AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

MINIREVIEW

Benzalkonium Chlorides: Uses, Regulatory Status, and Microbial Resistance

Beatriz Merchel Piovesan Pereira,[a,b] Ilias Tagkopoulos[b,c]

[a]Microbiology Graduate Group, University of California, Davis, Davis, California, USA
[b]Department of Computer Science, University of California, Davis, Davis, California, USA
[c]Genome Center, University of California, Davis, Davis, California, USA

ABSTRACT Benzalkonium chlorides (BACs) are chemicals with widespread applications due to their broad-spectrum antimicrobial properties against bacteria, fungi, and viruses. This review provides an overview of the market for BACs, as well as regulatory measures and available data on safety, toxicity, and environmental contamination. We focus on the effect of frequent exposure of microbial communities to BACs and the potential for cross-resistant phenotypes to emerge. Toward this goal, we review BAC concentrations in consumer products, their correlation with the emergence of tolerance in microbial populations, and the associated risk potential. Our analysis suggests that the ubiquitous and frequent use of BACs in commercial products can generate selective environments that favor microbial phenotypes potentially cross-resistant to a variety of compounds. An analysis of benefits versus risks should be the guidepost for regulatory actions regarding compounds such as BACs.

KEYWORDS alkyl dimethyl benzyl ammonium chlorides, antiseptic, BACs, benzalkonium chlorides, QACs, resistance

WIDESPREAD USE IN A MULTIBILLION-DOLLAR MARKET

Benzalkonium chlorides (BACs), also known as alkyl dimethyl benzyl ammonium chlorides, alkyl dimethyl (phenylmethyl) quaternary ammonium chlorides, ammonium alkyl dimethyl (phenylmethyl) chlorides, or ammonium alkyl dimethyl benzyl chlorides, are a class of quaternary ammonium compounds (QACs) (Fig. 1A). They are usually commercialized as a mixture of compounds with different lengths for the alkyl chain, ranging from C_8 to C_{18}, with higher biocide activity for C_{12} and C_{14} derivatives (1).

BACs were reported for the first time in 1935 by Gerhard Domagk, gaining the market as zephiran chlorides, and were marketed as promising and superior disinfectant and antiseptics (2). In 1947, the first product containing BACs was registered with the Environmental Protection Agency (EPA) in the United States (3). Since then, they have been used in a wide variety of products, both prescription and over the counter. Applications range from domestic to agricultural, industrial, and clinical (Fig. 1B). Domestic applications include fabric softeners (4), personal hygiene and cosmetic products, such as shampoos, conditioners, and body lotions (5), as well as ophthalmic solutions and medications that use the nasal route of delivery (6). BACs are also among the most common active ingredients in disinfectants (4) used in residential, industrial (7), agricultural, and clinical settings. Additional registered uses for BACs in the United States include applications on indoor and outdoor surfaces (walls, floors, toilets, etc.), agricultural tools and vehicles, humidifiers, water storage tanks, products for use in residential and commercial pools, decorative ponds and fountains, water lines and systems, pulp and paper products, and wood preservation (3). The recommended or allowed concentrations of BACs in different products vary considerably according to the application (Table 1). With perhaps the exception of countries which adopted

Citation Merchel Piovesan Pereira B, Tagkopoulos I. 2019. Benzalkonium chlorides: uses, regulatory status, and microbial resistance. Appl Environ Microbiol 85:e00377-19. https://doi.org/10.1128/AEM.00377-19.

Editor Claire Vieille, Michigan State University

Address correspondence to Ilias Tagkopoulos, itagkopoulos@ucdavis.edu.

