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Review

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Quaternary Ammonium Compounds: An Antimicrobial Mainstay and Platform for Innovation to Address Bacterial Resistance

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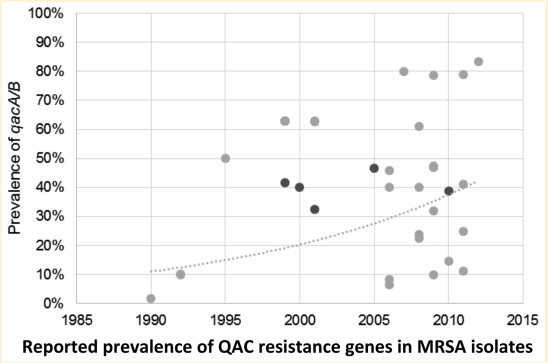
ABSTRACT: Quaternary ammonium compounds (QACs) have represented one of the most visible and effective classes of disinfectants for nearly a century. With simple preparation, wide structural variety, and versatile incorporation into consumer products, there have been manifold developments and applications of these structures. Generally operating via disruption of one of the most fundamental structures in bacteria—the cell membrane—leading to cell lysis and bacterial death, the QACs were once thought to be impervious to resistance. Developments over the past decades, however, have shown this to be far from the truth. It is now known that a large family of bacterial genes (generally termed *qac* genes) encode efflux pumps capable of expelling many QAC structures from bacterial cells, leading to a decrease in susceptibility to QACs; methods of regulation of *qac* transcription are also understood. Importantly, *qac* genes can be horizontally transferred via plasmids to other bacteria and are often transmitted alongside other antibiotic-resistant genes; this dual threat represents a significant danger to human health. In this review, both QAC development and QAC resistance are documented, and possible strategies for addressing and overcoming QAC-resistant bacteria are discussed.



KEYWORDS: *antibiotic resistance, antimicrobial, antiseptic, biocide, biofilm, disinfectant, MRSA, quaternary ammonium compound*

Quaternary ammonium compounds (QACs) have found ubiquitous use throughout society as surfactants, dyes, neuromuscular blocking agents, and disinfectants, the last of which will be the focus of this review. As broad-spectrum antibacterial agents, QACs are constantly applied in medical, industrial, household, and other settings,[1] raising debate in the literature as to the long-term effects of QAC disinfectants. It has been stated by the Cosmetic, Toiletry, and Fragrance Association that "Antibacterial products do not cause antibacterial resistance. They kill germs, thus breaking the circle of infection."[2] While this sentiment is shared by many, resistance to QACs has been developing at an alarming rate due to their robust chemical stability and heavy use. This issue is further exacerbated by the prevalence of biofilms, which are pervasive microbial communities capable of transferring QAC resistance via multi-drug-resistant plasmids at an increased rate. This review will focus on the structure and activity of QACs, discuss in detail the issues facing current QACs including emerging resistance, and highlight areas in which further studies are most promising.

1. BACKGROUND ON QACS

1.1. Physical Properties. QACs in a general sense contain a positively charged nitrogen "head" bearing four bonds; for the purposes of this review, only fully substituted QACs bearing four bonds to nonpolar alkyl or aryl tails will be considered, including imine- and imine-like QACs (Figure 1). It should be noted that QACs are often referred to as quaternary ammonium cations and quaternary ammonium surfactants (QAS) in the literature. This general amphiphilic scaffold provides a number of desired physical properties making it attractive to a number of markets, ranging from antiseptics to surfactants to anesthetics. Simple QACs tend to be water-soluble and are quite stable, lending themselves to straightforward and varied formulations. The counterion is viewed as less crucial to many applications, though it can influence solubility. Most reported QAC salts are composed of chloride or bromide; iodide salts tend to exhibit decreased solubility. Because of their amphiphilic nature, QACs are capable of forming micelles and thus are often tested for their critical micelle concentration (CMC).[3]

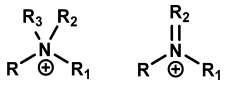


Figure 1. General structure of QACs.

1.2. Uses Other than Antimicrobial Agents. For the purposes of this review, focus will be placed on the antimicrobial activity of fully substituted quaternary ammonium compounds. It is worth mentioning, however, that QACs possess tremendous utility in a variety of applications, and many of their antimicrobial properties have been discovered

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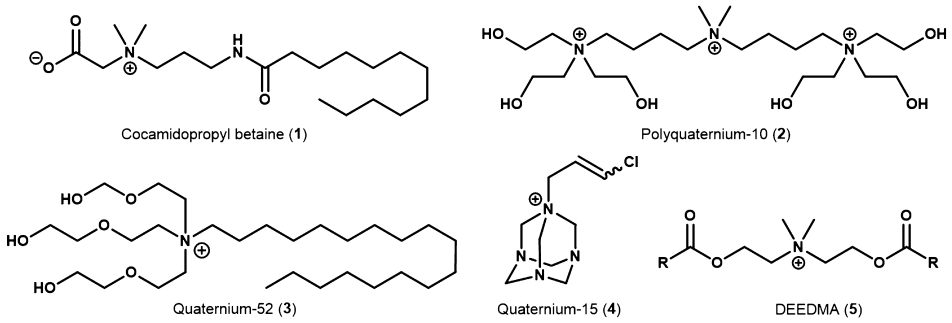

Figure 2. QACs used primarily as cationic surfactants.

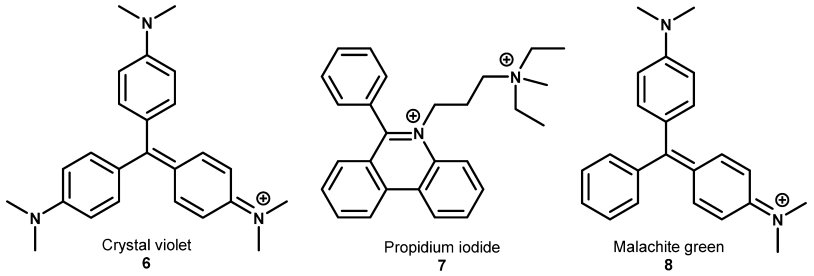

Figure 3. QACs used as biological stains and dyes.

through research efforts in these alternate areas, as enumerated below.

1.2.1. As Cationic Surfactants. The amphiphilic nature of QACs is simultaneously exploited for surfactant, preservative, and antimicrobial properties in a number of cosmetic and personal care products.[4] QACs such as cocamidopropyl betaine (**1**) and several quaternium and polyquaterniums scaffolds (**2**–**4**) (Figure 2) are incorporated into soaps, hair care products, cosmetics, and contact lens solutions[5,6] for their wetting, antistatic, and other properties. In 2002 alone, polyquaternium-10 was approved by the FDA for use in hundreds of products including shampoos and conditioners, eye makeup, hair dyes, soaps and detergents, shaving and skin care gels and lotions, and a number of suntan formulations.[7] Diesteralkonium products such as diethylesterdimethylammonium chloride (DEEDMA, **5**) (Figure 2) have been utilized since the 1950s as fabric softeners and in fact account for approximately 50% of all household QAC uses.[3,8]

1.2.2. As Biological Stains. QACs have found substantial use as biological stains and dyes. Crystal violet (**6**), propidium iodide (**7**), and malachite green (**8**) (Figure 3) are among several QACs that exist within highly conjugated aromatic systems, leading to their uses as biological indicators. These agents can either interact with bacterial cell wall components (i.e., crystal violet in Gram staining) or intercalate into DNA (i.e., propidium iodide in confocal microscopy imaging). It is important to note that these compounds do not affect bacterial viability within several-fold dilutions of the concentrations at which they are employed for staining purposes.

