8-2014

Synthesis and Antimicrobial Activity of Brominated Quaternary Ammonium Compounds

Wendy Pippin

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Wendy Pippin

Undergraduate Honors Thesis under the direction of
Dr. William T. Doerrler

August 2014

Louisiana State University &
Agricultural and Mechanical College
Baton Rouge, Louisiana

ACKNOWLEDGEMENTS:

There are many people to whom I owe a great debt of gratitude. I would first like to thank Dr. Randy Duran for helping me to find my love of organic chemistry, to attain my first research opportunity with Albemarle, and for encouraging me to take honors classes and to "go the extra mile."

Next I would like to thank my financial supporters. In particular, Hank Danos and the Board of Supervisors for the scholarship that has made my return to school possible, and the Tiger Athletic Foundation for the scholarship that has helped fund my research.

Dr. Joe Sauer deserves a huge amount of thanks for taking me on as an undergraduate researcher last spring and for being so generous with his time, attention, and credit. I also wish to acknowledge Albemarle for supporting me with four semesters of internships which has given me invaluable industry experience, along with the physical materials necessary to complete this project.

Of course significant recognition goes to Dr. Doerrler, my thesis advisor, for the substantial support, guidance, and flexibility he has provided me with throughout this process. Additionally, thanks goes to the other members of the Doerrler laboratory, in particular Sujeet Kumar and Megan Dugas for their generous assistance and advice.

I also greatly appreciate the time given by my final thesis committee member, Dr. Sue Bartlett, along with her support and advice during a particularly trying semester. Lastly, I could not have done this without the unwavering support of my family and friends. I cannot thank them enough for taking the time to be sounding boards and shoulders on which to lean.

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ABSTRACT:

Specific brominated quaternary ammonium compounds (QACs) were prepared by reacting tertiary amines with alkyl bromides via quaternization to produce water soluble quaternary ammonium salts. Their antibacterial activities against *Escherichia coli , Salmonella enterica* serovar *Typhimurium, Vibrio cholerae*, and *Pseudomonas aeruginosa* were explored by the determination of the minimum inhibitory concentrations (MICs). The results suggest potential new biocides for disinfectant use as well as a way to target specific antimicrobials by customizing QAC chain length for different environments. The results also raise a few important questions regarding the need for a better understanding of the details of the QAC mechanism.

INTRODUCTION:

Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) have a wide variety of uses: as disinfectants, surfactants, fabric softeners, antistatic agents, phase transfer catalysts, osmolytes, plant growth retardants, and in various other pharmaceuticals and cosmetics. In particular, the use of QACs as antimicrobials started in the 1930s (Bore et al., 2007) and has been increasing over the last several decades (Buffet-Bataillon, Tattevin, Bonnaure-Mallet, & Jolivet-Gougeon, 2012). Production of all forms of QACs totals greater than 1000 tons of pure compounds per year in the EU alone, with some of the individual compounds totaling more than 10 tons produced per year (Wessels & Ingmer, 2013).

QACs have a general structure of a cationic head group made up of a nitrogen atom with four R groups covalently bound, where an R group is a hydrogen atom, an alkyl group, or a substituted alkyl group. There is an associated anion, usually a halide such as a chloride or bromide ion (see Figure 1). The quaternary ammonium cations are permanently charged and relatively stable. They are also effective over a wide pH range, making them different from the structure of the ammonium ion (NH4+) and the primary, secondary, or tertiary ammonium cations (Bore et al., 2007).

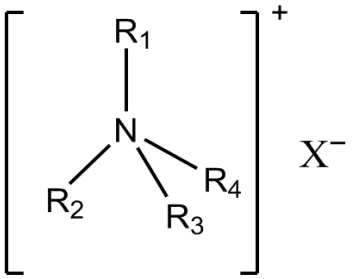

Figure 1: General structure of QACs: Cationic head group made up of a nitrogen atom with four R groups covalently bound, where an R group is a hydrogen atom, an alkyl group, or a substituted alkyl group. There is an associated anion, usually a halide such as a chloride or bromide ion

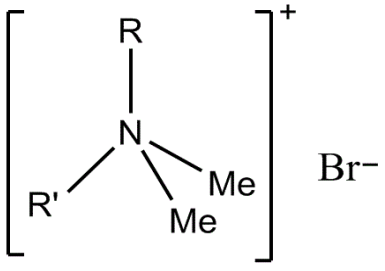

Figure 2: Dimethyl dialkyl ammonium bromide structure Two methyl groups take up two of the original R group positions, and the anion is a bromide ion.