Accepted manuscript posted online 26 April 2019
Published 17 June 2019

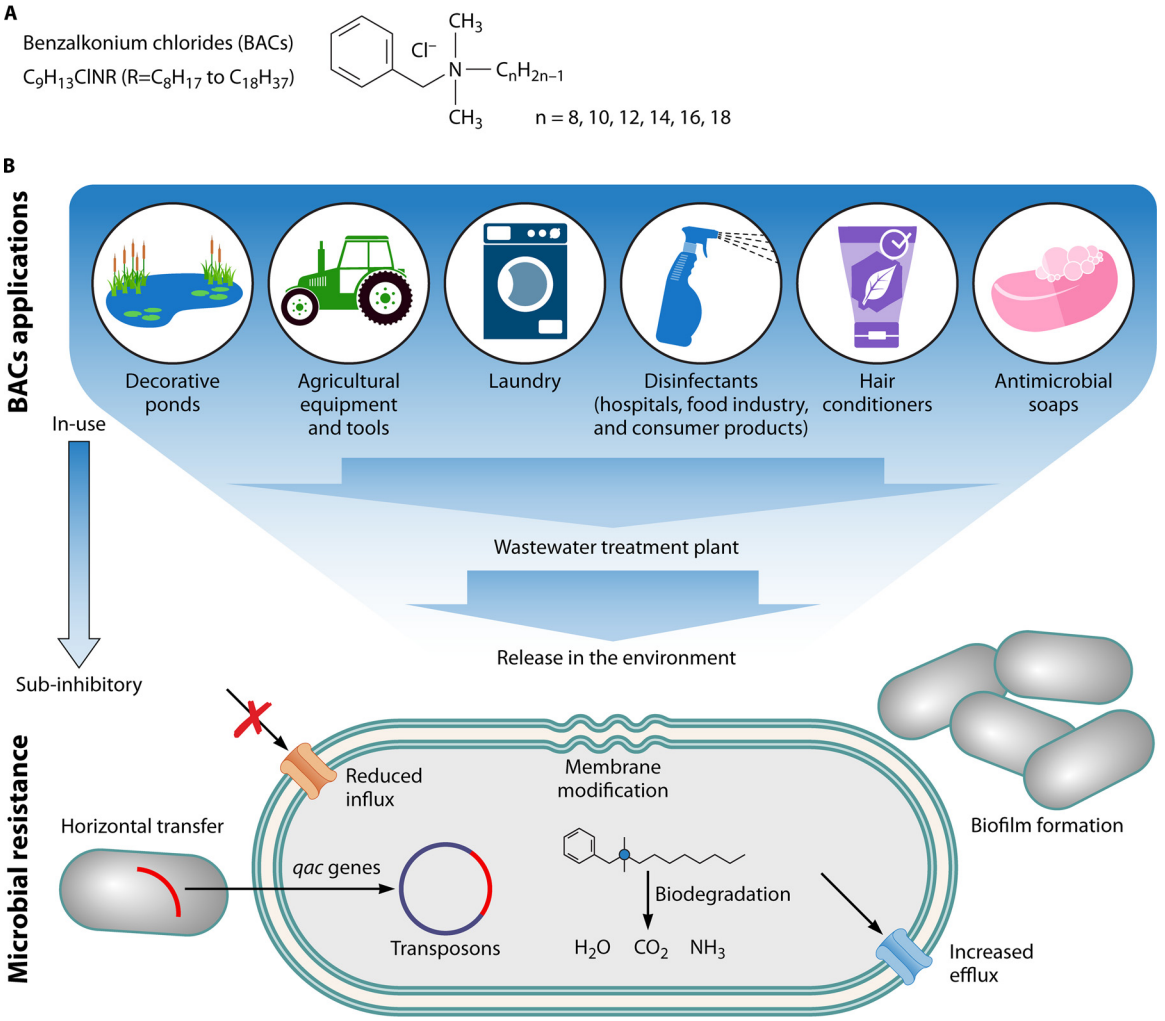

FIG 1 (A) Benzalkonium chloride (BAC) formula and structure. (B) Uses of BACs and six reported types of mechanisms of microbial resistance to BACs.

stricter regulations toward BAC use, discussed in the next section, the potential use of BACs is likely on the rise. The global market for disinfectants alone, which includes BACs, is expected to grow over 6% from 2016 and reach over $8 billion by 2021 (8).

CURRENT REGULATION

In Europe, the European Commission (EC) is involved in the regulation of BACs. Recent rules in the European market included a change in the maximum residual levels of BACs allowed in food products from 0.5 mg/kg to 0.1 mg/kg, values which will undergo an additional review by the end of 2019 (9). Additionally, recent changes in legislation, Decision (EU) 2016/1950 and the Biocidal Products Regulation (EU) no. 528/2012 (the BPR) (10, 11), meant that BACs are no longer approved for use in several biocidal products, such as consumer hand and body wash antiseptics, which is in contrast with current legislation in the United States.

In the United States, the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) share the role of regulating BACs. Such agencies regularly update regulations based on current scientific data, occasionally limiting the use of compounds found not to be safe or effective. Final determinations, however, can be delayed by requests from the industry sector that commercializes such products. As an example, the FDA recently published three proposed and final decisions regarding the use of chemicals as consumer hand rub antiseptics, consumer hand and body wash

TABLE 1 BAC concentrations found or allowed in commercial products, the environment, and substances to which microbes are resistant or tolerant and that cause toxic effects

