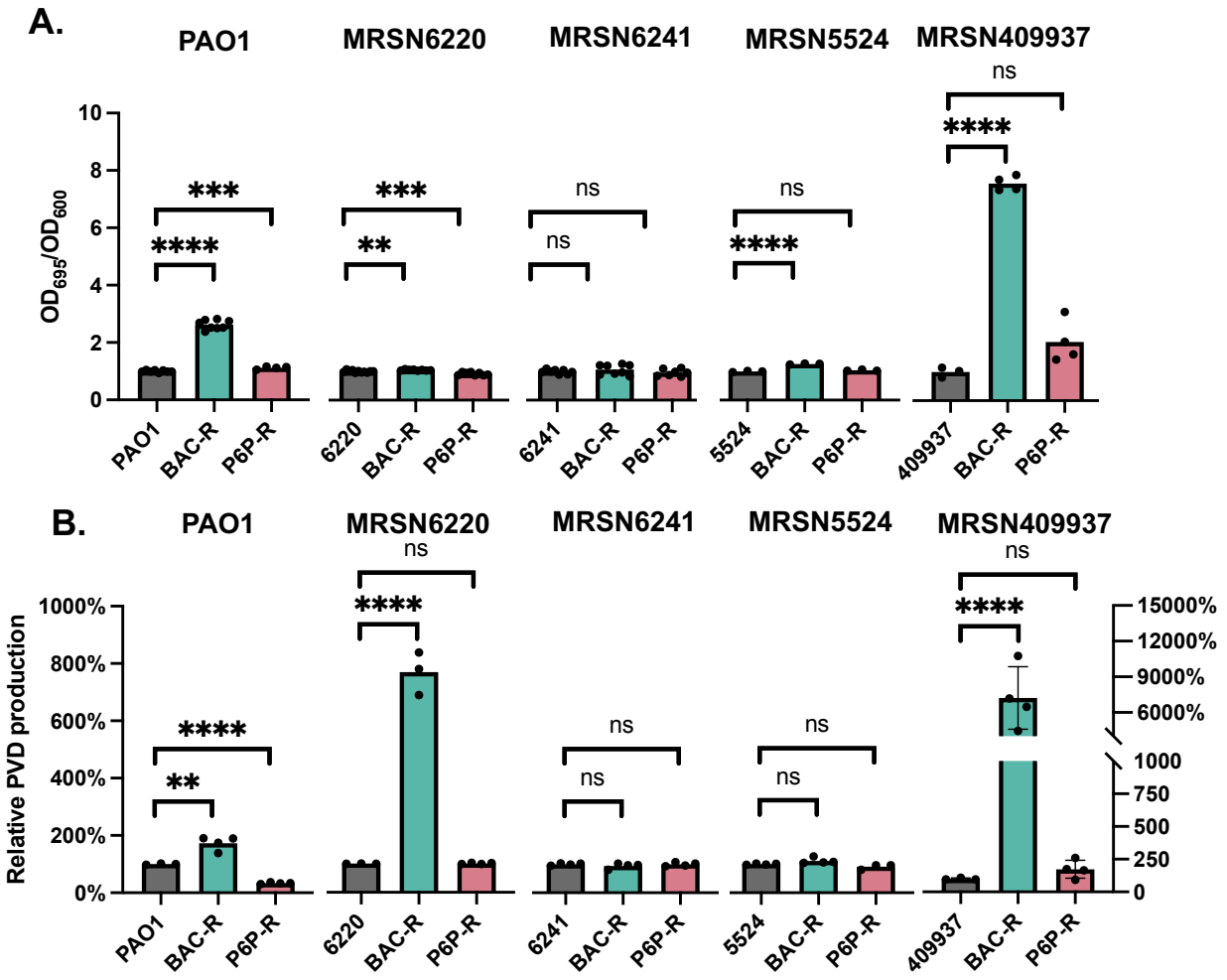

Figure 4.5. Evaluation of virulence-associated pigment production in *P. aeruginosa* CB-resistant strains. A) Pyocyanin (PYO) production was measured spectrophotometrically from supernatants at a wavelength of 695 nm and normalized to the cell density using OD_{600} values. The normalized values from 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.