1.3. As Antimicrobials: Mechanism of Action. Given their amphiphilic nature, QACs demonstrate a detergent-like mechanism of action against microbial life, notably against several of the ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) which are notorious for their pathogenicity.[9] Electrostatic interactions between the positively charged QAC head and the negatively charged bacterial cellular membrane are followed by permeation of the QAC side

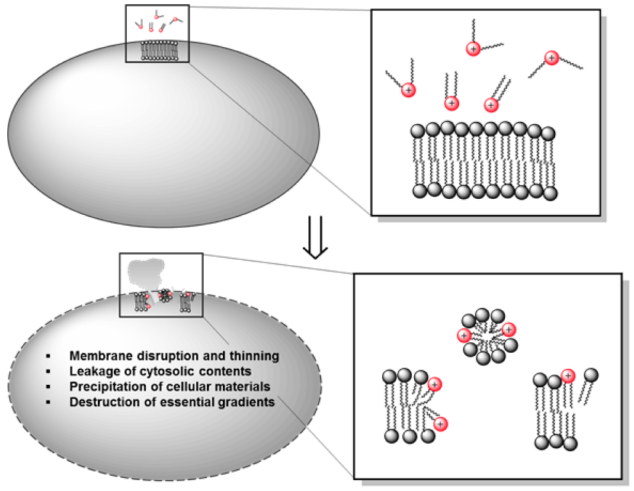

Figure 4. Mechanism of action of QACs against bacteria.

chains into the intramembrane region, ultimately leading to leakage of cytoplasmic material and cellular lysis (Figure 4).[10] Because QACs target the bacterial cell membrane, they can be considered to be broad-spectrum antibiotics though they exhibit markedly increased activity against Gram-positive bacteria. While Gram-positive bacteria possess a single phospholipid cellular membrane and a thicker cell wall composed of peptidoglycan, Gram-negative bacteria are encapsulated by two cellular membranes and a rather thin layer of peptidoglycan. It is due to the presence of this second membrane that QACs and other membrane-targeting antiseptics tend to exhibit decreased activity—typically on the order of 8-fold—against Gram-negative species. Though it is outside the scope of this review, it should be noted that QACs are also potent antifungal agents.[11]

2. COMMERCIALIZED QACS

For nearly a century, QACs have found use as leading antiseptics in virtually every residential, commercial, healthcare, industrial, and agricultural setting. Jacobs published the first

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account of QACs and their bactericidal activity in 1916, focusing on the benzylated and acylated quaternary salts of hexamethylenetetramine and ultimately concluding that the general quaternary ammonium nature of these classes of compounds is "bactericidogenic," or responsible for their bactericidal activity.[12−14] This work was followed by Hartmann and Kägi in 1928 with their paper titled "Saure Seifen", translating literally to acidic soap, wherein they document the antibacterial properties of esterified QACs.[15] Browning and co-workers published a series of papers in the 1920s regarding the bactericidal powers of quaternized derivatives of pyridine, quinolone, acridine, and phenazine. Shortly thereafter, Domagk published a landmark report on the germicidal properties of quaternary ammonium salts containing at least one long aliphatic side chain.[16] His work focused on a class of compounds deemed alkyl dimethyl benzyl ammonium chloride (ADBAC),[16,17] most notably benzyldimethyldodecylammonium chloride which in that era was marketed under the trade name Zephirol. A mixture of alkyl dimethyl benzylammonium chlorides, generally grouped as benzalkonium chloride (BAC, **9**: n = 1−11), was the first active-ingredient QAC approved by the Unites States Environmental Protection Agency in 1947[18] and remains to this day the main active ingredient in several leading antiseptics, including many formulations of Lysol. In tandem with this seminal registration, Shelton and co-workers discovered the potent antibacterial activity of cetyltrimethylammonium bromide (CTAB, **10**) and cetylpyridinium chloride (CPC, **11**),[19] QACs including amides and ester moieties,[20] and QACs derived from cyclic amines.[21]

Since their introduction to the market over 70 years ago, various QAC formulations have been utilized in combination with other ingredients such as ethanol to yield commercial antiseptics. In today's market, the most common commercial monocationic QACs include BAC (**9**), twin-chained QAC dimethyl dodecyl ammonium chloride or bromide (DDAC or DDAB, **12**), CTAB (**10**), and CPC (**11**) (Figure 5). The twin-chained compounds which replace the benzyl group of BAC with an additional long carbon side chain exhibit markedly better antimicrobial activity against Gram-positive *S. aureus* [minimum inhibitory concentration (MIC) of 1 μM for DDAC and 8 μM for BAC] along with somewhat increased toxicity as measured by red blood cell lysis (16 μM for DDAC, 63 μM for BAC). It is likely that the benzyl moiety has been incorporated into key formulations in order to mitigate toxicity; antibacterial activity is accordingly sacrificed.

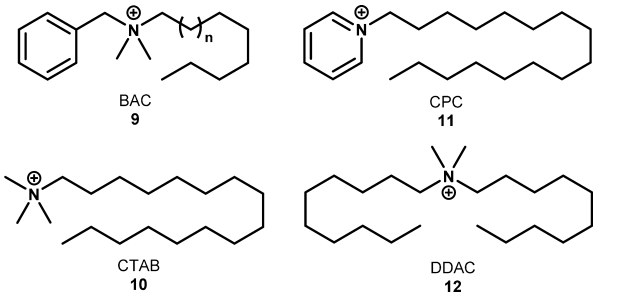

Figure 5. Lead commercial QACs.

Since their introduction to the market, commercial QACs have experienced global and exponential usage based on their potency, relatively low toxicity, simplicity, and ease of preparation. This heavy use has not come without consequences, however, and will be detailed in section 4.

3. ACADEMIC ENDEAVORS

In addition to the exploration of QACs in the industrial sector, significant progress has been made in the academic realm. A number of groups over the past few decades have actively explored the physical and biological effects of altering the structure of QACs, modifying the alkyl and aryl groups in QACs, and examining the importance of the charge state. Quite a variety of scaffolds as described below have been examined with diverse motivations including structural novelty, synthetic simplicity, an enhanced toxicity profile, and biological activity. Included in this section are a sampling of recent advances in the field; for a more thorough discussion see the reviews in refs 22−26.

3.1. Investigation of Alkyl QACs. Miklas and co-workers investigated the role of the hydrophobic portion of QACs by synthesizing binaphthyl QACs (**13**) and camphorsulfonamide (CSA) analogs bearing either a linear (**14**) or a cyclic QAC (**15**) (Figure 6).[27,28] The compounds exhibited a wide range of MICs, with the CSA C_{14} analog and C_{12} binaphthyl analogs being most active in their respective classes. As is common with QACs, all compounds demonstrated better activity against *S. aureus* (Gram +) than against *Escherichia coli* (Gram −) and *Candida albicans* (fungus).

Given the global membrane-targeting ability of QACs, toxicity is of obvious concern. In this vein, amino acid-derived QACs have been evaluated for antibacterial activity; one particular series consists of those derived from phenylalanine (**16**) and tyrosine.[29,30] The first report in this series investigated the effect of the ester alkyl chain length, confirming that C_{12} and C_{14} show optimal bioactivity, followed shortly thereafter by a report focusing on the selectivity of quaternized phenylalanine for bacterial cells over red blood cells. The authors found that hemolytic activity was more related to critical micelle concentration (CMC) whereas antibacterial activity was due to the action of monomeric QACs. In comparing the activity of the hydrochloride salt of phenylalanine to the activity of quaternized

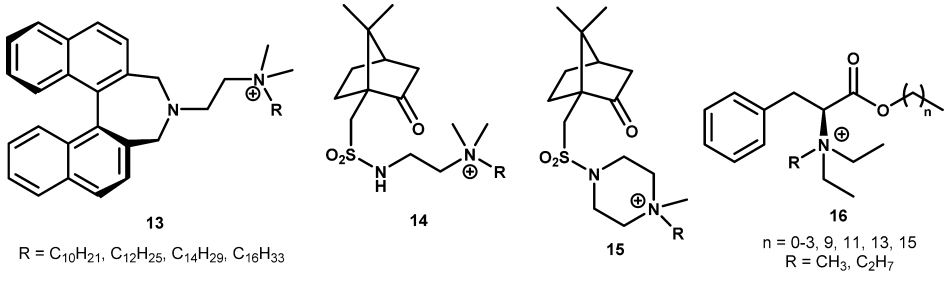

Figure 6. MonoQACs of various structures and alkyl chain lengths.

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derivatives, it was additionally confirmed that a permanent cationic nature confers superior antibacterial activity.

A series of long-chain alkyl and benzyl dihydroxy QACs (**17**, Figure 7) were prepared through the simple alkylation of

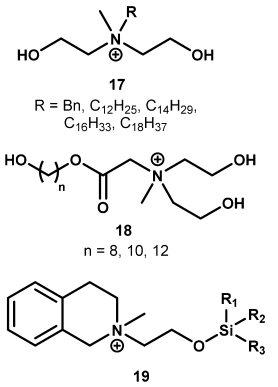

Figure 7. HydroxyQACs from the literature.

methyldiethanolamine (MDEA), with the authors' hypothesis being that the flexible hydroxyl groups would facilitate membrane permeation.[31] Again it was determined that C_{12} was the best analog out of long alkyl chain compounds, which exhibited antibacterial and antifungal properties. Cytotoxicity accordingly tracked with antimicrobial effects. MDEA has also been tethered to esters of various chain lengths (**18**) designed to probe the optimal length for antibacterial activity.[32] These hydroxyl esterQACs were active against Gram-positive pathogens and yeast, albeit with smaller zones of inhibition as compared to commercial standard CTAB; interestingly, they had no detectable activity against fungi. Following the introduction of ethanolamines, Zablotskaya and co-workers investigated the effect of silylated hydroxyls tethered to tetrahydroioquinolines (**19**) on biological activity.[33] They found enhanced activity of the silyl-modified compounds against bacteria and against tumor cell lines, though shorter alkyl chains surprisingly led to better antibacterial activity.