Description[a]	Category[b]	Reported BAC concn[c]	Reference[d]
BAC-C_{12} detected in wastewater treatment influents (maximum amt)	E	170 μg/liter	101
BACs detected in food samples (maximum amt)	E	14.4 mg/kg	38
Groundwater and reclaimed water	E	Up to 0.2 μg/liter	36
Wastewater samples	E	Up to 37 μg/liter	102
Wastewater sites that included hospital, laundry, dairy, swimming pools, paper production	E	Up to 2.8 mg/liter for BAC-C_{12}, 1.1 mg/liter for BAC-C_{14}, 0.027 mg/liter for BAC-C_{16}	33
Effluents from European hospitals	E	0.05 to 6.03 mg/liter	34
Sediment samples from metropolitan region of the lower Hudson Basin	E	1.5 mg/kg	37
Wastewater treatment plant	E	Up to 0.17 mg/liter	32
Wastewater from hospital and laundry	E	2.8 and 2.1 mg/liter	32
Municipal sewage sludge in China	E	0.09−191 mg/kg	103
Ophthalmological solutions	P	0.003–0.02%	29
Hair conditioner	P	0.5–2%	5
Zephiran chloride recommended concn after dilution of 17% product concentrate	P	0.03–0.2%	104
Mosquitocide	P	200 ppm	3
Legislation limits for food processing plants, equipment, and utensils	P	200–400 ppm	3
Maximum concn that can be safely used in contact products, according to the American College of Toxicology	P	0.10%	23
Disinfectant (Lysol)	P	0.1% (wt/vol)	35
Maximum allowed wastewater for rinse-off hair products (Europe)	P	3%	105
Disinfectant in food-processing areas (Denmark) before 2016	P	3%	106
Preservative (Denmark) before 2016	P	0.10%	106
Biocidal preservative in masonry (France/UK) before 2016	P	<2.5%	106
Water preservative for cooling systems (Denmark) before 2016	P	9–50%	106
Chemical toilets before 2016	P	10–15%	106
Antimicrobial soaps and antiseptics (Hy-G-Clenz; Global Industrial Advanced; Neosporin wound cleanser for kids, cleanse antibacterial hand soap)	P	0.13%	NA
Maximum allowed BACs in food products in Europe regulation 1119/2014/EU	P	0.1 mg/kg	9
MIC of BACs for *L. monocytogenes* isolates from food-processing plants	R	7 mg/liter	50
Pseudomonas strain resistant to ammonium acetate-buffered BACs	R	0.40%	43
MIC of BACs for methicillin-resistant *Staphylococcus aureus* strains resistant to BACs	R	5–10 mg/liter	52
MIC of BACs for *Escherichia coli* K-12 resistant to BACs	R	92 mg/liter	53
MIC of BACs for *Salmonella* strain resistant to BACs	R	256 mg/liter	54
MIC of BACs for *E. coli* O157 resistant to BACs	R	1,024 mg/liter	54
MIC of BACs for *L. monocytogenes* in BACs	R	40 mg/liter	107
MIC of BACs for *E. coli* HB101 parent strains and strain overexpressing *mdfA*	R	50 and 400 mg/liter	72
MIC of BACs for *E. coli* KAM32 parent strain and strain overexpressing *pmpM*	R	1.2 and 37.5 mg/liter	74
P. aeruginosa (nonadapted and adapted) tolerance to BACs	R	1,200 and 1,600 mg/liter	56
MIC of BACs for *L. monocytogenes* wild-type and with *emrE* deletion	R	30 and 10 mg/liter	71
MIC of BACs for multiple isolates of *P. aeruginosa* strains	R	70–625 mg/liter	93
MIC of BACs for *E. coli* strains before and after adaptation to BACs, respectively	R	25 and 150 mg/liter	89
MIC of BACs for *Listeria* spp. with BAC resistance	R	35–40 mg/liter	77
MIC of BACs for isolates of carbapenem-resistant *Acinetobacter baumannii*	R	4–64 μg/liter	92
MIC of BACs for *P. aeruginosa*	R	0.45 mM	84
MIC of BACs for *E. coli*	R	0.225 mM	84
MIC of BACs for *L. monocytogenes* after adaptation	R	30 mg/liter	55
MIC of BACs for *Campylobacter coli* after adaptation for 15 days	R	2 mg/liter	46
MIC of BACs for *P. aeruginosa* after adaptation to BACs for 30 days	R	350 mg/liter	60
MBC of BACs for MRSA and MRSP isolates	R	2.1–135 mg/liter	108
Inhibition of growth for planktonic cells of *S.* Typhimurium, no effect over mature biofilms from the same strain	R	0.01%	80
Causes skin irritation	T	0.5–1%	20
Toxic effects to human conjunctival cells (*in vitro*)	T	0.0001–0.1%	22
Algal growth inhibition	T	0.255 mg/liter	31

(Continued on next page)

TABLE 1 (Continued)

Description[a]	Category[b]	Reported BAC concn[c]	Reference[d]
NOAEC for aquatic invertebrates	T	4.15 μg ai/liter	3
Chronic NOAEC for mammals	T	44 mg/kg/day	3
Chronic effects in fish	T	32.2 μg ai/liter	3
Genotoxic effects in plant and mammalian cells (*in vitro*)	T	1 mg/liter	21
Toxic to *Ceriodaphnia dubia* (crustacean)	T	0.004 mg/liter	30
Superficial cell loss observed in the cornea of rabbits with an ophthalmological solution containing BACs	T	0.02%	25

