Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology

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Disinfectants play an important role in maintaining acceptable health standards by significantly reducing microbial loads as well as reducing, if not eliminating, pathogens. This review focuses on quaternary ammonium compounds (QACs), a widely used class of organic disinfectants. Specifically, it reviews the occurrence, microbial adaptation, and degradation of QACs, focusing on recent reports on the ecology of QAC-degraders, the pathways and mechanisms of microbial adaptation which lead to resistance to QACs, as well as to antibiotics. With the help of culture-dependent and nonculture-dependent tools, as well as advanced analytical techniques, a better understanding of the fate and effect of QACs and their biotransformation products is emerging. Understanding the underlying mechanisms and conditions that result in QAC resistance and biodegradation will be instrumental in the prudent use of existing QAC formulations and foster the development of safer disinfectants. Development and implementation of (bio)technologies for the elimination of QACs from treated wastewater effluents will lessen adverse impacts to both humans and the environment.

Introduction

Disinfectants are extensively used and their formulations contain active ingredients at levels well above the minimum inhibitory concentration (MIC) of targeted microorganisms. Inappropriate application of disinfectants, dilution in the environment after discharge and biodegradation result in biocide concentration gradients. Thus, microorganisms are frequently exposed to non-lethal (i.e., sub-inhibitory) concentrations of biocides. Recent studies suggest that exposure to sub-inhibitory biocide concentrations facilitates the evolution of resistance to the biocide, and may also lead to co-resistance and cross-resistance to other antimicrobial agents such as antibiotics [1*,2].

Quaternary ammonium compounds (QACs) are cationic surfactants introduced in the late 1930s along with the first antibiotics, sulfonamides. They are classified as ‘high production volume’ chemicals [3]. The chemical structure of QACs depends on the four aliphatic or aromatic moieties attached to the central nitrogen atom (R₁R₂N⁺R₃R₄). QACs are mainly used in disinfectant and antiseptic formulations utilized in homes, human and animal healthcare facilities, agriculture and industry. They are effective against a variety of bacteria, fungi and viruses at very low concentrations. When QACs are used as disinfectants, the applied concentration is typically between 400 and 500 ppm and almost always below 1000 ppm (e.g., 0.1% w/v in Lysol®) [3]. Domestic, hospital and industrial use of QACs results in QAC-bearing waste/wastewater. Because typical wastewater treatment plants are designed to remove major, easily degradable organics, most trace contaminants, including QACs, pass through wastewater treatment plants and are released into the environment. About 75% of QACs utilized annually are released into wastewater treatment systems, whereas the rest are discharged directly into the environment. The mean concentration of QACs in domestic wastewater, treated effluent wastewater, sewage sludge and surface water is reported to be around 0.5 mg/L, 0.05 mg/L, 5000 mg/kg dry weight, and 0.04 mg/L, respectively, which is significantly below applied concentrations [3–5].

Because QACs are biodegradable under aerobic conditions, their concentrations in indoor and outdoor environments continuously fluctuate. As a result, microorganisms are exposed to QACs dynamically over a wide range of concentrations (i.e., non-inhibitory, sub-inhibitory, over-inhibitory concentrations). In general, environmental concentrations of QACs are well below the MIC values. Sewage, biological wastewater treatment units, surface waters and sediments are environments where QACs are present at sub-inhibitory concentrations. When the high microbial diversity in these environments is coupled to sub-inhibitory QAC concentrations, such environments become selective, resulting in the emergence and dissemination of QAC resistance among different bacterial
genera, which may also include clinically important pathogens. Because many of the QAC resistance pathways and mechanisms are similar to those involved in antibiotic resistance, understanding QAC resistance and dissemination is very important in the context of the global antibiotic resistance problem. Although some suggest that there is no clear relationship between antibiotic resistance and exposure of microorganisms to QACs [6], many studies have shown that, for instance, exposure to QACs results in dissemination of integrons (i.e., promoterless mobile recombinational elements) which harbor resistance genes [7,8]. Evidence that soil bacteria and human pathogens share similar resistomes within integrons [9] suggests that there is a link between antibiotic resistance in nature and clinical settings, which is favored by exposure to QACs.