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).[49,50] 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.[51] In contrast, it has been reported that a QAC-adapted strain of *P. aeruginosa* decreased in pyocyanin production.[52] We observed a sharp increase in PYO production compared to the parental strains in BAC-resistant strains derived from PAO1 and MRSN409937 (Fig. 4.3A). 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. 4.3B). 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.

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

The following experimentation was led and designed by C.A.S.

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.[53] Co-infections have been shown to be more virulent than single species infections.[54,55] 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. 4.4). In plate assays, BAC- and P6P-10,10-resistant PAO1 strains had greater lysis zones than the parent PAO1 strain. These data indicate that CB resistance can affect important interbacterial relationships, which may have implications for human infections.

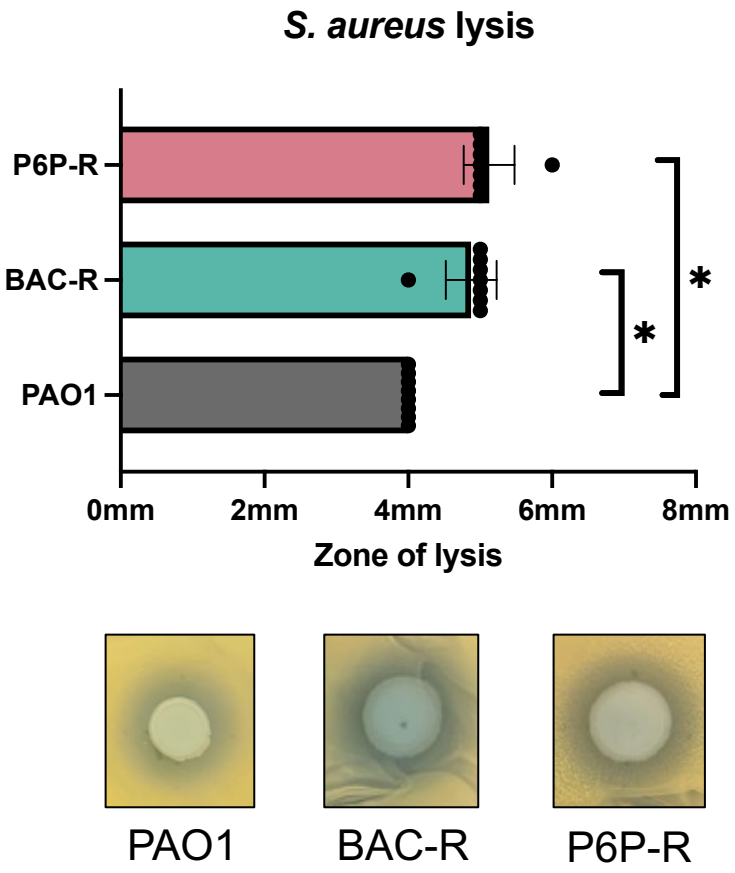

Figure 4.6. Lysis of *S. aureus* by *P. aeruginosa* PAO1 and CB-resistant strains. Lysis zones generated on *S. aureus* bacterial lawns by CB-resistant strains were measured and compared to the zones generated by parental isogenic strain PAO1. Top panel shows measurements of lysis zones (6 replicates 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.

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

The following experimentation was led and designed by G.V.C. and is included to provide context for the larger findings of the study.