QAC Formulations and synthesis

One of the most common QACs is dialkyl dimethyl ammonium chloride (DDAC). This is actually a generic name, due to the heterogeneous alkyl lengths in the formulation, usually because it is derived from natural sources such as soya bean oil (Gilbert & Moore, 2005). The mixture includes structures with two methyl groups and a variety of straight alkyl chains usually 8 to 18 carbons long (Buffet-Bataillon et al., 2012).

The synthesis of a wide variety of brominated dimethyl QACs is possible (see Figure 2), with customizable, specific alkyl lengths possible as bromine is a better leaving group than chlorine in S_N2 reactions due to its size and lower electronegativity (Bento & Bickelhaupt, 2008).

The synthesis of these specialized brominated QAC is a surprisingly facile process consisting of the quaternization, or exhaustive alkylation, of a tertiary amine with an unsubstituted alkyl halide (see Figure 3). This process allows specific alkyl chains to be determined with a very high yield in a relatively simple reaction. The specificity of this reaction is important in order to compare the effectiveness of these compounds in different environments and against individual pathogens.

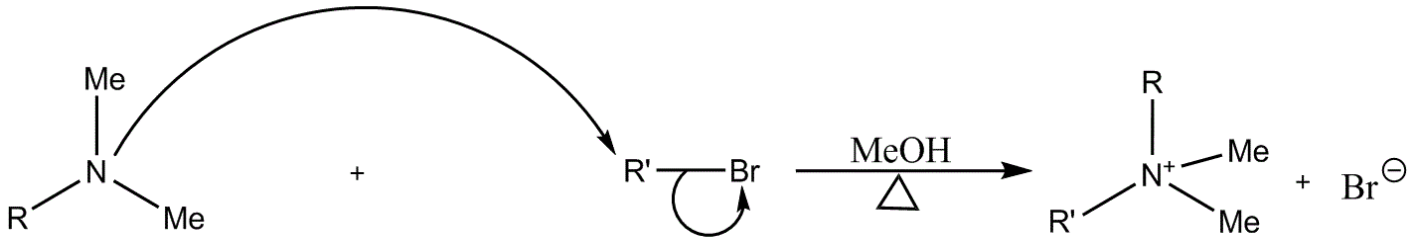

Figure 3: Mechanism of brominated dimethyl QAC synthesis: The lone pair of electrons on the nitrogen of the tertiary amine performs backside attack (S_N2 reaction) on the alkylbromide.

Mode of action

The general mode of action of QACs has been described as more of a physical reaction than a chemical one, with the QAC acting first as an ion exchanger and then as a "wedge" (Denyer & Maillard, 2002). The positively charged quaternary nitrogen of the QAC is attracted to and subsequently associated with the cell membrane, which has many negatively charged head groups that are part of acidic phospholipids (see Figure 4).