Many reviews exist on the fate and effect of QACs in the environment, QAC-related antimicrobial resistance, and implications of QAC resistance to human health [3,10,11]. In this review, we discuss the ecology of QACs-exposed microbial communities, as well as the pathways and mechanisms of microbial adaptation to sub-inhibitory QAC concentrations following the context of a recent review by Andersson and Hughes by linking the similarities of antibiotic and disinfectant resistance [12]. In addition, we consider the role of QAC biodegradation on the evolution and dissemination of QAC resistance by creating microenvironments with sub-inhibitory QAC concentrations. Moreover, technologies utilizing novel QAC-degraders for the alleviation of QAC contamination are discussed.

Pathways and mechanisms of QAC resistance at sub-inhibitory concentrations

The mode of action of QACs above MIC in bacteria is disruption of the cell membrane’s physical and ionic stability [13]. For example, benzalkonium chlorides (BACs) bind to the cell membrane of Pseudomonas fluorescens by ionic and hydrophobic interactions, bringing about changes of membrane properties and function, followed by cellular disruption, loss of membrane integrity, ultimately resulting in leakage of essential intracellular constituents [14,15]. Above MIC, bacteria with durable cell membrane are selected and proliferate. On the other hand, the mode of action of QACs at sub-MICs is complicated and always includes multiple processes such as loss of membrane osmoregulation, inhibition of respiratory enzymes, the dissipation of proton motive force and oxidative stress, which triggers SOS response, inducing error-prone DNA replication leading to mutations and gene transfers [16,17]. Adaptation to QACs at sub-MICs is achieved by modification of the outer membrane, cell membrane, density and structure of porins, regulatory hyperexpression of efflux pumps, and acquisition of QAC-specific efflux pumps through mobile recombinational elements, such as plasmids and integrons upon oxidative stress or (followed by) stress-induced mutagenesis [3].

Major pathways in the evolution of resistance at sub-inhibitory antibiotic concentrations have been recently reviewed [12,18]. Bacteria follow similar pathways while adapting to QACs. Exposure to QACs at sub-MIC creates (oxidative) stress. For instance, exposure of E. coli to cetyltrimethylammonium bromide resulted in intracellular production of superoxide and hydrogen peroxide [19]. Bacteria compensate for oxidative stress by SOS-response and induction of stress-response sigma factors rpoS, promoting cell survival by DNA repair, while nucleotide polymorphism may occur, resulting in mutations. Oxidative stress responses also boost gene transfer and recombination events via prophages, transposons, integrons and integrative-conjugative elements (ICE) (Figure 1). As a result, resistant sub-populations evolve and dominate in a microbial community upon exposure to any antimicrobial agent [20]. Major mechanisms of adaptation to QACs include modification of cell membrane structure and composition, enhanced biofilm formation, acquisition of efflux genes, overexpression of efflux pump systems, and biodegradation. Generally, multiple mechanisms co-develop during adaptation of bacteria to QACs [21].

Exposure to QACs at sub-MICs enhances biofilm formation [22]. QAC resistant strains of bacteria form biofilms faster and these species are less susceptible to QACs than planktonic species [23]. Presence of multiple species in biofilms increases QAC resistance [24,25]. Expression of certain genes enhanced biofilm formation by QAC resistant Listeria monocytogenes [26,27].

Several mutations result in selection of bacteria with reduced cell permeability. Resistant cells have modified cell membrane fatty acids, phospholipids, and outer membrane lipopolysaccharides [28], resulting in a more anionic and hydrophobic cell surface, thus restricting easy passage of the QACs through the cell surface. Other cell modifications in response to exposure to QACs are density reduction and composition change of the porins [29,30], as well as change of the outer membrane protein composition [31].