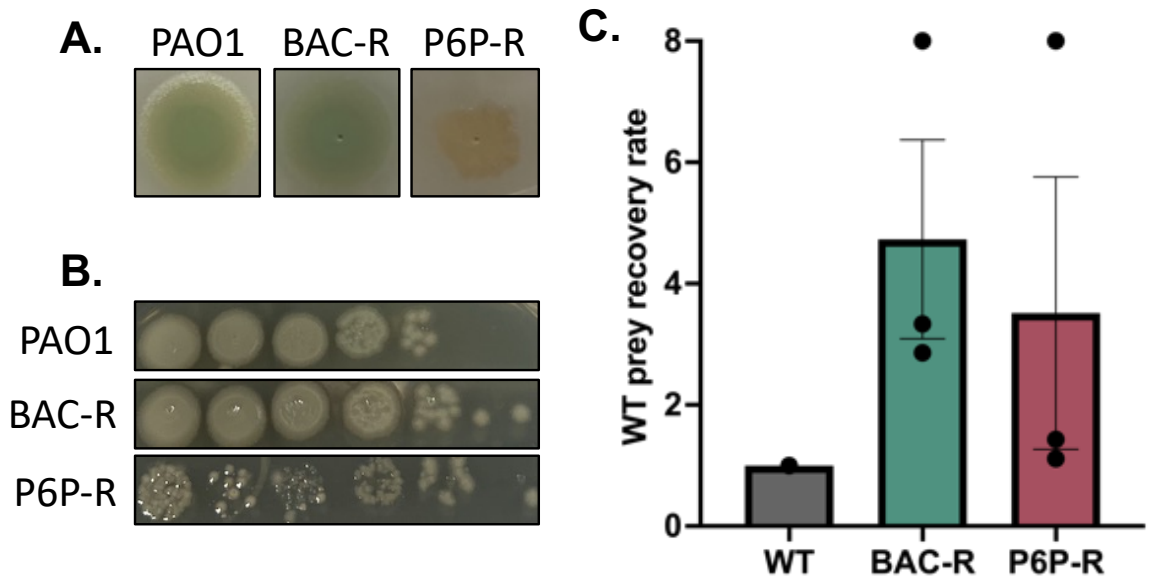

Figure 4.7. Bacterial competition assays of CB-resistant and PAO1 parental strain. A) Different morphologies observed in competition spots between attacker strains PAO1 (WT control), BAC-R and P6P-R strains versus PAO1 harboring a vector conferring gentamicin resistance (prey strain; GenR) on LB agar media after 24 hours using an attacker to prey ratio of 10:1. B) Spot plating for recovery of prey strain in LB gentamicin (60 μg/mL) plate for prey recovery rate determination after competition. C) Prey recovery rate of competition assays between *P. aeruginosa* PAO1, BAC-resistant and P6P-resistant strains against *P. aeruginosa* PAO1 GenR. The mean of three replicates is shown. No statistical significance was observed by two-tailed Student's t test.

Bacterial competition plays a critical role in infection and can occur between and within species. In *P. aeruginosa*, strains known as "social cheaters" can adapt to utilize the secreted factors by surrounding bacteria, forgoing the need to spend resources to produce them themselves. This in turn confers a competitive advantage, allowing them to thrive. We utilized a prey recovery rate competition assay to investigate this.[56,57] 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. From this assay, we can gain

additional information on the fitness of the strains under mixed culture conditions. We observed phenotypic differences during our competition incubation period, especially with our P6P-10,10-resistant strain (Fig. 4.5A). The overall prey recovery rate indicated no significant difference compared to the wildtype control (Fig. 4.5B). 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. 4.5C). These data suggest that resistance to CBs affects competitive fitness within these isogenic strains.

4.3.5 Evaluation of *P. aeruginosa* virulence in *Galleria mellonella* infection model.

The following experimentation was led and designed by G.V.C. and is included to provide context for the larger findings of the study.