[a]MBC, minimal bactericidal concentration; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; NOAEC, no observable adverse effect concentration.
[b]P, commercial products; E, environment; R, substance to which microbes are resistant or tolerant; T, causes toxic effects.
[c]ai, active ingredient.
[d]NA, not applicable.

antiseptics, and health care antiseptics (12–14). The rules banned specific biocides, such as triclosan, or added additional and stricter regulatory approvals for several others, such as chlorhexidine, regarding the applications mentioned above. In all instances, however, BACs were excluded from the decisions and granted deferral letters as requested by manufacturers. The decisions granted manufacturers extra time to provide data to fill gaps related to safety and efficacy. Since 2015, letters and recommendations have been moving back and forth between the FDA and manufacturers and their representatives, such as the American Cleaning Institute, Lonza America, and Henkel Consumer Goods, Inc. (15–19). Decisions to postpone any action regarding the regulation of BACs were taken based on the affirmation of lack of sufficient data in the literature. Yet, multiple researchers have studied the safety aspects of BACs over the years, which include data on the toxicity to humans and the environment, as we discuss next.

TOXICITY TO HUMANS

The toxicity of BACs to humans and other animals has been described in the literature, even though discordant conclusions arise from differences in experimental conditions. As reviewed elsewhere (20), BACs are known skin irritants, with occasional, rarer reports as allergens (skin sensitizers). Regarding acute toxicology data, BACs are classified by the EPA as toxicity category II by the oral and inhalation routes and toxicity category III via the dermal route (3). They are also considered to be highly irritating to the eyes and skin (toxicity category I) (3). Small but significant genotoxic effects in both plant and mammalian cells were observed *in vitro* for BAC concentrations as low as 1 mg/liter, which is lower than those reported to be found in the environment (21). Considerable cell toxicity was observed *in vitro* for human ocular cells exposed to BAC concentrations as low as 0.0001% (22).

In contrast, a few reports in the literature found BACs to be considered safe. A report from 2006 by the EPA did not recognize BACs as being carcinogenic, mutagenic, or genotoxic (3). Regarding their addition to intranasal products, a review of 18 studies from the literature revealed no major safety concerns when BACs were used in concentrations ranging from 0.00045% to 0.1% (23). A recent review of BAC safety in cosmetic products (5) regarded their use as possibly safe, based on calculations of the margin of safety (MOS), a formula which considered the concentration of BACs in products, use frequency, and amount, and estimated parameters such as no observed adverse effect level (NOAEL) and dermal absorption ratios.

For the specific application of ophthalmological solutions, a study sponsored by Alcon Laboratories concluded that there was no safety difference between those with or without the addition of BACs (24), even though multiple researchers reported pathological effects when ophthalmological solutions containing BACs as a preservative were used, compared to preservative-free solutions (25, 26). Multiple reports of BAC toxicity for such application have even motivated the development of preservative-free ocular solutions (27). Labeling recommendations from the European Commission for

medicinal products containing BACs have also recognized eye irritation as a toxic effect from BACs (28).

In summary, most studies and governmental agencies agree that BACs are not innocuous substances, even when used in small concentrations (3, 20–22, 25, 26, 28). Safety concerns regarding their use are frequently associated with long-term contact product use, such as in preservatives in medications used by glaucoma patients, which can be chronically exposed to BACs (22, 25, 26, 29).

ENVIRONMENTAL CONTAMINATION

In a 2006 report, the EPA recognized the toxicity of BACs to the aquatic environment and its inhabitants, such as fish, oysters, shrimp, and invertebrates, advising against the release of BACs into lakes, oceans, or other waters (3). Since then, their toxicity to aquatic organisms, as well as other animals, has been well established by several research groups (30, 31). Despite that, BACs have been detected in wastewater effluents and other environments (Table 1).

Data regarding the detection of BACs in the environment are sparse in the literature, and recent measurements are lacking. BACs were reported in wastewater effluents from hospitals, reaching concentrations in the milligram-per-liter range (32–34). Other effluents, such as those from laundry, dairy, community pools, also had the presence of BACs (32, 33) at various concentrations that were generally lower than those originating from hospitals. Typical wastewater treatment plants are not designed to treat QAC contaminants, resulting in the release of at least a portion of them into the environment as micropollutants (35). Concentrations varying in the ranges of microgram per liter or microgram per gram were found in ground and reclaimed water (36), as well as soil samples (37). BACs were also detected in up to 3.5% of over 4,000 food samples analyzed by the European Food Safety Authority (EFSA) (38).