Though monoQACs have been heavily investigated, it is surprising that less work has been published on the antibacterial and antibiofilm activity of bisQACs. Notable contributions to this field include work by Bhattacharya,[34] Menger,[35] Devinksy,[36] and Clardy.[37] Moreover, the literature presents little work on QACs possessing an even higher charge state (multiQACs). This is at odds with the notion that increased cationic nature drives selectivity for bacterial cells over eukaryotic cells,[38] as the former are more negatively charged than the latter. This prompted our group to launch an investigation into the importance of the charge state, distribution of charge, and nature of side chains in the antibacterial, antibiofilm, and hemolytic activity of QACs. Beginning with tetramethylethylenediamine (TMEDA)-derived QACs (**20**, n = 1, Figure 8), we initially examined the effects of alkyl chain length and symmetry on antibacterial activity, finding that symmetrical compounds bearing C_{12} side chains exhibited optimal bacteriolytic activity.[39] We then extended the polyamine backbone to generate additional bis- (**21**, **22**), tris- (**23**), and tetraQACs (**24**) as well as bipyridine structures with various substitution patterns (**25**–**27**, Figure 8).[40−42] Each of these classes possesses MICs in the low micromolar range against several of the Gram-positive and Gram-negative ESKAPE pathogens and are simple and economical to prepare. Furthermore, these QACs represent some of the most potent biofilm-eradicating molecules published to date, demonstrating minimum biofilm eradication concentrations (MBEC) well under 100 μM against Gram-positive bacterial biofilms.[43]

3.2. Incorporation into Aromatic Systems. In addition to the bipyridine structures above, QACs have been incorporated into aromatic systems through a variety of strategies. 6-Hydroxyquinolinium QACs bearing alkyl chains of varied length (**28**, Figure 9)[44] and bisQAC pyridinium compounds were synthesized and found to possess broad antibacterial activity.[45] The bioactivity of QACs arising from pyridine and isoquinoline scaffolds bearing hydrophobic cholesterol, menthol, and borneol groups was likewise investigated.[46] Select analogs, such as pyridinium-cholesterol QAC **29** (Figure 9), inhibited Gram-positive bacterial growth below 4 μM yet were less active against Gram-negative species. Several analogs were also active against mycobacterial strains. Fadda and El-Mekawy also incorporated quaternized pyridines into their novel methane cyanine dyes (**30**, **31**).[47] The most active compounds, perhaps not coincidentally, were those that contain permanent quaternary centers. The mechanism of action for these dyes has yet to be determined, though it should be noted that they also showed anticancer activity.

Through the synthesis of quaternary ammonium compounds for use as neuromuscular blocking agents (section 1.2), Collier and co-workers discovered that decamethylene bisisoquinolinium bromide possessed remarkable antibacterial activity.[48] Because of this observation, a number of bisisoquinolinium derivatives were synthesized and found to possess superior activity against *S. aureus* and *Mycobacterium phlei* with MICs in

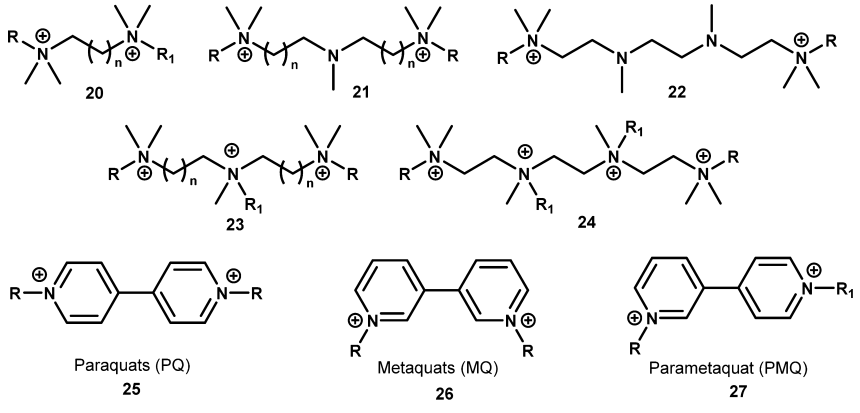

Figure 8. Bis-, tris-, and tetraQACs developed in our laboratories.

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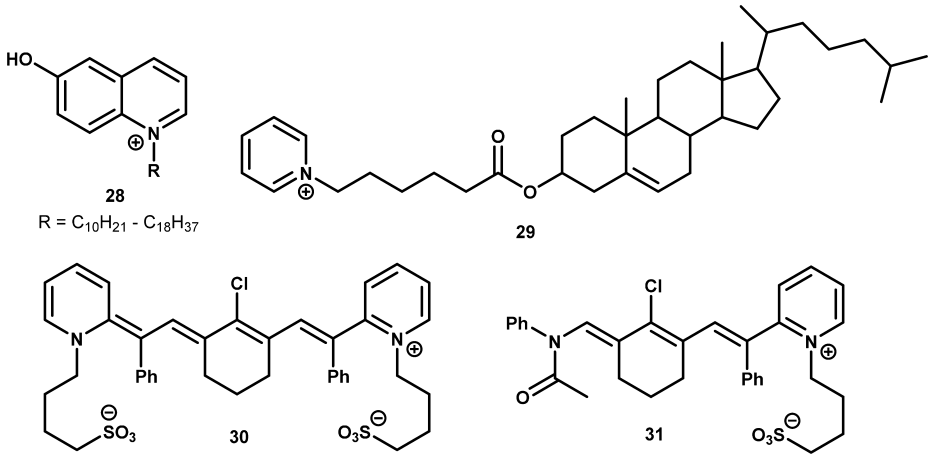

Figure 9. Select QACs incorporating pyridine.

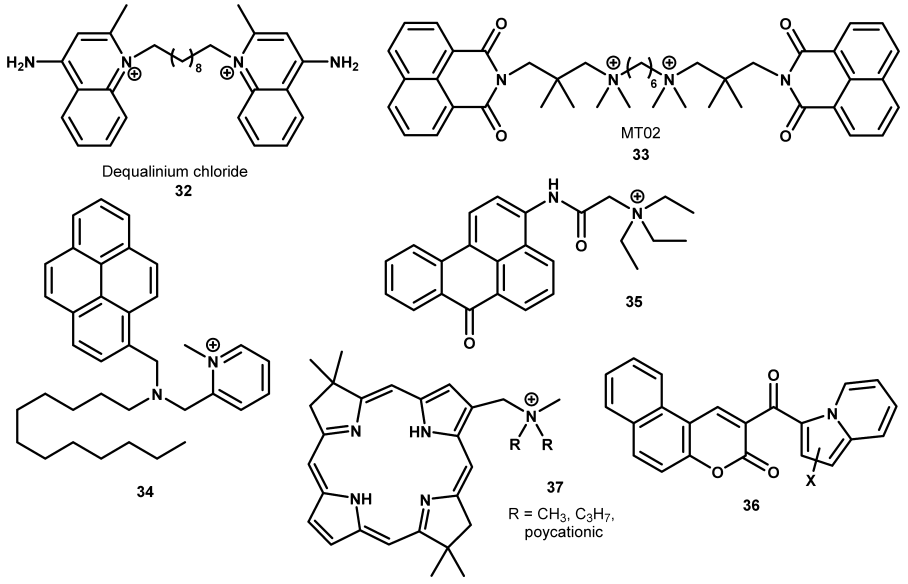

Figure 10. Aromatic and arylQACs with various mechanisms of action.

the single-digit micromolar range. This study was expanded, ultimately leading to the discovery of dequalinium chloride (**32**, Figure 10), which inhibited the growth of all of the microbial species tested yet failed to exhibit toxic effects when applied topically and subcutaneously to rodents.[49] Dequalinium chloride has since been shown to be antibacterial, antifungal, and antiparasitic through membrane disruption[50] as well as through the precipitation of cytosolic components, namely, nucleic acids.[51] This compound completely inhibits Gram-positive bacterial growth at concentrations of as low as 2 μM and possesses IC_{50} values in the submicromolar range against various parasites.[24] Extensive work with the isoquinolinium linker has confirmed that a 10- to 12-carbon linker is optimal.[24] Originally intended as anticancer agents, bisnaphthalimides such as MT02 (**33**) have since been found to possess remarkable activity against Gram-positive bacteria, especially against methicillin-resistant *S. aureus* (MRSA), though their mechanism of action appears to be the inhibition of DNA replication rather than membrane damage.