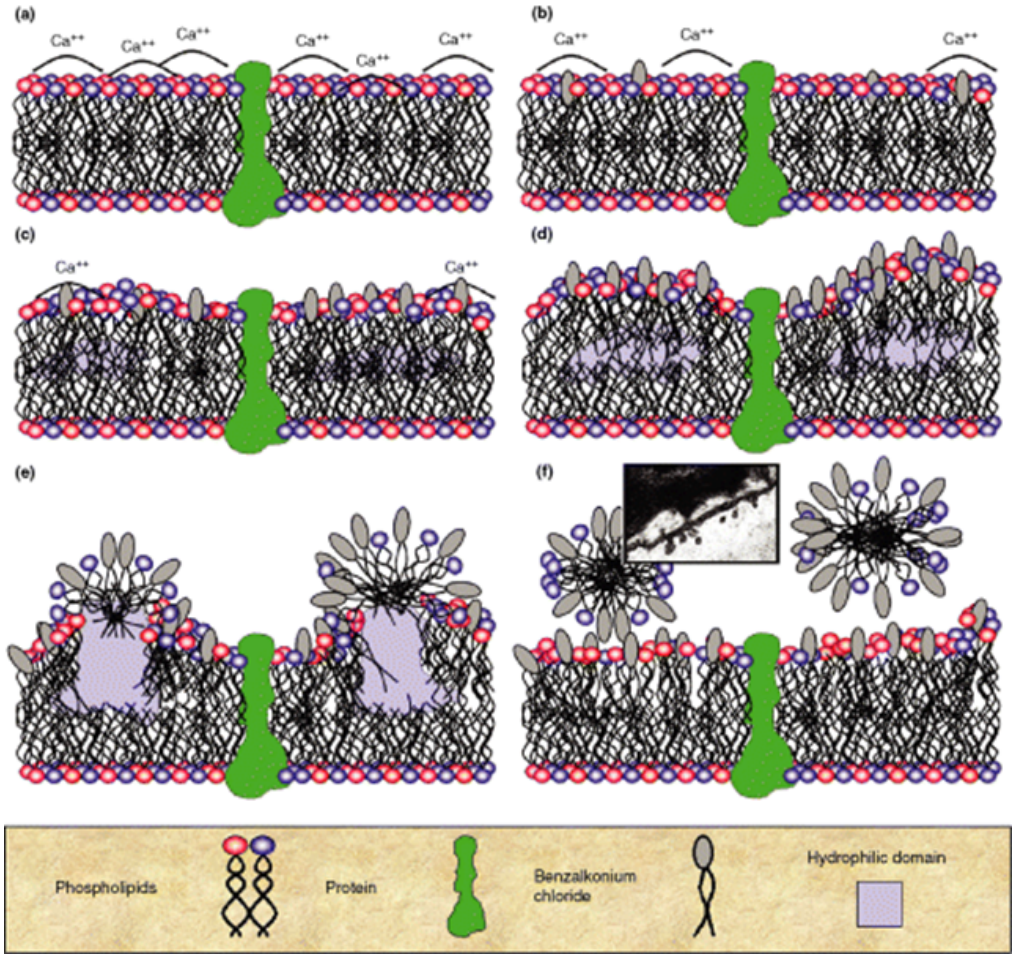

Figure 4: QAC Mode of Action Cartoon showing the mechanism of action for quaternary ammonium biocides. The segments (a–f) show progressive adsorption of the quaternary headgroup to acidic phospholipids in the membrane with increasing QAC exposure/concentration. This leads to decreased fluidity of the bilayers and the creation of hydrophilic voids in the membrane. Protein function is perturbed with an eventual lysis of the cell, and solubilization of phospholipids and proteins into QAC/phospholipid micelles. Inset micrograph shows vesicle formation from outer membrane caused by QAC treatment (Gilbert & Moore, 2005).

The hydrophobic N-alkyl chain "tails" of the QAC can integrate into the hydrophobic bacterial membrane core once the positively charged "head" of the QAC has associated with the membrane. However, it is the N-alkyl chain that provides the bulk of the antimicrobial activity due to its lipophilicity.

Indeed, it is the length of the N-alkyl chain that determines the QAC's activity. QACs have optimal activity against Gram-negative bacteria when their alkyl chains have 14-16 carbons making up the length. This is similar to the optimal chain length of 12-14 carbons against Gram-positive bacteria and yeast (Buffet-Bataillon et al., 2012).