Efflux-mediated QAC resistance has received significant interest because it has a genetic origin, confers co-resistance to antibiotics and is transferable among species through horizontal gene transfer. Multidrug efflux pumps mediate the transfer of biocides from the inside to the outside of the cell through an energy or proton-dependent mechanism. Efflux determinants, which confer resistance to QACs, are given in Table 1. QAC resistance via efflux pumps follows two mechanisms. First, QAC resistance is induced by overexpression of efflux pumps upon exposure to QAC or as a result of QAC-induced stress. Such stress either triggers a regulatory system that controls the
expression of an efflux determinant or causes a mutation resulting in the overexpression of the efflux determinant or increase in its extrusion efficiency [32]. These efflux pumps are generally chromosomally encoded and act against a wide array of antimicrobial agents. Overexpression of these efflux pumps results in a two-fold to eight-fold increased tolerance of the adapted bacteria to QACs and other substrates of these pumps [11*,33–36]. For instance, the study by Mc Cay et al. [11*] showed that *Pseudomonas aeruginosa*, variants of which are pathogenic, grown in continuous culture at subinhibitory concentrations of BAC resulted in increased resistance to both BAC and ciprofloxacin; its antibiotic resistance was attributed to amino acid substitution (Val-51AAla) in nfxB, the Mex efflux system regulator gene.

The second mechanism of QAC resistance is through acquisition of genes for specialized QAC efflux pumps,

**Table 1**

<table>
<thead>
<tr>
<th>Efflux determinants conferring QAC resistance (adapted from Ref. [81])</th>
<th>Efflux proteins extruding QACs</th>
<th>Typical antibiotic substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance nodulation division (RND)</td>
<td>YhiUV-ToIC, AcrAB-ToIC, MexAB-OprM, CmeABC, CmeDEF, SdeXY, OqxAB</td>
<td>Aminoglycosides, β-lactams, Chloramphenicol, Erythromycin, Fluoroquinolones, Novobiocin, Rifampin, Tetracyclines, Trimethoprim</td>
</tr>
<tr>
<td>Major facilitator superfamily (MFS)</td>
<td>QacA, QacB, NorA, NorB, MdeA, EmcA, MdfA</td>
<td>Aminoglycosides, Chloramphenicol, Erythromycin, Fluoroquinolones, Lincosamides, Novobiocin, Rifampin, Tetracyclines</td>
</tr>
<tr>
<td>Multidrug and toxic compound extrusion (MATE)</td>
<td>MepA, NorM, PmpM</td>
<td>Aminoglycosides, Fluoroquinolones</td>
</tr>
<tr>
<td>Small multidrug resistance (SMR)</td>
<td>QacE, QacE31, QacF, QacG, QacH, Qacl, QacJ, smr, EmrE, SugE</td>
<td>Aminoglycosides, Chloramphenicol, Erythromycin, Tetracyclines</td>
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which belong to the SMR family. Among them, EmrE, smr and SugE are multidrug efflux pumps [37], whereas QacE, QacEΔ, QacF, QacG, QacH, QacI, QacJ and QacZ are QAC-specific efflux determinants [38]. The genes of these efflux proteins are mainly found in mobile genetic elements such as transposons, ICEs, plasmids and integrons. As a result, they can be horizontally transferred between bacteria of the same or different genera. Such genes are abundant in the environment.

Acquisition of QAC efflux genes by bacteria involves integration of genes to integrons and plasmids or recombination of efflux gene containing ICE and transposons into the recipient genome. Oxidative stress response plays a crucial role in both QAC resistance mechanisms. Integrons play a significant role in the acquisition and mobilization of QAC resistance genes [39]; they are found in many environmental bacterial species, particularly in those exposed to QACs and/or antibiotic residues [40,41]. QAC contamination is also responsible for the stabilization of integrons and their gene cassettes [42]. A direct link between SOS response and the expression of integron integrases was demonstrated. SOS regulation enhances cassette swapping and capture under stressful conditions, such as during QAC exposure [43].