From all the QACs tested by different research groups, BACs, mainly C_{12}, C_{14}, and C_{16}, were found in higher concentrations than in other QACs (32, 33). The high incidence of BACs could be attributed, at least in part, to their popularity in various applications, from consumer products such as eye drops, shampoos, and mosquito insecticides, to disinfectants and antiseptics used in hospitals and food industries. Whether the widespread use of these compounds, a lack of proper disposal, or a combination of both contributed to the observed incidence in the environment is unknown. We estimate that BAC disposal in the environment is still considerable, especially in countries that have less-restrictive legislation, such as the United States. Deeper investigations, however, are required to establish the current levels of BACs in the environment, as well as the potential links for the development of resistant microbial strains, which we discuss next.

MICROBIAL TOLERANCE AND RESISTANCE

The use of BACs for multiple applications, many of which unavoidably result in the generation and release of residual biocide, can result in the presence of environments in which there is a selection pressure over microbes to evolve resistance to such chemicals (35). The capacity of bacteria to survive and thrive in BACs has been demonstrated by tracking outbreaks usually associated with misuse or improper dilution and storage of disinfectants and antiseptic solutions (39). In fact, multiple outbreaks were associated with BACs throughout 4 decades (39), motivating a series of recommendations to discontinue their use as an antiseptic (40, 41).

Concerns about the use of BACs as antiseptics are not novel, and researchers have observed resistant strains capable of surviving in BAC solutions (0.1 to 0.4%) as early as the 1960s (42, 43). It is known that bacteria can adapt and increase their tolerance to stressful chemicals (44, 45), and such phenomena have been shown repeatedly for BACs. Frequently, the adaptive mutations that select for increased tolerance or resistance are stable at a population level and can be still observed for evolved strains even after the selection pressure has been lifted (46). Even though the reported concentrations vary depending on the study and the bacterial genera (Table 1), it has been

demonstrated that bacteria can evolve to survive to BAC concentrations similar to those found in the environment and in consumer products (Table 1).

It is important to highlight that the terms "tolerance" and "resistance" have been used interchangeably, especially when related to biocides, which could lead to misinterpretation of data (47, 48). Resistance is broadly understood as the "insusceptibility of a microorganism to a particular treatment under a particular set of conditions" (47, 48). Multiple researchers defined resistance based solely on an increase in the MIC (49, 50). The term tolerance has been used on several distinct occasions. Tolerant strains were defined as those in which the antimicrobial's MIC for them did not increase, but the strain was able to survive killing by, for example, reducing growth (51). Tolerant strains were also defined as those in which the antimicrobial's MIC for them increased compared to the controls (48). We believe that the broad term "decrease in susceptibility" is often more appropriate to describe the observed increases in MIC for biocides, including the following examples.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were evolved in BACs (52), doubling the MIC of BACs from 5 to 10 mg/liter, after a period of adaptation. The MICs of BACs increased 4-fold for *Campylobacter coli* after exposure to the chemical for 15 days (46). *Escherichia coli* K-12 strains exposed to increasing concentrations of BACs were able to survive in a concentration of 92 mg/liter BACs, which was eight times higher than the concentration in which the parent strain could survive (53). Another study showed that the MICs of BACs changed from 4 to 256 mg/liter for *Salmonella enterica* serovar Virchow and reached over 1,000 mg/liter for *E. coli* O157 (54). The leading foodborne pathogen in the United States, *Listeria monocytogenes*, is also capable of decreasing its susceptibility to BACs. Three different strains (H7550, SK2802, and J0161) of *L. monocytogenes* from outbreaks and disease cases were exposed to BACs, and isolates with up to 3-fold increases (10 to 30 mg/liter) in the MICs of BACs were obtained for all strains (55).

The *Pseudomonas* sp. strains can naturally withstand the highest concentrations of BACs. *Pseudomonas aeruginosa* survives at up to 1,600 and 1,200 mg/liter BACs with or without a previous adaptation to the chemical, respectively (56). The MIC of BACs for the isolated strain *Pseudomonas* sp. BIOMIG1 was 1,024 mg/liter (57). The higher recalcitrance of *Pseudomonas* spp. may explain why, after exposing complex microbial communities to BACs, there was an enrichment in *Pseudomonas* species, with a decrease in microbial diversity (58, 59). In another study, *P. aeruginosa* NCIMB 10421 was cultivated in continuous culture, and the BAC concentration was progressively increased for about 30 days. The MICs of BACs increased from 25 mg/liter to over 350 mg/liter, and the adapted strain had higher fitness when competed with the parent strain in the presence of BACs, especially with magnesium depletion and the presence of glucose in the medium (60).

A recent study has questioned the use of aqueous solutions of BACs to determine their activity against microorganisms, demonstrating that BACs in real-use formulations (with surfactants and chelating agents) is more effective to control microbial growth (59). Despite this finding, strains with decreased susceptibility to BACs can not only develop and be selected for under controlled laboratory conditions, but they have also been isolated directly from real-case scenarios, environments in which BACs is frequently used as a biocide. Strains of the pathogen *L. monocytogenes* isolated from diverse environments, such as food-processing plants, food products, patients, and animals, were reported as having decreased susceptibility to BACs. Such strains ranged from 8% (49) and 10% (50) up to 40% (61) and 45% (62) of the total number of isolates in these environments.