A clever melding of these two mechanisms of action has led to the production of a dual-warhead QAC that bears a pyridinyl quaternary center and a tertiary amine—a pH-dependent quaternary center—along with a 12-carbon alkyl chain and a DNA-intercalating fluorescent pyrene moiety (**34**, Figure 10).[52] While this compound was active against Gram-positive bacteria, its four-carbon alkyl chain counterpart exhibited no antibacterial activity, confirming the importance of the alkyl-side-chain length in conferring antibacterial behavior.

Recently, the modest activity of a benzanthrone-derived QAC (**35**) was reported.[53] This fluorescent, water-soluble QAC showed zones of inhibition against several bacterial and fungal strains comparable to those of gentamicin, and when it was incorporated into a thin polylactic acid film, slow release of the photostable QAC was achieved. The authors suggest the potential for using such compounds as additives to biomedical devices and in the agricultural industry. An alternate fluorescent scaffold that incorporates the QAC directly into the aromatic system is composed of coumarin indolizines (**36**).[54] While such moieties may not initially appear to be QACs, their resonance forms, generated by the donation of nitrogen's lone pairs to the aromatic system, yield quaternary ammonium centers. These "QACs by resonance" exhibit moderate antimicrobial activity against bacteria and multiple fungal species though the specific mechanism of action has yet to be elucidated.

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Of special note are bacteriochlorins appended with QACs (**37**), which possess an alternative mechanism of action.[55] It was found that several analogs with various substitutions possess potent activity against several of the ESKAPE pathogens and induce greater than 6 log killing at concentrations below 200 nm for Gram-positive species and 1 to 2 μM for Gram-negative species. Interestingly, the activity was highly dependent on the substituent nature and incubation time. Such compounds that potentially combine mechanisms of action would be of great interest for further study.

3.3. Natural Products Containing QACs. Though each of the leading QAC antiseptics currently on the market is synthetic, a number of natural products bearing QAC moieties have been shown to possess antibacterial activity, including the inhibition of biofilm formation. These include chelerythrine (**38**) and closely related structural analogs sanguinarine (**39**) and berberine (**40**) as well as ageloxime D (**41**) (Figure 11).[56]

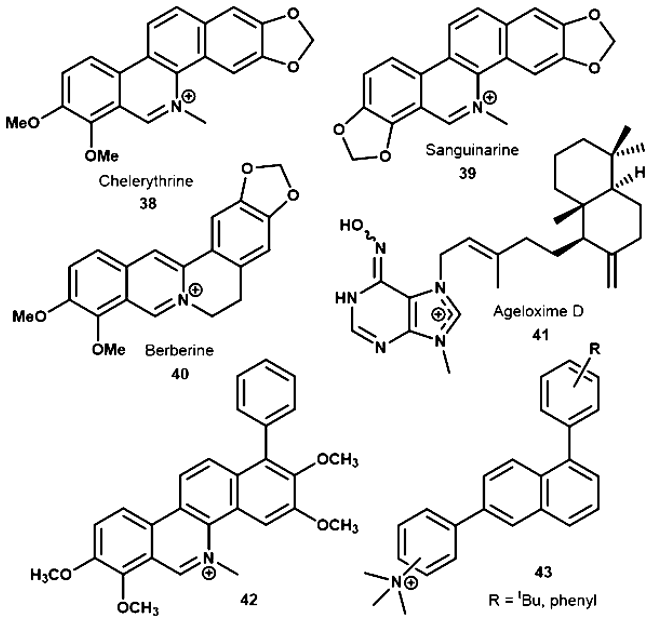

Figure 11. Natural product QACs and derivatives thereof.

As an extension of antimicrobial studies on sanguinarine and chelerythrine, phenyl substitution of the naphthalene core (**42**) was found to significantly enhance antibacterial activity. This finding was further explored through appending amines to 1,6-diphenylnaphthalenes (**43**), the quaternized analogs of which further increased antibacterial activity to submicromolar concentrations.[57] The authors postulate that the antibacterial activity of 1,6-diphenylnaphthalenes is associated with their effects on FtsZ polymerization, an essential bacterial cytokinesis protein.

3.4. Guanyl and Guanidine QACs. In addition to QACs containing a central nitrogen connected to four alkyl or aryl groups, positively charged nitrogens in the form of guanyl groups have been employed in the antiseptic market. In the guanidine class, the most prevalent product is chlorhexidine (**44**), a chlorinated bisguanide compound that at physiological pH exists as a bis-cation (Figure 12). Though often described as possessing a similar mechanism of action to traditional long-chain alkyl QACs, chlorhexidine and similar compounds lacking significant side chains may additionally be biocidal through the precipitation of cytosolic materials and the inhibition of the

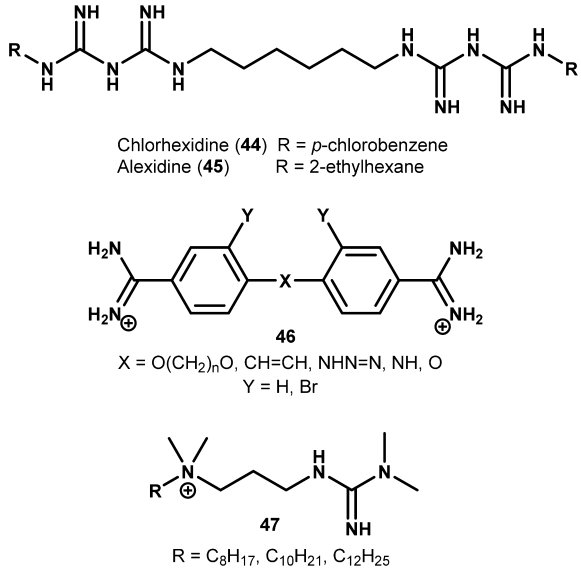

Figure 12. Guanyl and guanidine QACs.

production of ATP.[10,58,59] Interestingly, it has been reported that chlorhexidine exhibits superior activity in alkaline pH as opposed to an acidic pH environment and is bacteriocidal at high concentrations but bacteriostatic at sub-MIC concentrations.[59] Analogs with differing side chains such as alexidine (**45**) exhibit similar activity.[60] Amidines linked to various functional groups and different substitution patterns (**46**) have likewise been employed as antibacterial agents.[60] Not surprisingly in light of recent work examining the role of bromine substitution in antimicrobial activity,[61] bromination ortho to the linker yielded the most active amidine compound in its class.

The dicephalic hybrids (**47**) created by Song and co-workers (Figure 12) are an interesting combination of QACs with guanidines.[62] The role of alkyl chain length was investigated in a pilot study testing the inhibition of *S. aureus*, *E. coli*, and *C. albicans*; each of the compounds significantly inhibited growth at 50 ppm (corresponding to approximately 100 μM), though it would be interesting to know if efficacy is retained at lower concentrations.

3.5. Polycyclic Guanidine Alkaloids. Though not traditionally defined as QACs, some guanidine alkaloids can indeed be considered to be QACs based on resonance and/or pH. Guanidine alkaloids are a structurally diverse class of metabolites that possess broad biological activity. In this vein, the antibacterial activity of several high-energy intermediates initially designed as fungal ergosterol biosynthesis inhibitors has been demonstrated.[63] Structurally, these intermediates are 8,13,15-triazasteroid analogs with various aryl and alkyl substitution (**48**, Figure 13) that possess micromolar MICs against Gram-positive pathogens, though these analogs are noticeably less active against Gram-negative *E. coli*, *K. pneumonia*, and *P. aeruginosa*.