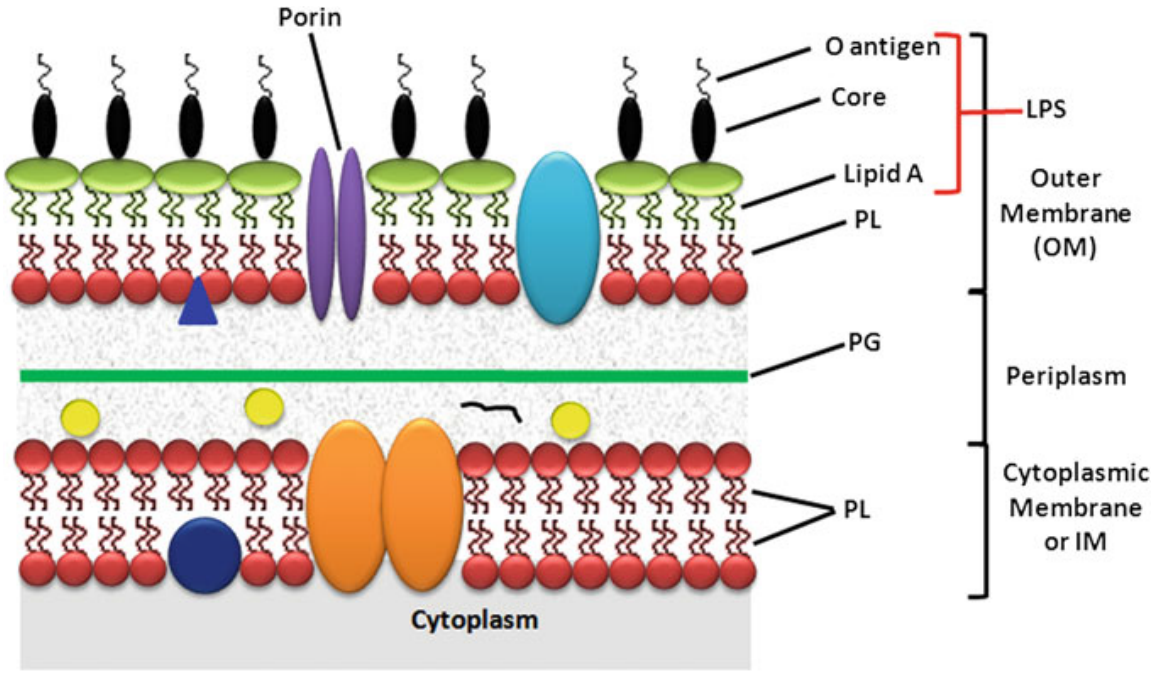

Figure 5: Schematic diagram of the outer membrane (OM), cytoplasmic or IM, and the intermediate periplasmic layer containing the peptidoglycan (PG). The IM consists of the two phospholipid (PL) leaflets and different lipoproteins. The outer membrane consists of two leaflets, the inner leaflet being composed of one phospholipid layer and the outer leaflet of lipid-A, core polysaccharide, and the O-antigen polysaccharide chains projecting outward (Chatterjee & Chaudhuri, 2012).

Closer examination of the structure of the Gram-negative bacterial cell membrane sheds some light as to why the QAC optimal activity occurs with alkyl chain length of 14-16 carbons. The outer

leaflet of the outer membrane (OM) consists primarily of lipopolysaccharide (LPS) which is composed of three major components (see Figure 5): lipid-A, the core polysaccharide, and the O-specific polysaccharide chains. It is possible to see a strong resemblance between the cation of the dialkyl dimethyl QACs and the structure of LPS lipid-A , in particular, the acyl chains attached to the nitrogen of the glucosamine units (see Figure 6). The six acyl chains of lipid-A are often 10-16 carbons. For example, one of the possible lipid-A compositions in *E. coli* has five 14-carbon chains, and one 12-carbon chain (Chatterjee & Chaudhuri, 2012). Thus the alkyl chain of QAC could interact with the OM of the Gram-negative cell in the same way as its inner membrane, or the OM of a Gram-positive cell (Denyer & Maillard, 2002).

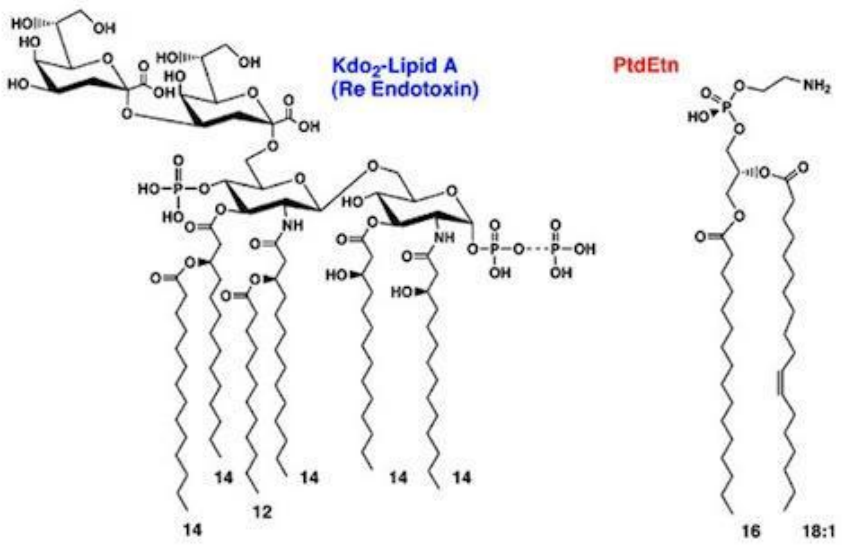

Figure 6: Structure of Kdo2-lipid A in E. coli K-12 compared to phosphatidylethanolamine, the major membrane phospholipid. Lipid A is much more complex in structure than phosphatidylethanolamine. Lipid A is glucosamine-based, and contains no glycerol. The acyl chains are shorter, fully saturated and hydroxylated if attached to glucosamine. Both Kdo and lipid A are required for growth, possibly because they are required for outer membrane protein folding (Raetz, n.d.).