Plasmids also play a significant role in harboring and disseminating genes related to resistance to QACs and other biocides. Plasmids of the incompatibility (Inc) group IncP-1, also called IncP, as extrachromosomal genetic elements can be transferred and replicated virtually in all Gram-negative bacteria. IncP-1 group plasmids are broadly distributed in environments with sub-inhibitory QAC concentrations, such as soils and wastewater treatment plants. IncP plasmids commonly harbor QAC resistance genes along with many other resistance genes [44–47]. In addition, plasmid-associated QAC resistance genes were transferred between non-pathogenic and pathogenic bacteria exposed to QACs, a process that also leads to the co-selection of resistance to other contaminants [48].

Using the SOS-response, ICEs are transferred from one bacterial chromosome to another. ICEs containing multiple QAC resistance genes were identified in strains of Vibrio scophthalmi, V. splendidus, V. alginolyticus, Shearawella halniotis, and Enterocibrio nigricans isolated from fish grown in marine aquaculture systems [49]. Exposure to biocides below inhibitory concentrations also facilitates the conjugation of transposons [50]. Novel transposons carrying QAC resistance genes have been identified in both Gram-negative and positive bacteria [51**,52].

**QAC biodegradation: ecology, mechanism and genetics**

Exposure of a microbial community to QACs results in the development of resistance, as well as selection and proliferation of resistant bacteria. A very recent study showed that when a microbial community, fed with an organic carbon source was exposed continuously to QACs, its diversity decreased substantially and was dominated by Proteobacteria, mainly composed of Pseudomonads. In addition, a versatile repertoire of antimicrobial resistance genes, such as efflux pumps (e.g., RND family and SMR family) and cell envelope modification systems (e.g., aminoarabinose lipid A modification) were amplified during a long-term exposure to BACs [53,54].

Aerobic biodegradation of QACs has been attributed mainly to bacterial species in the genera of Xanthomonas, Aeromonas, and Pseudomonas [3]. More recently, several bacteria have been identified that are capable of QAC degradation, such as Pseudomonas putida ATCC 12633 [55], Pseudomonas nitroreducens B and DB [56*], Pseudomonas sp. BIOM1G1 (Ertelkin and Tezel, 114th General Meeting of American Society for Microbiology, Abstract no: Q352) and Stenotrophomonas sp. B27 (unpublished). In addition, non-culture-based techniques, such as cloning, metagenomics and pyrosequencing have been used to elucidate the composition of QAC degrading, enriched microbial communities. A microbial community originating from a river sediment, utilizing BACs as the sole carbon and energy source had a dramatically decreased phylogenetic diversity as compared to the control (without BAC exposure), and was dominated by Pseudomonas species (>50% of the total) [53,54,57]. Metagenomic analysis revealed that community adaptation to BACs occurred primarily via selective enrichment of BAC-degrading Pseudomonas species, particularly P. nitroreducens, and secondarily via amino acid substitutions and horizontal transfer of selected genes, including a gene encoding a PAS/PAC sensor protein and ring-hydroxylating dioxygenase genes [54,56*]. In addition, multi-drug efflux pump genes such as sugE, PmpM, mexAB-oprM and mexEF-oprN were enriched in the BAC-degrading community [53].

Based on the phylogenetic tree prepared using the 16S rDNA sequences of QAC degrading isolates and predominant species in QAC degrading microbial communities, Pseudomonas spp., particularly the P. putida and P. aeruginosa group, are key species in QAC degradation (Figure 2a). In addition Stenotrophomonas spp. (γ-Proteobacteria), and Achromobacter spp. (α-Proteobacteria) are frequently identified in communities degrading QACs, indicating that they play a role in the biodegradation of QACs [54].