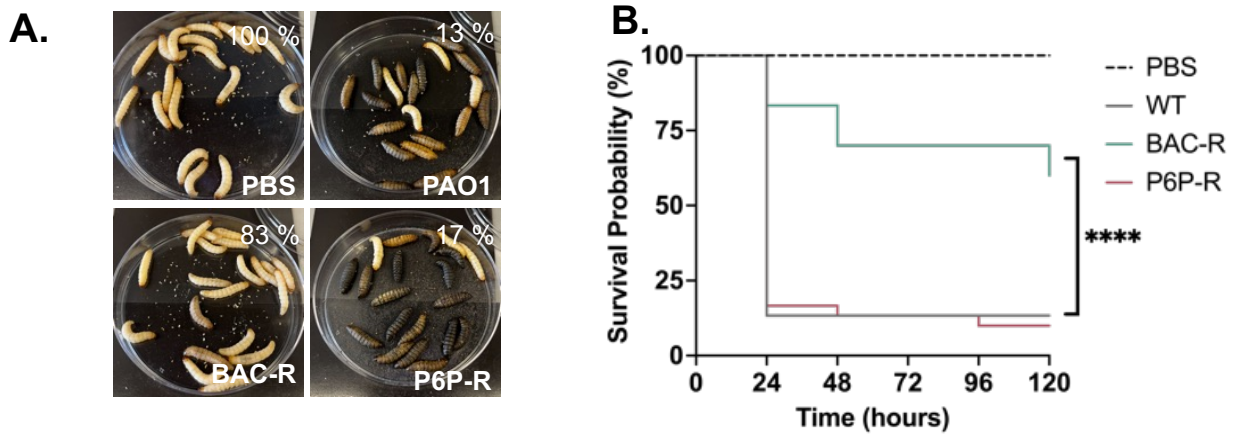

Figure 4.8. *Galleria. mellonella* larvae infected with CB-resistant and parental PAO1 strains. A) Morbidity of *G. mellonella* larvae 24 hours post-infection. B) Kaplan-Meier survival curves of *G. mellonella* larvae monitored over a 5-day period. Two independent experiments were performed with a total of 120 larvae with PBS-injected larvae used as mock infection control (n = 30/group).

We utilized the *Galleria mellonella* larvae infection model in order to evaluate how our *in vitro* studies translated to an infection model. Because we observed increases in virulence factor production in BAC-resistant PAO1, we hypothesized that it would result in a higher rate of morbidity in an infection model. The *G. mellonella* larvae infection model is ideal model system

due to its ease of use and the minimal amount of maintenance required. This system has been used previously to study virulence factor production and virulence in *P. aeruginosa*.[58] Consistent with previous reports, we observed most larvae infected with 5 CFU/larvae of PAO1 died within a 24 hour period with only 13% of the infected larvae surviving.[59] P6P-10,10-resistant strains displayed similar results to larvae infected to PAO1 with 10% of the infected larvae surviving the infection (Fig. 4.6A). Interestingly, the larvae infected with the BAC-resistant PAO1 strain displayed decreased morbidity (57 %) (Fig. 6B). This was unexpected since the BAC-resistant strain consistently showed the higher production of various virulence factors compared to wildtype and P6P-10,10 resistant strains. From this result, we hypothesize that the fitness cost imposed by mechanisms of BAC resistance decreases virulence *in vivo*.

4.4. Conclusion

Disinfectants are an important part of our arsenal against infections as they represent the first lines of defense against the spread of pathogens. Cationic biocides 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-10,10 mutants were 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.

4.5 References

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5.0 Supporting Information

Methods

Biological Assays. For all biological assays, laboratory strains of MSSA (SH1000), *E. faecalis* (OG1RF), *E. coli* (MC4100), *P. aeruginosa* (PAO1), *A. baumannii* (ATCC 17948), 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, OG1RF, MC4100, USA300-0114, and PAO1 were grown in BD Mueller–Hinton broth (MHB), whereas ATCC 33591 was grown in BD tryptic soy broth (TSB). Optical density (OD) measurements were obtained using a SpectraMax iD3 plate reader (Molecular Devices, United States).

Minimum inhibitory concentrations (MICs). Compounds were serially diluted 2-fold from stock solutions (1.0 mM) to yield 12 100 μL test concentrations, wherein the starting concentration of DMSO was 2.5%. Overnight, *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. baumannii*, USA300-0114 (CA-MRSA), and ATCC 33591 (HA-MRSA) cultures were diluted to ca. 106 CFU/mL in MHB or TSB and regrown to mid-exponential phase, as determined by OD recorded at 600 nm (OD600). All cultures were then diluted again to *ca*. 106 CFU/mL and 100 μL and were inoculated into each well of a U-bottom 96-well plate containing 100 μL of compound solution. Plates were incubated statically at 37 °C for 48 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 highest value in three independent experiments. Aqueous DMSO controls were conducted as appropriate for each compound.