MICROBIAL MECHANISMS OF TOLERANCE AND RESISTANCE

The mode of action of QACs, including BACs, involves the perturbation and disruption of the membrane bilayers by the alkyl chains and disruption of charge distribution of the membrane by the charged nitrogen (63). Accordingly, susceptibility to BACs may emerge through a combination of mechanisms (56), with many of those related to the

cell membrane. The mechanisms proposed in the literature include changes in the overall membrane composition, downregulation of porins, overexpression or modification of efflux pumps, horizontal gene transfer of transposon elements and stress factors, biofilm formation, and biodegradation (Fig. 1B).

Changes in the membrane composition have long been associated with decreased susceptibility to BACs (64, 65). Resistant strains of *P. aeruginosa* were shown to have different phospholipid and fatty acid compositions compared to a susceptible strain (64). Other work has demonstrated that exposure of *Bacillus cereus* to BACs induced genes involved in fatty acid metabolism and caused changes in the fatty acid composition of the membrane (66). The authors, however, did not evaluate whether exposed strains exhibited a tolerant phenotype. A strain of *E. coli* with reduced susceptibility to BACs was shown to have a lipopolysaccharide composition diverse from that of the susceptible strain (64). Recently, it was suggested that *Pseudomonas* strains could partially adapt to BACs by stabilizing the membrane charge through the increase in polyamine synthesis gene expression and mutations in *pmrB* (56).

The reduced influx of BACs has been suggested to collaborate to decreased susceptibility to the biocide. Since adsorption of QACs is believed to occur through porins (63), decreased susceptibility could be achieved, in theory, by the downregulation of porins. In accordance, the downregulation of genes for multiple porins has been associated with *Pseudomonas* (56, 67) and *E. coli* (53) strains less susceptible to BACs. A lower level of the porin OmpF in the *E. coli* membrane decreased the strain susceptibility to BACs (64). A causal relationship between a disinfectant product containing BACs and the downregulation of porins was demonstrated for *Mycobacterium smegmatis*; knockout mutants for Msp porins were less susceptible to the biocide than was the wild type (68). The use of a disinfectant formulation by the authors, however, limits the extent to which the observed effect can be attributed to BACs, other components of the formula, or the mixture. Further studies are required to reinforce the link between tolerance to BACs and the downregulation of porins.

The presence or upregulation of certain families of efflux pumps has been associated with multidrug resistance and decreased susceptibility to BACs across several genera of bacteria. Resistance via increased efflux lowers the concentration of biocide inside the cell, allowing the bacteria to survive against higher environmental concentrations of the chemical. One such case is the Qac proteins, a group of multidrug efflux proteins frequently associated with resistance to BACs (69). In the foodborne pathogen *L. monocytogenes*, the efflux pump MrdI (70) and the efflux pump EmrE (71) have been associated with resistance to BACs. In isolates of *L. monocytogenes*, the susceptibility to BACs and antimicrobials could be restored when the efflux inhibitor was added to the medium containing a previously adapted and resistant strain. This suggested at least a partial role of efflux pumps for resistance to BACs in this organism (55). The efflux protein MdfA contributed to increased resistance to BACs in *E. coli* (72). For the plant pathogen *Pseudomonas syringae*, the resistance-nodulation-division (RND)-type pump MexAB-OprM knockout mutant showed increased sensitivity to BACs (73). Another efflux pump, the PmpM of the multidrug and toxin extrusion (MATE) family, from *P. aeruginosa*, contributed to decreased susceptibility to BACs when expressed in a plasmid in *E. coli* (74). Accordingly, the exposure of *Pseudomonas* strains to BACs for a long time resulted in the overexpression of multidrug efflux pump genes (56). Mutations in the *nfxB*, a regulator for the Mex efflux system, as well as overexpression of both MexAB-OprM and MexCD-OprJ efflux systems and downregulation of *mexR*, a repressor of the Mex system, was also correlated to decreased sensitivity to BACs in *P. aeruginosa* (60).

Resistance elements, like efflux pumps, often appear to be associated with other genes, such as mobile elements and transposases (75), which contributes to their dissemination in bacterial populations and maintenance of tolerant and resistant phenotypes. The transposon Tn*6188* was associated with strains of *L. monocytogenes* with increased tolerance to BACs. It included three transposases and a protein which was similar to the Smr, EmrE, and Qac efflux proteins (75). Strains of *L. monocytogenes*

responsible for outbreaks in Canada had a genomic island containing multiple resistance, stress response, and virulence-associated genes (76), which included an efflux pump involved in resistance to BACs (71). Successful horizontal gene transfer of resistance-associated genes from nonpathogenic BAC-resistant *Listeria innocua* and *Listeria welshimeri* to the pathogenic *L. monocytogenes* does occur, and it suggests that more common nonpathogenic strains frequently exposed to the biocide in food-processing plants can act as resistance reservoirs (77).