A number of guanidine alkaloids produced by marine sponges demonstrate broad bioactivity, ranging from cytotoxicity to antimicrobial and antiviral properties. Several structural variants have been isolated from related marine sponge species, including the batzelladines and crambescidins (Figure 13). Select analogs exhibit low micromolar to high nanomolar IC_{50} values against a range of microorganisms including *S. aureus*, MRSA, *C. albicans*, and *P. aeruginosa*.[64] The structurally related

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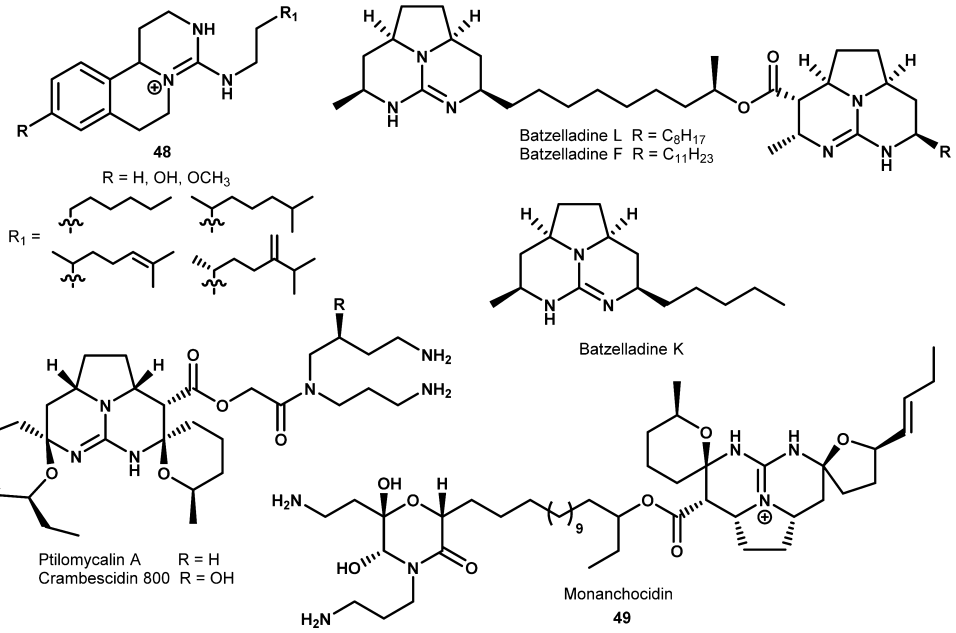

Figure 13. Guanidine alkaloids possessing QACs by resonance.

monanchocidin (**49**), isolated from marine sponge *Monanchora pulchra*, possesses cytotoxicity against human leukemia THP-1, carcinoma, and mouse epidermal cell lines at low micromolar IC_{50} values[65] but has yet to be tested for antimicrobial activity.

Another class of guanidine alkaloids includes the saxitoxins (**50**) and zetekitoxins (**51**) (Figure 14), which have been found

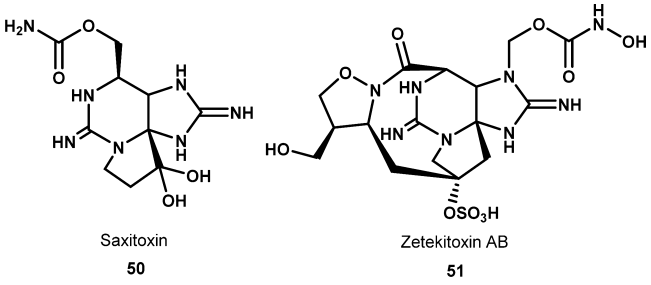

Figure 14. Saxitoxin and analog zetekitoxin AB, which are guanidine alkaloid QACs.

to be potent sodium channel blockers[66] and may also act through inhibiting copper uptake in microbial species.[67] Detailed antimicrobial activity has yet to be established for such compounds, prompting further investigation. Such natural product scaffolds serve as an interesting platform for the development of novel bacteriocidal QACs because they may mitigate toxicity and off-target effects and have thus far been the subject of limited antimicrobial investigations.

3.6. Cleavable QACs. Given their high stability and low reactivity, bioaccumulation and environmental leaching of QACs are of obvious concern. It is estimated that over 700 000 tons of QACs are used annually, which, based on their typical uses, make their way to wastewater treatment facilities or into the environment. Here, they can continue to exhibit their toxic behavior, leading to environmental concerns. Additionally, they adsorb onto negatively charged surfaces such as soil and sediments,[3] which host microbial communities with tremendous biodiversity, driving antibacterial resistance (to be discussed in detail in section 4). Possibly due to these concerns, efforts have been made toward the construction of biodegradable QACs. The most common method employed in cleavable QACs is the incorporation of ester moieties, which undergo hydrolysis to nontoxic fatty acids and QAC-diols at a substantial rate while maintaining their antibacterial activity.[8,68] Such QACs are thus referred to as soft antimicrobials.[8] Though used more extensively in fabric softeners rather than as an antimicrobial agent, DEEDMA (**5**, Figure 2) has a half-life of 0.8 to 18 days after application;[3] similar esteralkonium species have been shown to achieve greater than 99.9% killing within 2 min of application while hydrolyzing 50% within 5 h.[68] Some classic examples of antimicrobial QAC-esters are the C_{10}, C_{12}, and C_{14} alkanoylcholines (**52**)[69] and compounds incorporating the imidazolinium headgroup (**53**) or the 1,4-diazabicyclo[2.2.2]-octane (DABCO) core (**54**) (Figure 15).[70]

In addition, QACs bearing either trimethylammonium (**55**) or pyridinium (**56**) heads connected through cleavable ester moieties (Figure 15) have been synthesized and tested for antibacterial activity against Gram-positive *S. aureus* and *Enterococcus faecalis* and Gram-negative *E. coli* and *Shigella sonnei*.[71] The antibacterial activity of these compounds appears to be directly correlated to the number of QAC headgroups present on the molecule, with minimum bacteriocidal concentrations (MBC) in the 5 to 30 μM range. This trend may be due to the lower solubility of compounds possessing one charged QAC headgroup and/or their greater ability to form micelles, which would allow these less-charged QAC derivatives to bind to a lower number of target cells and therefore inflict less damage at a given concentration. Overall, the authors showed that such esterQACs are readily hydrolyzable at physiological pH yet are able to kill the majority of bacterial cells present within 20 to 40 min of application.[71]

In a similar vein, cleavable amide-containing QACs exhibit MICs of as low as 10 to 13 μM against *S. aureus* and *E. coli*, respectively, with a hexamethylene linker (**57**, $n = 6$, Figure 15) producing optimal antibacterial activity.[72] These compounds were also tested for the disruption of HeLa cell lines and yield decent selectivity indexes of HL_{50}/MIC. A similar scaffold of amido-amine QACs (**58**) demonstrated comparable or superior

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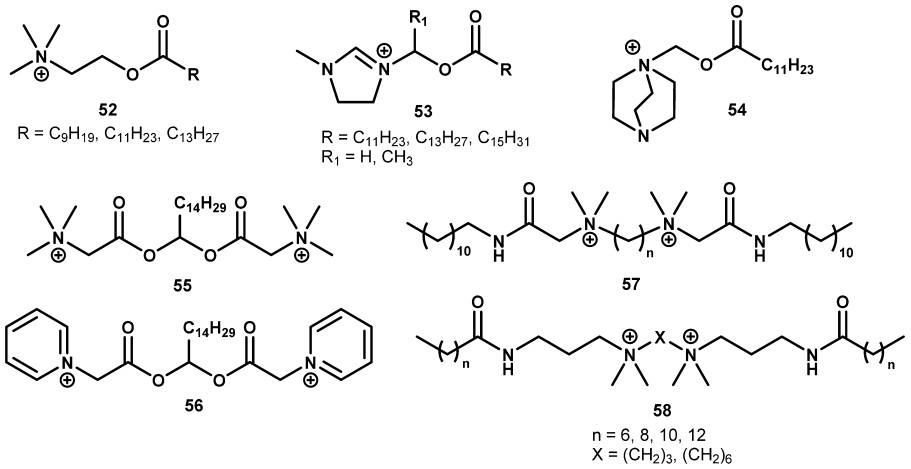

Figure 15. Cleavable QACs from the literature.

activity against *Bacillus subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa* as compared to commercial CTAB.[73]

The majority of cleavable QACs made their debut in the 1980s and early 1990s as new data regarding bacterial resistance to QACs was emerging, as will be detailed in the next section. Though it is not clear in the literature if cleavable QACs were initially developed to curtail the development of QAC resistance, their biodegradability serves as an innovative venture through which to combat current and future QAC resistance nonetheless.