Once the QAC tail has adsorbed to the bacterial membrane there is decreasing fluidity in the bilayers and hydrophilic voids are created, leading to change in protein function and cell lysis. The phospholipids and proteins of the membrane form mixed aggregates with the QAC called micelles (Wessels & Ingmer, 2013). These protuberances, or "blebs" on the cell wall have been documented on both Gram-positive and -negative cells (Bragg, Jansen, Coetzee, van der Westhuizen, & Boucher, 2014), (Yoshimatsu & Hiyama, 2007).

It is important to note that while the general mode of action is essentially the same for most QACs, there are potentially some important differences between specific types of QACs. The mode of action represented in Figure 3 utilizes a cartoon representation of benzalkonium chloride (BAC) as the acting QAC. The chemical structure of BAC can be seen in Figure 7 where it is contrasted with DDAC. The large benzyl group in BAC is replaced with an additional alkyl chain in DDAC, which could decrease steric hindrance at adsorption sites. This could explain the difference in their uptake isotherm classifications.

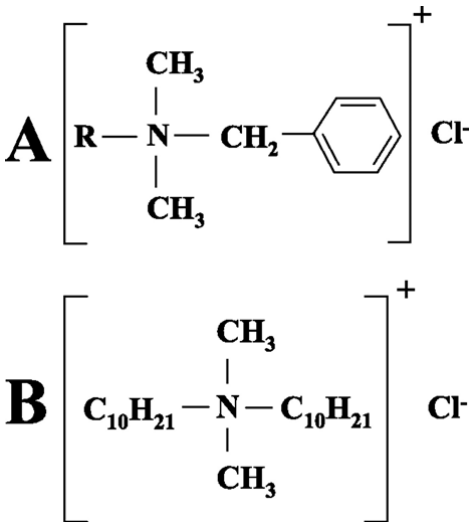

Figure 7: Structures of ADBAC (A), where R is a monoalkyl chain length varying in carbon number (50% C14, 40% C12, and 10% C16), and **DDAC (B)** (Ioannou, Hanlon, & Denyer, 2007).

In a study by Ioannou et al., BAC showed Langmuirian uptake to *S. aureus* cells, which is when molecules are adsorbed flat or vertically onto the surface, and uptake is limited by the number of binding sites available (see Figure 8). DDAC showed high-affinity binding, which is when solutes are adsorbed as ionic micelles or by ion exchange. Also, DDAC was shown to be the more potent agent and was less sensitive to changes in temperature (Ioannou et al., 2007). However, both of these uptake isotherm models assume a passive sorptive surface, and do not account for any active cellular intervention (Denyer & Maillard, 2002).

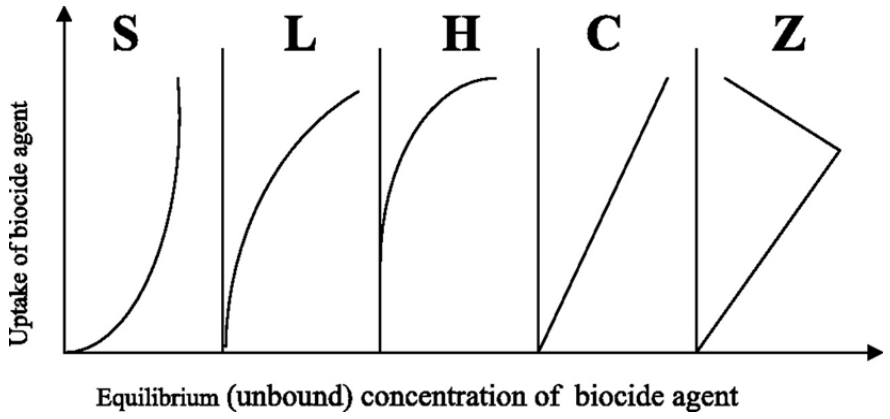

Figure 8: S-shaped, Langmuir (L), high (H), C-shaped, and Z-shaped uptake isotherm profiles. Subclass groups 1 to 4 are not shown (Ioannou et al., 2007).

Bacterial Resistance to QACs

Efflux transporters provide the primary means of resistance to disinfectants by lowering the concentration of the compound in the cell or cell membrane. In the case of QACs, pumps with a wide range of substrates are necessary in order to confer resistance. These substrates can be unrelated structurally and can include antibiotics (Poole, 2005).