Factors such as the presence of biofilms can affect the ability of a biocide to control and eliminate microorganisms (78). Biofilms are communities of single- or multispecies microorganisms attached to solid surfaces surrounded by their secreted exopolysaccharide matrix. Biofilm formation represents one of the mechanisms of resistance and tolerance explored by bacteria to avoid and protect themselves against stressful environments (79). Bacterial communities in biofilms have increased ability to survive antiseptics and disinfectants, such as BACs, compared to planktonic cells (80). Exposure of *Salmonella enterica* to 0.02% of BACs (2-fold higher than the MIC for planktonic cells) for between 10 and 90 min, though it reduced the cell number, failed to eradicate the biofilm (79).

Tolerance to BACs can be greater for multispecies biofilms than for single-species biofilms, as was the case for a dual-species biofilm with *L. monocytogenes* and *Pseudomonas putida* (78, 81). This result can be partially explained by the selection pressure for the strain with higher intrinsic resistance to the biocide (78). As mentioned before, *Pseudomonas* species naturally have a better capacity to survive in higher concentrations of BACs (56, 57). Their presence in the biofilm community could contribute to the increased tolerance compared to other single-species biofilms.

The cases mentioned above demonstrate the better capacity of both single cells and multispecies cells to survive the presence of biocides when in biofilms versus planktonic cells. In addition, the exposure to the biocide can occasionally increase biofilm formation by bacteria (82–84). Continuous exposure of bacteria to BACs resulted in thicker biofilms, as observed with scanning electron microscopy (SEM) (84). Strains of *E. coli* isolated from the dairy industry which were less susceptible to BACs and antibiotics also had an increased ability to form biofilms (82). The susceptible strains became strong biofilm formers as well after a period of adaptation (exposure) to BACs (82). Exposure to BACs induced biofilm formation by *Staphylococcus epidermidis* CIP53124, although the same effect was not observed for other species tested (83).

Last, some microbial communities and species such as *Pseudomonas* spp. are capable of degrading BACs, converting them into less toxic chemicals and utilizing them as secondary substrates and energy sources (58, 85). Degradation of BACs by dealkylation decreases its toxicity to microorganisms (86). A study of microbial communities suggested that *Pseudomonas* sp. strain BIOMIG1 was responsible for the biodegradation of BACs, possibly via dioxygenase (57). Degradation of BACs under nitrate-reducing conditions in the presence of a methanogenic culture obtained from an anaerobic digester has also been demonstrated (87). The transformation was determined to be abiotic by a nucleophilic substitution with nitrite that generated benzonitrile (87).

Given the mode of action of BACs through membrane disruption (63) and the above-described general mechanisms of bacterial response by membrane modification (64, 65), overexpression of multidrug efflux pumps (56, 70–74), and biofilm formation (78, 79, 81), we expect some level of cross-resistance to other antimicrobials, which is described next.

CROSS-RESISTANCE TO ANTIBIOTICS

Cross-resistance is the phenomenon in which exposure to one chemical grants an advantage for survival in a distinct chemical (44, 45). Cross-resistance between antiseptics, disinfectants, and antibiotics has been thoroughly described in the literature, including cases involving BACs.

The antibiotics oxacillin, cefazolin, and ofloxacin had higher MICs in methicillin-

resistant *S. aureus* (MRSA) strains evolved in the presence of BACs (52). MRSA strains nonadapted to BACs were already resistant to ofloxacin, as defined by EUCAST standards (88), and the MICs of the antibiotic increased up to 4-fold for the adapted strains (52). Similar results were observed with *E. coli* (53, 54, 89). The laboratory strain *E. coli* K-12 adapted to increasing concentrations of BACs. This resulted in several antibiotics, such as ampicillin, ciprofloxacin, and nalidixic acid, increasing the MIC on such a strain (53). MICs for multiple antibiotics also increased after adaptation to BACs for the pathogen strain *E. coli* O157 (54), and the same was observed for *E. coli* ATCC 11775 and DSM 682 (89). In some cases, *E. coli* strains adapted to BACs became resistant to antibiotics, as defined by EUCAST (88), such as chloramphenicol (54, 89) and ampicillin (89). The MICs of multiple antibiotics also increased after adaptation to BACs for the bacteria of *Salmonella* serovar Virchow (54). Such strains became resistant to amoxicillin, as defined by EUCAST (88), after exposure to BACs. *L. monocytogenes* strains adapted to BACs showed decreased sensitivities to both ciprofloxacin and gentamicin (55). *P. aeruginosa* evolved in the presence of BACs in continuous culture, on the other hand, exhibited varied sensitivities to antibiotics. The adapted strain PA-29 was less sensitive to ciprofloxacin but more sensitive to minocycline, which is an antibiotic similar to tetracycline (60). The authors believed that the increased sensitivity to minocycline was due to a decrease in the expression of the MexXY-OprM efflux pump system observed for the adapted strain (60), which plays a role in the resistance to an analogue of minocycline (90). They did not confirm this hypothesis, however.