4. RESISTANCE TO QACS

Because commercial antimicrobial QACs have low reactivity and therefore remarkable longevity, their accumulation in the environment is inevitable. Thus, bacterial populations are subjected to diluted, sublethal doses of QACs. Unfortunately, such exposure can serve as a driver for the development of resistance, which has indeed been observed in recent decades, finally ending any hope that QACs would be immune to resistance by virtue of their molecular target. Of even greater concern, however, is the observation that the "widespread use of biocides co-selects for antibiotic-resistant genes and could promote the spread of multi-drug-resistant plasmids."[74]

4.1. Concerns with Resistant Bacteria. Antibiotic-resistant bacteria have been a major concern since the dawn of widely distributed antibiotics; rarely has more than 10 years passed between the introduction of an antibiotic to the market and the development of resistance to that antibiotic.[75] The application of antibiotics has been excessive in the agricultural and food industries as well as in the medical community, wherein up to 50% of prescribed antibiotics are not necessary or appropriate to treat the ailments for which they are prescribed.[75] This overuse has contributed to the development of antibiotic resistance. According to the CDC, at least 2 million severe bacterial infections that cannot be treated with traditional antibiotics occur in the United States alone; at least 23 000 people die each year from these antibiotic-resistant infections.[75] Bacteria may possess intrinsic resistance or may acquire resistance through alterations of their genetic composition. Intrinsic resistance refers to a component or characteristic of a bacterium that renders that bacterium innately immune to an antibiotic, such as the dual cellular membrane in Gram-negative bacteria, which could result in elevated MICs for QACs. Acquired resistance may come in the form of genetic mutations and/or the transfer of plasmids and integrons, as exemplified in the transfer of plasmids that contain QAC-resistant genes.

4.2. Adaptation or Resistance? Because the mechanism of action of QACs, namely, the disruption of bacterial membranes, has a target so fundamental to bacterial survival, the development or acquirement of resistance to QACs initially seemed improbable. Perhaps surprisingly, bacteria have developed a diverse set of resistance mechanisms to mono- and bisQACs ranging from alterations of the cell wall or membrane composition to the efflux of cationic antibacterial agents.[76] A few initial reports of bacterial tolerance to QACs were published in the 1950s and 1960s, showing that Gram-negative species were capable of growing at elevated concentrations over several passages with BAC (**9**, Figure 5), though tolerance was lost upon removal of QAC from growth media.[77,78] The genetic basis of this and related phenomena did not emerge, however, until the late 1980s,[79−83] and publications in this field have rapidly expanded since then as shown in Figure 16. It should be noted that the terms "adaptation" and

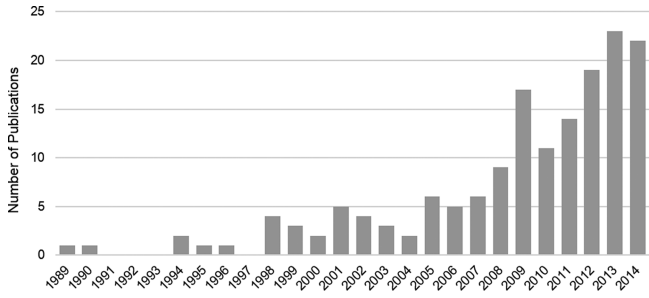

Figure 16. Number of publications involving the study of qacA, including its prevalence, mechanism, and effects, in bacterial cells.

"tolerance" to QACs refer to a reversible process that allows bacteria to persist while subjected to an increased concentration of biocide, which is lost upon removal of biocide. The mechanism by which this occurs has yet to be determined. This is in contrast to true resistance, which is rooted in genetic alterations of the bacteria that confer the permanent ability to grow at elevated concentrations of QAC.

4.3. Genetic Basis of QAC Resistance. The *qacA* gene, which remains the most prevalent QAC-resistant mechanism in Gram-positive bacteria, was the first proton motive force

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Table 1. QAC-Resistant Genes Documented (by Reference) in Select ESKAPE Pathogens

	qacA	qacB	qacC	qacD	qacE	qacEΔ1	qacG	qacH	qacJ	qacZ	smr	norA	mepA
S. aureus	84, 119, 138-148	25, 86, 87, 90, 117-120, 122-124, 143, 145, 147-150	86, 87, 138, 139, 146	138, 139	138	138, 139	107	106, 138, 144, 146	108, 139		83, 84, 119, 142, 145	89, 138, 139	89
MRSA	25, 86, 87, 90, 117-120, 122-124, 143, 145, 147-150	25, 86, 87, 93, 119, 122-124, 143, 145, 147, 148, 151, 152	90, 142, 143				121				118, 119, 121, 122, 124, 152	90, 121	
E. faecalis			138			138				102			
K. pneumoniae					138	138							
A. baumannii	149	149			153	149, 153	149						
P. aeruginosa					138	138	138						
E. coli				88	88, 138	88	88						

(PMF)-dependent multi-drug-resistant gene to be reported in *S. aureus*.[79,84,85] Along with its nine homologues *qacB-H/J/Z*, *qacA* controls the production of membrane-affiliated efflux pumps that are evidently specific to mono- and bis-cationic substrates. Each of the QAC-resistant genes (*qac*) and the species in which they have been documented are detailed in Table 1. This is a cursory survey of the literature and is meant to be representative and not exhaustive. While other multidrug efflux pumps have been shown to confer resistance to QACs,[86−89] this review will discuss only *qac* genes.

QAC-resistant genes are typically found on plasmids that harbor several multi-drug-resistant genes. For example, the pSK1 family of multidrug resistant plasmids contain 12 putative gene products including QAC efflux pumps, teichoic acid translocation permease, and several open reading frames that code for various surface proteins.[76] In clinical MRSA isolates, it has been shown that most strains carry a plethora of resistant genes ranging from bacitracin resistance and heavy-metal resistance to a number of drug transporters including those from the quinolone, ABC, and EmrB/QacA transporter families.[90] In particular, *qacA* is believed to share common ancestry with tetracycline and sugar transport proteins.[83] Given that many plant alkaloids are structurally similar to synthetic multi-drug-resistant substrates, *qacA* may have evolved recently from the same genetic material that protects microbes from naturally produced antimicrobials.[91]

4.4. Mechanism of Resistance. As noted in Table 1, the most prevalent QAC-resistant genes among Gram-positive bacteria are the *qacA/B* gene system, both members of the major facilitator superfamily (MFS). There exists some ambiguity in the literature as to the differentiation between these genes because they are more often than not reported as *qacA/B*. However, *qacA* and *qacB* are in fact closely related yet distinct genes, which gives rise to difficulties in distinguishing between them in genetic assays. Specifically, *qacA* codes for the production of a transmembrane efflux protein of the major facilitator superfamily named QacA. Bearing 14 transmembrane helical coils, QacA utilizes a key acidic aspartic acid residue (D323) to recognize and expel both mono- and bisQACs via the proton motive force.[60,84,85] Closely related to QacA is QacB, which shares a high degree of homology—only seven nucleotides differ between *qacA* and *qacB*—yet is more specific in recognizing monoQACs due to the presence of an uncharged residue, alanine, instead of aspartic acid as found in QacA.[92] It has been shown that the mutation of this alanine to an acidic residue allows for greater recognition of divalent substrates.[84,93] A rather simple method of probing the efflux of substrates by proteins coded by *qac* is to treat bacteria with both the QAC substrate and a known efflux pump inhibitor such as resperine or with a protonophore such as carbonyl cyanide *m*-clorophenylhydrazone (CCCP).[94,95]

Though *qacA/B* are found nearly exclusively on plasmids transferred between Gram-positive *Staphylococci* such as

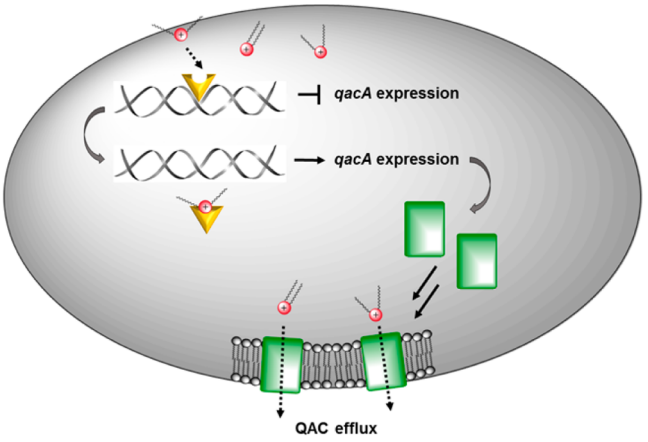

Figure 17. Resistance mechanism to QACs conferred by the *qacA/R* system. QACs in the cytoplasm (red) encounter and bind QacR (yellow), the negative transcriptional regulator of qacA. Upon binding the QAC substrate, QacR induces QacA production (green). QacA, a membrane-affiliated efflux pump, utilizes proton motive force (PMF) to combat QACs in the cell and within the membrane.