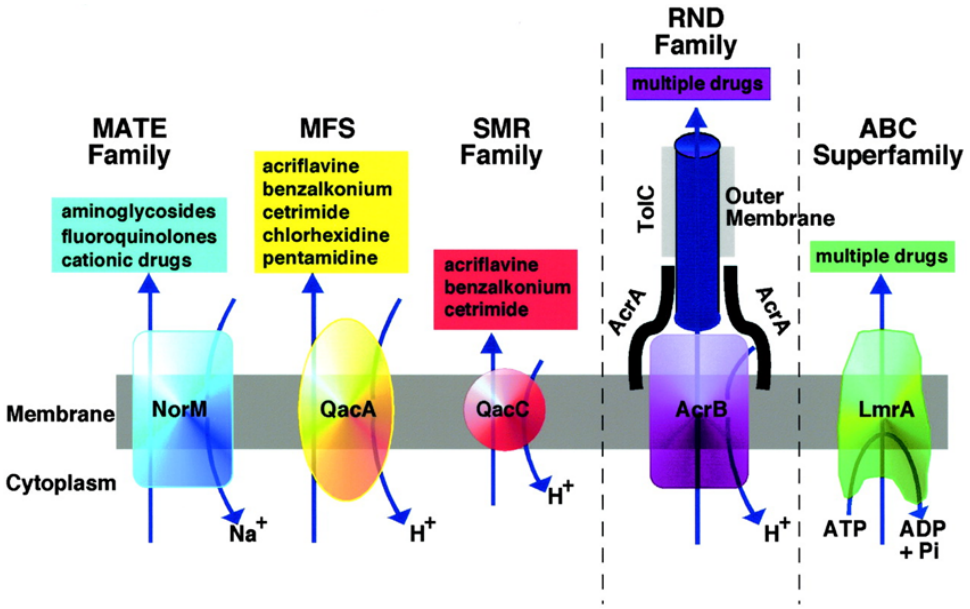

Figure 9: Diagrammatic comparison of the five families of efflux pumps. There are five families of multidrug-resistance efflux pumps: the multidrug and toxic-compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance nodulation division (RND) family, and the ATP-binding cassette (ABC) superfamily. A diagrammatic representation of the structure and membrane location of efflux pumps from each of these families is shown. Common examples of the individual proteins that form each class of efflux pump are indicated. Antibiotic substrates and examples of other substrates are also listed for each class of efflux pump. (Piddock, 2006).

There are five families of multidrug efflux transporters (see Figure 9): adenosine triphosphate binding cassette (ABC) transporters, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the resistance-nodulation- division (RND) family, and the multidrug and toxic compounds extrusion (MATE) family (Bragg et al., 2014). Gram-negative bacteria can possess multiple efflux pumps. The biocide pumps present, along with their associated substrates, can vary across species (Piddock, 2006), as can be seen in Table 1.

For this study, the Gram-negative bacteria *Escherichia coli* (W3110 & BC202), *Vibrio cholerae* (N16961), *Salmonella enterica* serovar *Typhimurium* (ATCC 14028), and *Pseudomonas Aeruginosa* (PAO1) were used to determine the minimal inhibitory concentrations (MICs) of ten

synthesized QACs. Each of these Gram-negative bacteria has pathogenic strains with notable virulence factors which make them clinically relevant.

E. coli is a bacterium commonly found in the lower intestines of humans and other mammals. Pathogenic strains cause diarrhea and are transmitted fecal-orally through contaminated water or food, or through contact. Shiga toxin-producing *E. coli* (STEC) cause approximately 265,000 infections each year in the United States alone ("CDC - General Information - E. coli,"). BC202 is an *E.coli* mutant that is hypersensitive to QACs and other biocides used here as a positive control. (Kumar & Doerrler, 2014)

S. typhimurium can also be found in the intestines, and can also cause diarrhea. It has a broad host range and is an intracellular pathogen that replicates within host-cell vacuoles using its two virulence-associated type III secretion systems (T3SS) (Yu, McGourty, Liu, Unsworth, & Holden, 2010), (Haraga, Ohlson, & Miller, 2008).

P. aeruginosa virulence factors include the LPS, a T3SS, type IV pili, extracellular products such as exotoxin A (similar to diphtheria toxin), flagellum, and biofilm formation which has been shown to additionally inhibit QACs through "quenching" (Morita, Tomida, & Kawamura, 2014). Biofilms can form on medical equipment such catheters and are involved in the pathogenesis of this opportunistic pathogen, making them especially dangerous to burn victims and cystic fibrosis patients (Aeschlimann, 2003).

V. cholerae is noted for its cholera toxin (CT) and is a cause of acute gastroenteritis in humans (Ceccarelli, Salvia, Sami, Cappuccinelli, & Colombo, 2006). The CDC estimates over three million cases of cholera illness causing over 100,000 deaths occur each year around the world ("CDC - General Information - Cholera,").