RBC Lysis Assay ($lysis_{20}$). RBC lysis assays were performed on mechanically defibrinated sheep blood (Hemostat Labs: DSB030). An aliquot of 1.5 mL blood was placed into a microcentrifuge tube and centrifuged at 3800 rpm for 10 min. The supernatant was removed, and the cells were resuspended with 1 mL of phosphate-buffered saline (PBS). The suspension was centrifuged as described above, the supernatant was removed, and the cells were resuspended four additional times in 1 mL of PBS. The final cell suspension was diluted 20-fold with PBS. Compounds were serially diluted with PBS 2-fold from stock solutions (1.0 mM) to yield 100 μL of 12 welve test concentrations on a flat-bottom 96-well plate (Corning, 351172), wherein the starting concentration of DMSO was 2.5%. To each of the wells, 100 μL of the 20-fold suspension dilution was then inoculated. The concentration of DMSO in the first well was 2.5%, resulting in DMSO-induced lysis at all concentrations > 63 μM. TritonX (1% by volume) served as a positive control (100% lysis marker), and sterile PBS served as a negative control (0% lysis marker). Samples were then placed in an incubator at 37 °C and shaken at 200 rpm. After 1 h, the samples were centrifuged at 3,800 rpm for 10 min. The absorbance of the supernatant was measured with a UV spectrometer at 540 nm wavelength. The concentration inducing 20% RBC lysis was then calculated for each compound based upon the absorbances of the TritonX and PBS controls. Aqueous DMSO controls were conducted as appropriate for each compound.

Mitochondrial Toxicity Assay. Mitochondrial toxicity was evaluated using a Promega Mitochondrial ToxGlo kit. Human hepatocellular carcinoma cells (HepG2) were cultured in RPMI-1640 medium containing 10% FBS at 37 °C and 5% CO_2. Cells were seeded at a density of 2500 cells/well in 384 well tissue culture plates in either glucose (10 mM) or galactose (10 mM) supplemented media and were incubated overnight to allow for cell adherence. Cells were rinsed and replaced with serum-free media prior to experimentation. The Mitochondrial ToxGlo assay

was performed in accordance with the manufacturer's instructions. Cells were incubated with test compounds for 90 min prior to assay per the manufacturer's protocol. Cells were incubated for 30 min with a cell impermeable, fluorogenic substrate. Cell integrity was measured by subsequent fluorescence (Ex/Em 485/525 nm) produced by necrosis-associated protease activity upon the substrate. Lysis buffer was then added, and net ATP levels were determined by luminescence measurement from a luciferase reporter. Compounds were serially diluted 2-fold from stock solutions (1.0 mM) to yield 12 100 μL test concentrations, wherein the starting concentration of DMSO was 0.5%. Mitochondrial toxicity, as per the manufacturer guidelines, was defined as a greater than 20% decrease in the ATP measure with a less than 20% increase in cytotoxicity.

Minimum Biofilm Eradication Concentration (MBEC) Assays. To determine the MBEC values, 8.0 µL of overnight culture was added to 200 µL of MHB in a 96-well flat-bottom plates (Biologix ®, 07-6096). The plates were then statically incubated for 24 hours at 37ºC to establish the biofilms. After incubation, unadhered cultures were removed by inverting the plates and gently shaking, careful not to disturb the adhered biofilms, and wells were carefully washed with PBS three times to remove planktonic bacteria. The biofilms were then treated using 200 µL of a pre-mixed 1:1 solution of MHB to diluted compound at twelve test concentrations, in which the highest final compound concentration tested was 4.0 mM. The plates were then incubated statically at 37ºC for 24 hours, after which all media was removed, the biofilms were carefully washed three times with PBS to remove planktonic cells, and 200 µL of fresh MHB media was added to each well. The plates were incubated at 37ºC for 24 hours to re-grow the surviving biofilm bacteria. The optical density (OD600) was then measured for each well and the concentrations of compound yielding an OD below 0.100 corresponded to the MBEC. The highest MBEC value of three individual experiments was reported. Aqueous DMSO controls were conducted for each strain.

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.

Bacterial strains and growth conditions. P. aeruginosa strains were streaked onto lysogeny broth (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated at 37 ºC overnight. Single colonies were used to inoculate liquid cultures and incubated for 18-24 hours at 37 ºC. P. aeruginosa clinical isolates were obtained from the Multi-drug resistant organism Repository and Surveillance Network (MRSN).

Growth Curves. Growth curves were performed in 96-well flat-bottom plate (Falcon®, 351172) with shaking. OD600 was measured 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.52 Synthetic cystic fibrosis medium (SCFM) was prepared as previously described with addition of N-acetyl glucosamine.53,54

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.55 First, an overnight culture of PAO1 in Difco™ Mueller-Hinton Broth (Sigma Aldrich, DF0757-17-6) was diluted to a concentration of 106 CFU/mL, according to the OD600 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 triplicate.