S. aureus, *S. epidermidis*, *S. saprophyticus*, and *S. hominis*,[96] they were recently detected for the first time in *E. faecalis*.[97] Related but much less studied systems are those belonging to the small multidrug resistance (SMR) family found primarily on class 1 integrons: *qacC* through *qacH*, *qacJ*, and newly discovered *qacZ*. Second in prevalence to the *qacA/B* pair are *qacC* and *qacD*, which are often grouped together as *qacC/D*, as the two are identical in the coding region yet have different flanking regions;[98] based on their similarity, it is postulated that *qacC* may have evolved from *qacD*.[99] Vasquez and co-workers reported that *qacC* found in a natural *S. epidermidis* isolate was tied to resistance to *β*-lactam antibiotics when expressed in Gram-negative bacteria, though the antibiotic activity of QacC was (somewhat surprisingly) not dependent on outer membrane proteins.[95] More common among Gram-negative strains are *qacE* and its deletion mutation homologue *qacEΔ1*, first described by Skurray and co-workers on an integron of broad-host-range origin.[100] They have since been reported in a number of Gram-negative strains, namely, those belonging to *Enterobacter*, *Pseudomonas*, and *Vibrio* species,[101,102] as well as *Klebsiella pneumonia*.[103] Both *qacE* and *qacEΔ1* are associated with elevated MICs against dyes such as ethidium bromide, crystal violet, proflavine, and rhodamine 6G as well as commercial QACs including BAC, CTAB, CPC, and, to a lesser extent, dequalinium chloride.[88,100] The first report of these two genes showed that the presence of *qacE* generally correlates to a greater efflux of these substrates as compared to *qacEΔ1*, and neither *qacE* nor *qacEΔ1* appears to confer resistance to diamidines.[100] One study investigating a variety of Gram-negative bacterial strains detected *qacE* and *qacJ* only in *Enterobacter* strains;[104] *qacH* was most often found among

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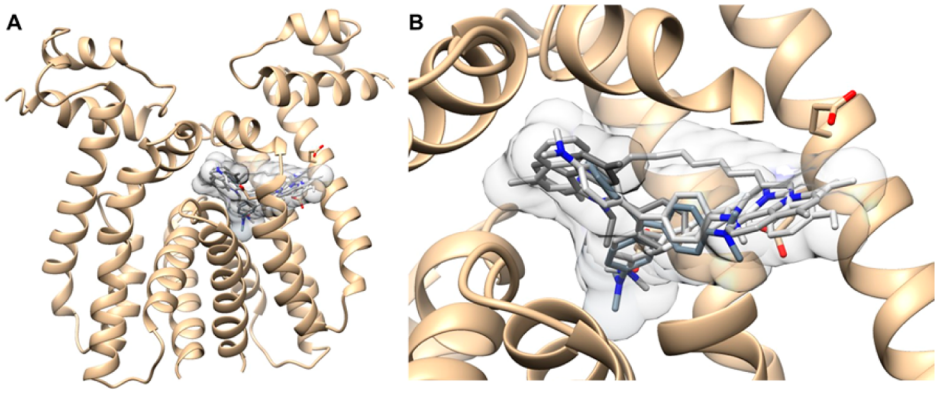

Figure 18. Overlay of several QAC-QacR complexes. (A) QacR dimer that binds DNA on the bottom face and (B) diffuse binding pocket with an overlay of various QAC substrates, shown in gray with quaternary nitrogens highlighted in blue. Key glutamic acid residues are shown.

Enterobacter.[102] A high degree of similarity is shared between *qacF* and *qacE* (67.8%) and therefore QacF and QacE, whereas QacC and QacF exhibit a lower degree of similarity.[88,105] Heir and co-workers identified *qacG* and *qacH* in *Staphylococci*, but these genes have since been reported among Gram-negative strains.[106,107] Initially found among equine *Staphylococci*, QacJ, the gene product of *qacJ*, bears high similarity to other SMR proteins such as QacC, QacG, and QacH yet has been shown to confer even greater resistance to BAC than strains carrying any of these homologous genes.[108] Each of these genes has been reported both on multi-drug-resistance plasmids in Gram-positive *Staphylococci* and on class 1 integrons in Gram-negative bacteria.[102,109,110] The most recently identified *qac* gene, *qacZ*, was found in *E. faecalis* and appears to be more specific for BAC over chlorhexidine and ethidium bromide.[111]

The production of each QAC efflux pump is regulated by QacR, a negative transcriptional regulator protein that cooperatively binds intergenic region IR1 of *qacA* as a pair of dimers (Figure 18A).[112] Once presented with a QAC substrate, QacR utilizes several acidic residues (E57, E58, E90, and E120) to guide cationic substrates into its diffuse and flexible binding pocket, as shown in Figure 18B. Brennan and co-workers presented crystal structures for six QacR/QAC-dye substrate complexes (select structures shown in Figure 3), highlighting the variability in substrate binding to the same protein.[113,114] Once in the binding pocket, the affinity for the substrate is enhanced by interactions with a number of aromatic residues as well as backbone amide hydrogen bonding (T39, L54, Y93, G96, Y123). QacR appears to have two subpockets within its binding site, allowing this negative transcriptional regulator to recognize and bind a variety of structurally dissimilar substrates.[113] The binding of a QAC substrate induces conformational changes in the α-helical structure of QacR, leading to its dissociation from DNA and thus allowing for the transcription of *qacA*, the production of QacA efflux pumps, and the efflux of toxic QACs from the cell and the cellular membrane (Figure 17).[115] In this sense, the *qacA/qacR* system allows the bacterium to control metabolic activity with respect to the conservation of energy as well as protect its own membrane integrity.[112] When *qacR* is removed from the genome, QAC efflux pumps are produced indiscriminately.[84] It has been shown that several of these same cationic lipophilic molecules induce *qacA* expression yet have no generalizable effects on *qacR* expression.[116]

4.4. Emergence of Resistance. The rate at which bacteria have evolved resistance mechanisms to QACs is quite alarming,

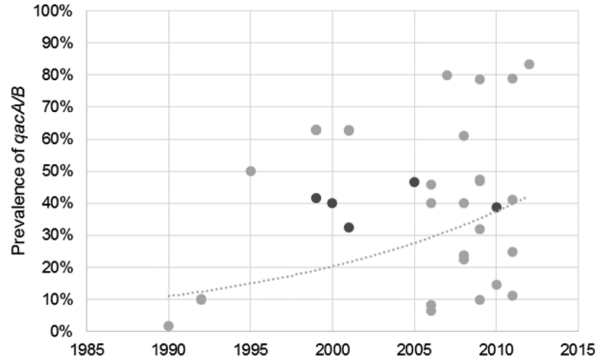

Figure 19. Prevalence of *qacA/B* genes detected in MRSA isolates from representative reports. Dark-gray points represent studies consisting of more than 500 isolates.

as evidenced in a collection of studies focused on the prevalence of *qacA/B* in MRSA isolates (Figure 19). In 1990, little to no resistance was observed for commercial QACs. In one longitudinal study of MRSA isolates collected from a Taiwanese hospital, 50% of the strains developed elevated MICs to chlorhexidine (**44**, Figure 12) by 1995, a proportion that held steady over their 10-year investigation.[117] Within this study, *qacA/B*-resistant genes were first detected in one clone in 1995 and by 2005 had spread to several different clones. A separate investigation composed of nearly 900 MRSA isolates collected from several Asian countries from 1998 through 1999 found *qacA/B* in 41.6% of the strains.[118] Similar results were found for over 500 MRSA isolates obtained from Japanese hospitals between 1993 and 2001, with nearly 33% possessing *qacA/B*.[119] A few reports have found a lower prevalence of *qacA/B* in MRSA isolates, as highlighted in the 7% prevalence among Japanese patients[120] and 8.3% among patients in the U.K. in 2006.[121] Moreover, some studies have shown a marked difference in QAC-resistant gene distribution across the globe: 10 to 20% in the United Kingdom, 80% in Brazil, and 32−41% in Asia;[122] these discrepancies have yet to be rationalized or resolved. The overall trend of *qac* detection over the past few decades, however, has been seemingly exponential, as shown in Figure 19.