Three aerobic QAC biotransformation pathways, which differ in the location of the initial reaction, have been observed (Figure 3): (a) hydroxylation of the terminal C of the alkyl chain (ω-hydroxylation), followed by multiple β-oxidation cycles, progressing toward the hydrophilic moiety; (b) hydroxylation of the C adjacent to the central
N (α-hydroxylation), followed by central fission, resulting in the separation of the hydrophobic from the hydrophilic moiety; and (c) hydroxylation of the methyl-C-attached to the central N, followed by fission of the methyl group [3]. In spite of the fact that QACs have a relatively high adsorption capacity resulting in their transfer to anoxic/anaerobic environments, such as anaerobic digesters and aquatic sediments, limited information exists relatively to the fate of QACs under such conditions. The transformation of BACs under anoxic, nitrate-reducing conditions was recently reported to be initiated by means of an abiotic nitrite nitrile nucleophilic substitution reaction (modified Hofmann reaction) producing alkyl dimethyl amines (tertiary amines) [3,58]. Under anaerobic conditions, there is no evidence of mineralization of QACs that contain alkyl or benzyl groups [3].

In spite of the fact that the energetic burden of the above-discussed QAC biotransformation pathways (initial reaction) is the same, pathway-b starting with the cleavage of the Calkyl–N bond is the most predominant. Although, pathway-a was the first identified QAC biotransformation pathway, it could not be demonstrated in later studies for similar QACs. Moreover, the product of pathway-b, a tertiary amine, is less toxic than the products of pathway-a and pathway-c [57]. The combination of these two observations suggests that pathway-b is naturally selected as a mechanism for QAC biotransformation to cope with the toxic effects of QACs and to eliminate the detrimental consequences of the other biotransformation pathways. As a result, QAC biotransformation may have evolved as a QAC-resistance mechanism.

None of the isolates described above, except Pseudomonas sp. BIOMIG1, was able to grow on the non-alkyl containing amines, such as trimethyl amine and dimethyl amine, after dealkylation. Activation of the C–H bond in the QAC alkyl group was thought to commence with NADH-dependent hydroxylation by a monoxygenase in the presence of oxygen [3]. Recently, two enzymes responsible for QAC dealkylation have been identified. Tetradecyl trimethyl ammonium bromide monoxygenase (TTABMO) identified in P. putida ATCC 12633 is a typical flavoprotein that utilizes NADPH and FAD as cofactor [55]. On the other hand, the enzyme responsible for dealkylating BACs by Pseudomonas nitroreducens was identified as a FAD-using amine oxidase (AOx-BAC).
Aerobic QAC biotransformation pathways.

[56]. AOX-BAC is phylogenetically more closely related to *P. putida* amine oxidase and *Pseudomonas* sp. pseudoxynicotine amine oxidase than TTABMO (Figure 2b), which suggests that there may be multiple enzymes responsible for the initial steps in QAC biotransformation.

With the exception of certain dimeric gemini QACs, almost all QACs are biodegradable under aerobic conditions [59]. QAC degraders play an important role in mitigating QAC contamination in the environment. However, proliferation of QAC degraders and their transfer in indoor environments are not desired because it would decrease their antimicrobial efficacy, resulting in public health problems. Efforts should be devoted to develop technologies based on immobilized QAC degraders or their enzymes for the treatment of QAC-bearing wastewater. For instance, *P. putida* ATCC 12633 immobilized using alginate entrapment was able to remove 80% of QACs within 48 hours at a concentration range between 35 and 315 mg/L [60]. To this end, QAC degraders have to be physiologically and metabolically well characterized and factors affecting QAC biodegradation identified. In case QAC detoxifying enzymes are used, strategies to enhance enzyme functionality by optimizing cofactor requirements using directed enzyme evolution approaches will need to be evaluated.

**Conclusion and outlook**

QACs are effective disinfectants that have contributed to maintaining acceptable health standards by ensuring a significantly reduced microbial load, and thus the likelihood that pathogenic bacteria are also reduced, if not eliminated. Because QACs are extensively used in domestic, agricultural, industrial and clinical applications, humans and microorganisms are in constant contact with them. Misuse, environmental dilution and biodegradation of QACs often create environments with low QAC concentrations. At sub-inhibitory QAC concentrations, QAC resistance emerges mainly as a bacterial response to QAC-induced oxidative stress, often causing mutations or facilitating gene transfer, ultimately leading to evolution and selection of QAC-resistant bacteria.