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. Pseudomonas aeruginosa strains were streaked onto lysogeny (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated 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 optical density at 600 nm (OD600). All cultures were diluted to ca. 104 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), gentamycin 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.56,57 Pseudomonas aeruginosa strains were streaked onto lysogeny (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. Pseudomonas aeruginosa strains were streaked onto lysogeny (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated (NuAire, Plymouth, MN) for 18 h at 37oC. 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).58 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 buffer 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 H2O. 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 H2O 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.

Motility Assay. Motility assays were performed following the procedure described by Cullen et al. with minor modifications.59 Pseudomonas aeruginosa strains were streaked onto lysogeny (LB) agar (Sigma-Aldrich, 1102830500) plates and incubated 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. 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 h. 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 h. 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 lysogeny (LB) plate (Sigma-Aldrich, 1102830500) with an overnight culture of S. aureus.60 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.61 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. Pseudomonas aeruginosa strains were streaked onto lysogeny (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. 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.48,49 P. aeruginosa PAO1 pUCP30T (GenR) 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 OD600 of 1. Cultures were normalized to an OD600 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 (GenR) prey strain was selected in LB supplemented with gentamicin 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 lysogeny (LB) medium (VWR, TS61187-5000) and grown with shaking at 37 °C for 20 h.62 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 OD695 were measured on a BioTek Synergy H1 Hybrid plate reader (Santa Clara, CA). The pellets

were resuspended in PBS, and OD600 was measured to determine the cell density of each sample. Pyocyanin production was determined by normalizing OD695 of the clarified supernatant to the OD600 of the resuspended pellet.

Calculation of IC_{90} Values. To determine the IC90 values for each compound against the clinical isolates, the OD600 for each compound concentration against each strain was recorded. Using Prism 9 (GraphPad software, v. 9.3.1), the IC90 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 OD600 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 OD600 of 0.500. Cells were harvested by centrifugation (4000 rpm, 25ºC, 10 min), washed twice with assay buffer (5mM HEPES, 5 mM glucose, pH 7.2), and resuspended in assay buffer to a final OD600 of 1. Then, 100 uL of washed cells and 100 uL of assay buffer containing 20 uM NPN were together and incubated for 10-30 min. 198 ul of cells and NPN added to a 96-well optical-bottom black plate. Either 2 uL 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 uM 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 mL of bacterial suspension was added to a 96-well plate containing 100 mL 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 mL 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 OD600 after mixing) and every 10 s after stabilization for 30 s. Relative values were normalized to PBS as 0% and 99% isopropanol as 100%.

$DISC_3(5)$ 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 mL of 10 mM EDTA was added to 5 mL of resuspended cells for a final concentration of 200 mM EDTA. The bacterial solution was then gently mixed and then let sit for 2 minutes. Afterwards, 5 mL of 0.75 mM $DISC_3(5)$ was added to the solution for a final

concentration of 0.75 mM. Following another gentle mix, the solution was left to incubate in the dark at 37ºC. After incubation, 125 mL of 4M KCl was added to the cells for a final concentration of 100 mM KCl. Finally, 198 mL of cells and $DISC_3(5)$ added to a 96-well optical-bottom black plate. Either 2 mL 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 OD600 of 0.600. *Pa* cells were harvested (4000 rpm, 25°C, 10 min), washed, and resuspended in PBS buffer at pH 7.2. Then, 50 mL 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 mL 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 OD600 = 0.4 and diluted to 105 CFU/mL in HEPES buffer, followed by 60 min incubation with Laurdan 2.5 mM 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 mM and Spd (5mM) 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. 106 CFU/mL in MHB and as determined by optical density recorded at 600 nm (OD600). 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 (45). 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. All genetic variants are reported in **Table S2 and S3.** 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.

Bacterial strains and plasmids. All strains and plasmids are listed in **Table S1**. 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 selected in LB agar plates supplemented with gentamycin (60 μg/mL) for selection of transformants (Emory Integrated Genomics Core) (46). 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 Bio-Rad GenePulserXcell™) into *P. aeruginosa* PAO1 electrocompetent cells prepared as previously described (47).

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). Cultures were diluted (1:100 dilution) in fresh MHB 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.

Hoechst 33342 dye accumulation assay. Hoechst 33342 accumulation assays were performed as previously described (48). 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.