Interestingly, Ho and Branley found no significant increase of *qac* genes among MRSA isolates in Australia despite the introduction of a hand hygiene campaign that increased the amount of QACs consumed nearly 20-fold.[123] They did, however, find an average yearly prevalence of *qacA/B* from

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2000 to 2009 of nearly 79%, which is higher than some numbers reported in Asia and the U.K. Some of the most recent reports have detected *qacA/B* in anywhere from 25% to over 80% of hospital isolates,[124] with hospital workers carrying a significantly higher portion of QAC-resistant strains within their microbiome as compared to the general population.[125] Though the majority of isolates have come from patient swabs or hospitals, samples taken from automated teller machines in Hong Kong revealed the presence of MRSA possessing *qacA/B*,[126] highlighting the pervasiveness of QAC resistance both in medical settings and in the greater community.

Given these unsettling statistics, it may come as a surprise that a survey of the most recent literature shows little work in overcoming QAC resistance and its increasing ubiquity. This is perhaps due to the oft-dismissed significance and impact of QAC resistance, which has not garnered much attention over the years in comparison to other more well-known resistance systems. It is likely that efflux-based QAC resistance has some limitations, given the metabolic toll and spatial limitations of efflux pumps on the cell surface. It is important to note, however, that QAC-resistant genes are often harbored on plasmids that carry a plethora of antibiotic gene clusters, leading to cross- and coresistance.[74,95,109]

4.5. Quantitative Studies on Resistance. Bacterial strains possessing *qac* genes have been shown to harbor at least 4-fold resistance to BAC,[127] the most prominent QAC currently on the market. The presence of *qacF* in particular has been shown to increase MICs 2- to 4-fold for several commercial QACs such as BAC, CTAB, CPC, and hexadecyltrimethylammonium bromide.[88] In one study, several staphylococci isolates collected from surfaces frequently treated with QACs were found to be resistant to BAC, while isolates collected from nontreated surfaces remained susceptible to the compound.[110] All of the resistant strains also exhibited resistance to erythromycin, ampicillin, and penicillin, and several were additionally resistant to ciprofloxacin, methicillin, and chloramphenicol. It has been found that MRSA isolates possessing *qac* genes exhibit significantly higher MBCs ($P < 0.01$),[128] with the induction of *qac* genes upon exposure to QAC biocides.[129]

An iterative study by Zhang and co-workers highlighted the decrease in susceptibility for both coagulase-positive and coagulase-negative *S. aureus* carrying the *qacA/B* system and at least 4-fold resistance to DDAC.[125] Similar increases in MIC were found for DDAC against a *qacA/B*-positive strain of *E. faecalis*.[97] Moreover, Zhang et al. showed that bacterial resistance seems to increase synergistically when *qacA/B* are combined with the presence of the *smr* gene. A study conducted by McBain et al. showed a species-specific development of resistance to commercial disinfectant Bardac, which is composed of a mixture of ADBACs and twin-chain QACs.[130] Over a period of 14 consecutive serial passages at sublethal doses with this QAC disinfectant, several Gram-negative bacterial strains exhibited 2- to 8-fold increases in MICs. Likewise, HA-MRSA developed tolerance to QACs following exposure to sublethal concentrations of QAC.[129] When subjected to biofilms commonly found in sink drains—an area that receives perhaps the greatest contact with commercial QACs—the QAC under study was ineffective, and after long-term exposure to the QAC, no changes in susceptibility were noted, though this may be due to the biofilm phenotype. Furthermore, no changes in susceptibility were observed for other antibiotics after long-term exposure to Bardac, though the biofilm state likely affects results as well as innate resistance.[131] In a large study composed of over 1600 clinical isolates of *S. aureus*, it was found that BAC MICs were increased in the presence of *qacA*, *qacB*, and *qacG* while chlorhexidine MICs were increased by *qacA* and *qacB* only.[132]

Though there are several reports describing the substrate scope of the battery of *qac* genes toward commercial antiseptics, others have focused on further investigation of the mechanism of action of QAC tolerance or resistance. In one recent publication involving *Listeria monocytogenes*, the bacterium that causes illnesses such as meningitis and cerebritis, more than one in four isolates was deemed tolerant of BAC. When cotested with known efflux pump inhibitor resperine, 13 out of the 19 strains deemed BAC-tolerant were rendered more susceptible to BAC, underscoring the role that efflux pumps play in BAC resistance.[94] In a separate report, it was found that when treated repeatedly with sublethal doses of didecylammonium bromide (DDAB), *P. aeruginosa* was able to grow at 5 times the original MIC, and significant changes in membrane fatty acid composition were noted during treatment.[133] This ability to grow at increased concentrations of QAC, however, was lost upon transfer to media lacking QAC, demonstrating that this instance was "phenotypic adaptation" of the bacteria in response to the QAC, rather than true resistance.[134] How bacteria are able to accomplish this is a question that remains unanswered.

As a continuation of our QAC studies, we wished to determine the propensity of our bis-, tris-, and tetraQACs to trigger resistance mechanisms in *S. aureus*.[135] Our results show the development of resistance to commercial monoQACs over a period of 24 days yet no resistance to alkyl bis-, tris-, or tetraQACs. Within a few hundred generations, *S. aureus* experienced a 2- to 4-fold increase in MIC for BAC, CPC, and novel bisQAC PQ-12,Bn (**25**, Figure 8), which is an N-substituted bipyridinium; all of these possess aromatic regions. These results provide insight as to the mechanism and substrate scope of QAC resistance, namely, that the incorporation of aromatic moieties allows for greater recognition by the bacteria. This may be a result of specific interactions between the substrate and QacR or may be due to potential greater cell permeation of QACs possessing aromatic moieties.[136] Further investigation of these findings will shed light on the resistance machinery and its interactions with a variety of novel QAC substrates.

4.6. Biodegradation of QACs. A small number of studies have reported the microbial degradation of lead commercial QAC BAC.[137] Specifically, several *Pseudomonas* strains such as *P. nitroreducens* and *Aeromonas hydrophila* and *Bacillus niabensis* have been shown to biodegrade BAC via dealkylation to nontoxic benzyldimethylamine by amine oxidase and related enzymes. Further characterization of this biodegradation mechanism as well as the discovery of additional strains that are capable of executing these reactions will reveal insights into the microbial response and processing of toxic QACs as well as provide a platform for new approaches to antibacterial agents that retain efficacy yet are biodegradable.

5. SUMMARY AND OUTLOOK

The structure and activity of QACs published to date is quite diverse, though some clear trends have emerged. Permanent cationic charge coupled with hydrophobic regions lead to antibacterial behavior with optimal activity for alkyl chains typically ranging from 12 to 14 carbons. Compounds possessing increased cationic charge and/or delocalized charge

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likewise confer antimicrobial activity. Given the remarkable stability of QACs in general, the design of QACs that are able to self- or biodegrade yet retain efficacy against microbes is of great interest. As QACs possess a global mechanism of action, further investigation of QACs that are more selective for microbial cells over eukaryotic cells is needed. Perhaps the incorporation of natural product scaffolds, increased charge, or combinations of mechanisms of action can accomplish this goal. Furthermore, QACs that are active against bacterial biofilms need to be developed in order to combat these pervasive and persistent infections, especially given the increased rate of resistance transfer among bacteria in the biofilm state. Though QAC resistance currently confers modest protection against such cationic agents, a plethora of data shows that resistance is only spreading and may have more dire consequences in the future. Co- and cross-resistance to a variety of antibiotics have been increasingly linked to QAC resistance, underscoring the high need for further investigation of this field and the development of strategies to overcome and evade these issues. The mitigation of QAC resistance does not appear to be a major area of study at present but is an emerging area that will be key in confronting future issues with heavily used commercial QACs. As chemists and biologists, it is our responsibility to society to communicate the potential downstream effects of antiseptic use as well as call for new strategies to overcome and evade these issues. As the most recent CDC report on antibiotic resistance states, "Bacteria will inevitably find ways of resisting the antibiotics we develop, which is why aggressive action is needed now to keep new resistance from developing and to prevent the resistance that already exists from spreading."[75] Therefore, the development of next-generation QACs as antimicrobial agents is imperative to stay on top of such issues and promote a healthier, safer society.

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Notes

The authors declare the following competing financial interest(s): K.P.C.M. and W.M.W. are equity shareholders in NovaLyse BioSolutions, which works in this arena.

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ABBREVIATIONS

quaternary ammonium compound, QAC; minimum inhibitory concentration, MIC; benzalkonium chloride, BAC; didecyldimethylammonium chloride, DDAC; cetylpyridinium chloride, CPC; methicillin-resistant *Staphyloccocus aureus*, MRSA

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