Table 1: Efflux pumps and determinants of relevant bacteria. Adapted from (Poole, 2005)

Efflux System/ Determinant	Pump Family	Substrate(s)	Organism
PmpM	MATE	BAC, FQ	*P. aeruginosa*
VcrM, VcmA	MATE	BAC, QAC	*V. Cholerae*
MdfA	MFS	BAC, CAM, ERM, TET, Aminoglycoside	*E. coli*
EvgA	RND	QAC	*E. coli*
YhiUV-TolC	RND	BAC	*E. coli*
AcrAB-TolC	RND	QAC, PHN, macrolides, Glycylcyclines, Oxazolidinones, FQ	*E. coli*
MexAB-OprM	RND	Macrolides, β-lactams,, aromatic hydrocarbons, other biocides, TET, CAM, β-lactamase inhibitors, other antibiotics	*P. aeruginosa*
MexCD-OprJ	RND	aromatic hydrocarbons, other biocides, TET CAM, other antibiotics	*P. aeruginosa*
MexEF-OprN	RND	Trimethoprim, TRI, CAM, FQ, aromatic hydrocarbons	*P. aeruginosa*
QacE	SMR	QAC	*P. aeruginosa*
QacEΔ1	SMR	QAC	*P. aeruginosa, E. coli, S. typhimurium, Vibrio spp.*
QacG	SMR	QAC	*P. aeruginosa*
EmrE	SMR	BAC	*E. coli*
SugE	SMR	QAC	*E. coli*

CAM = chloramphenicol, ERM = erythromycin, FQ = fluoroquinolones, TET = tetracyline, TRI = triclosan

Table 2: Bacterial strains

Strain	Relevant markers	Source or reference
E. coli W3110	Wild type, F^-, λ^-, *IN(rrnD-rrnE)1*, *rph-1*	*E. coli* Genetic Stock Center, Yale University
E. coli BC202	W3110, ΔyqjA::Tet^r ΔyghB781::Kan^r	Thompkins, Chattopadhyay, Xiao, Henk, & Doerrler, 2008
Vibrio cholerae (N16961)	Antigenic Properties: biovar eltor, serov O:1, serotype Inaba biovar eltor	ATCC 39315
Salmonella enterica serovar *Typhimurium*	Antigenic Properties: I4,5,12: i:1,2	ATCC 14028
Pseudomonas Aeruginosa (PAO1)	Produces pyocyanin	ATCC 15692

MATERIALS AND METHODS

Synthesis of QACs. All chemicals were reagent grade. The tertiary amines were provided by Albemarle and the alkyl bromides were purchased from commercially available sources. The QACs were synthesized by the reaction of an alkyl bromide with a tertiary amine with a ratio of ~0.9:1 using methanol (or ethanol) as a solvent as needed. Most of the reactions utilized a ratio of reagents to solvent of about 4:1, with the reaction stirring at a temperature high enough to maintain the reaction mixture as a liquid, as adapted from U.S. Patent 5599990 (Joseph H. Miller, Joe D. Sauer, Dru L. DeLaet, 1997). Analytics were performed by Albemarle Corporation.

Bacterial growth conditions. Bacterial cultures were grown in Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract, and 1%NaCl) unless otherwise stated. LB medium of pH 6.0 was buffered with 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES). When required, the medium was supplemented with kanamycin (Kan) at 30 μg/ml. All cultures were grown at 30°C or 37°C as appropriate. (Kumar & Doerrler, 2014)

MIC determination. The MICs of biocides were carried out using a 2-fold dilution technique either in 1.5- by 15-cm glass tubes in liquid medium. Overnight cultures were freshly diluted 1:100 into LB medium with suitable antibiotics and additives. Exponentially growing cultures at an OD600 of ~1.0 were inoculated at a density of 105 cells per ml into LB medium supplemented with a series of 2-fold dilutions of indicated biocide. Cell growth was determined visually after incubation at 30°C or 37°C as appropriate for 20-24 h. All experiments were repeated at least three times (Kumar & Doerrler, 2014). The lowest concentration of QAC that completely inhibited growth was identified as the MIC. A >4-fold difference in susceptibility was considered significant.

RESULTS AND DISCUSSION:

Ten QACs were selected from the library of QACs synthesized in conjunction with Dr. Joe Sauer at Albemarle Corporation (see Table 3). These QACs were used in determination of MICs against the Gram-negative bacteria *Escherichia coli* (W3110 & BC202), *Vibrio cholerae* (ATCC 39315), *Salmonella enterica* serovar *Typhimurium*, and *Pseudomonas Aeruginosa* (PAO1). The MIC results are summarized in Table 4.