Wastewater, wastewater treatment plants, wastewater sludge, sludge applied soil, surface waters, and aquatic sediments are environments where QAC resistance evolves and proliferates. QAC resistant bacteria may be transferred from outdoor environments to indoors such as homes and healthcare facilities. QAC resistance genes, especially efflux genes, are frequently found in bacterial isolates obtained from human/animal healthcare facilities. These genes either confer resistance to multiple biocides and antibiotics (co-resistance) or mobile genetic elements that they are attached to carry resistance genes to other biocides and antibiotics. Therefore, QAC resistant bacteria, if they are pathogenic, may pose a serious threat to human health. In addition, QAC resistant bacteria may decrease the efficacy of QAC disinfectants by creating microenvironments habitable by QAC susceptible bacteria, as for example in the case of biofilms. Moreover,
QAC-degrading bacteria create QAC gradients in which susceptible species survive or even develop QAC resistance and proliferate.

The connection between QAC degradation and resistance is not well understood. Is QAC degradation a blessing or a curse? Given that QACs degrade, at least under aerobic conditions, QACs could be considered as relatively low risk disinfectants. However, QAC biodegradation creates sub-inhibitory concentrations where otherwise susceptible species may develop QAC resistance through various pathways and mechanisms as described above. Many species are phylogenetically closely related to QAC-degraders. Upon chronic exposure to QACs, these species could become resistant, but unfortunately some of them are pathogenic (e.g., *P. aeruginosa*) [1*,30]. If efficient QAC degraders are used in treatment technologies and then integrated into wastewater treatment plants, release of QACs into the environment may be avoided or at least minimized. As a result, QAC resistance and QAC-induced antibiotic resistance might be reduced.

In conclusion, QACs are widely used in many disinfectant formulations and will continue to be used in the future. The underlying mechanisms and conditions that result in QAC resistance and biodegradation, especially in the environment, must be better understood for the prudent use of existing QAC formulations, as well as the development of safer disinfectants. Development and implementation of (bio)technologies for the elimination of QACs from treated wastewater effluents, along with many emerging microorganisms, will greatly reduce adverse impacts to both humans and the environment.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as: **of special interest** and **of outstanding interest**


This study demonstrated increased resistance to both BAC and antibiotics of a potentially pathogenic bacterial species grown in continuous culture at subinhibitory BAC concentrations; resistance was attributed to genetic modifications.


This study tested the durability of biofilm composed of dual species on benzalkonium chloride (BAC) disfunction and resistance of each biofilm after biofilm was continuously exposed to BACs. Biofilms were developed on stainless steel coupons, exposed to BACs and species isolated from biofilm were tested for BAC MICs.


van der Veen S, Abee T: HrCa and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in Listeria monocytogenes. Microbiology-SGM 2010, 156:3782-3790.


Outer membrane proteins of Pseudomonas aeruginosa adapted to 2 mM BAC by stepwise exposure to lower concentrations was extracted and analyzed with 2D gel electrophoresis. Proteins with different abundance in adapted cells compared to wild-type cells were identified using mass spectroscopy.


44. Popowska M, Krawczyk-Balska A: Broad-host-range IncP-1 plasmids and their resistance potential. Front Microbiol 2013, 4:44.


A novel transposon containing qacH gene was identified in Listeria monocytogenes chromosome using shotgun sequencing. MIC assays performed with strains having and not having transposon and qacH gene as well as qacH expression assays showed that transposon associated qacH gene confers resistance to BACs in Listeria monocytogenes.


The gene responsible for dealkylation of tetradeacytrimethylammonium bromide was identified and its product, a flavoprotein, was purified from *Pseudomonas putida* for the first time.


Metatranscriptomic profile of a BAC-degrading microbial community was obtained during BAC degradation. Candidate genes responsible for BAC degradation were determined and cloned in *E. coli*. A novel amine oxidase gene responsible for transformation of BAC to benzyl dimethyl amine was identified.