Table 3: Selected QACs

Code	R, R'	Mol. Formula	Mol. Wt	%QAC
10.10	Didecyl	$C_{22}H_{48}BrN$	406.54	80.0
12.12	Didodecyl	$C_{26}H_{56}BrN$	462.65	79.1
12.10	Dodecyl, decyl	$C_{24}H_{52}BrN$	434.59	81.8
12.8	Dodecyl, octyl	$C_{22}H_{48}BrN$	406.54	100
12.6	Dodecyl, hexyl	$C_{20}H_{44}BrN$	378.48	64.0
12.4	Dodecyl, butyl	$C_{18}H_{40}BrN$	350.43	79.8
14.10	Tetradecyl, decyl	$C_{26}H_{56}BrN$	462.65	80.0
14.8	Tetradecyl, octyl	$C_{24}H_{52}BrN$	434.59	78.7
14.4	Tetradecyl, butyl	$C_{20}H_{44}BrN$	378.48	100
16.3	Hexadecyl, propyl	$C_{21}H_{46}BrN$	392.51	82.7

Table 4: Minimum inhibitory concentrations (MICs) of selected microbes (ug/mL)

QAC	BC202	W3110 (30°C)	W3110 (37°C)	*S. enterica* serovar *Typhimurium*	*V. cholerae*	*P. Aeruginosa*
10.10	≦2.5	5	5	5	≦0.625	25
12.12	25	>100	100	5	2.5	>100
12.10	5	5	5	5	≦0.625	>100
12.8	≦2.5	5	5	5	≦0.625	25
12.6	6.25	25	25	25	6.25	50
12.4	50	100	100	50	50	>100
14.10	50	>100	>100	12.5	1.25	>100
14.8	≦2.5	5	≦5	≦2.5	1.25	>100
14.4	≦6.25	12.5	12.5	12.5	6.25	50
16.3	5	6.25	10	6.25	2.5	>100

Generally, the MIC values were as expected with a few notable exceptions. For QAC 10.10 against W3110, a value of 5 ug/mL was obtained, which corresponds to values found in the literature for the chlorinated version (DDAC) against *E.coli* (Dadekian, 1974), (Walsh et al., 2003). This value was also found against W3110 for QACs 12.10, 12.8, 14.8, and 16.3. The QACs that did not show effectiveness against W3110 are QAC 12.12 and 14.10, with QAC 12.4 being nearly ineffective. QACs 12.6 and 14.4 are effective against W3110 at essentially the same value, which is interesting considering they have the same total number of carbons between the two alkyl chains. This pattern also applies to the previously mentioned 10.10 and 12.8, as well as the 12.12 and 14.10.

BC202 and *V. cholerae* showed the lowest values across the board as expected. BC202 has been shown to be hypersensitive to biocides (Kumar & Doerrler, 2014), and the *V. cholerae* strain used has been shown to lack any resistance to QACs (Sjölund-Karlsson et al., 2011), which validated comparisons of MIC comparisons between QACs and other bacteria.

S. enterica serovar *Typhimurium* showed MIC values similar to W3110 for eight of the ten selected QACs: 10.10, 12.10, 12.8, 12.6, 12.4, 14.8, 14.4, & 16.3. These MICs for both strains were either exactly the same, or the differences were deemed insignificant. The two QACs that showed a significant difference in MIC values between W3110 and *S. enterica* were the 12.12 and the 14.10.

P. aeruginosa showed the highest values across the board as expected, since it is known to be hardest to kill. There were four QACs which were shown to be effective against *P. aeruginosa*: 10.10, 12.8, 12.6, and 14.4. Out of these, the 12.6 and the 14.4 are surprising, considering that they are somewhat less effective against the other bacteria, which may indicate different efflux pumps are in play in these cases.

CONCLUSIONS:

The results show comparable MICs of the dialkyl dimethyl brominated QACs to what is currently available using chlorinated dialkyl dimethyl QACs, showing that both types are effective as biocides, and that further development of dialkyl dimethyl brominated QACs for biocide use is possible. BC202 shows increased sensitivity to most of these brominated QACs as expected. Differences in pathogen QAC sensitivity can help us investigate the QAC mechanism and resistance, and lead us towards optimization of biocides stemming from correlation of alkyl chain length to Gram-negative bacterial lipid-A composition. The results suggest potential new biocides for disinfectant use as well as a pathway for better understanding of QAC mechanism and more targeted antimicrobial activity.

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