Besides isolated strains, evidence of cross-resistance between BACs and antibiotics has been shown for microbial communities. The exposure of complex microbial communities to BACs not only decreased the overall diversity of the population but also resulted in decreased susceptibility to three clinically relevant antibiotics, penicillin, tetracycline, and ciprofloxacin (58).

Evidence of cross-resistance between BACs and antibiotics is not exclusively limited to controlled laboratory experiments and strains. Following the isolation of *S. aureus* strains from patients, the MIC of BACs increased for over 100 isolates, which corresponded to approximately half of the isolates. BAC-resistant isolates harboring plasmids with *qacA* and *qacB* genes were also less sensitive to multiple antibiotics than were BAC-sensitive ones. The incidence of *qac* and β-lactamase *bla* genes in the same plasmids provided strong evidence of a linkage between the selection pressure for resistance to disinfectants, such as BACs, and antibiotics, such as penicillin (91). A similar association occurred for over 50 isolates of carbapenem-resistant *Acinetobacter baumannii*. Strains obtained from four different hospitals had a high prevalence of both *qac* and *bla* genes (92).

In contrast, a study conducted by the Unilever group (93) questioned the correlation between biocide use and cross-resistance to antibiotics for *P. aeruginosa*. Their statistical analysis revealed a stronger link between biocides and antibiotic susceptibility between strains isolated from clinical settings than from industrial settings, which made the authors conclude that misuse of antibiotics, and not disinfectants, were driving the results. Though interesting, additional studies would be necessary to demonstrate such a conclusion. It is also not clear whether such a correlation would hold for other bacterial species.

The exposure and adaptation to BACs can result in decreased susceptibility to several clinically relevant antibiotics in some species (52, 54, 55, 58, 89, 91), but not all, and several studies have also reported the opposite result, i.e., increased susceptibility to antibiotics (60, 94, 95). Most studies do not report whether the observed increases in MIC for the antibiotics are within the definition of resistance according to clinical standards (88, 96). Such a fact often motivates questioning of the relevance of such studies (48, 93). However, an increase in MIC by itself demonstrates the existence of a cross-resistance effect and should not be ignored. Researchers showed that bacteria that are merely tolerant to antibiotics can develop resistance to them faster (51). The ability of bacteria to survive the presence of the antibiotics, even before the MIC has

reached clinical standards, helps keep and accumulate mutations that can eventually result in the emergence of strains resistant to the antibiotics (51).

CONCLUSION

This review of the literature explored the data currently available on the potential implications of BACs to human safety and the environment in general. There is evidence that the continuous use of biocides and their release to the environment in subinhibitory concentrations may lead to the emergence of tolerant, resistant, and cross-resistant microbial strains, even though there are occasional controversial reports in the literature. Given the reported side effects of BACs, we believe that a thorough analysis of benefits versus risks should be the guidepost for future regulatory and manufacturing use of the compound. Based on the analysis presented here, we have a few recommendations.

We propose restrictions for BAC use in consumer products. Currently, the Centers for Disease Control and Prevention (CDC) recommends (97), and the FDA endorses (98), the use of only water and plain soap by regular consumers (which does not include professionals in health care settings). Despite that, BACs are still commercialized in over-the-counter antimicrobial soaps in the United States. The FDA has recently regulated other chemicals, such as triclosan and chlorhexidine, postponing any decisions regarding the use of BACs (12, 13).

Additionally, updated data regarding the presence of BACs in the environment, water, and soil are required to determine the need for monitoring such a compound and establishing a baseline of its concentration in various environments. Based on the available data, bacteria can survive BAC concentrations found in the environment (Table 1), and cross-resistance between BACs and antibiotics has been reported (52–55, 58, 89, 91).

Finally, we urge further research on the effect of BAC exposure, both in free form and as part of consumer products, to microbial populations and tissues to elucidate its toxigenic and long-term potential to alter the microbial flora in both a clinical and environmental context. We still have a limited understanding of the mechanistic underpinnings and basis of adaptation and how these link to the emergence of global health challenges like antibiotic resistance. Another link that remains to be determined is the impact of BACs and QACs in general to the human microbiota of the skin, gut, and others, which are lately associated with numerous diseases and performance outcomes (99, 100).

Balancing the concentrations that effectively inhibit bacteria in products, are not toxic to users, and will not leave residual pollutants after disposal is certainly challenging. Limiting the use and regulating and monitoring chemicals such as BACs are important to reduce the negative impacts on humans and the environment.

ACKNOWLEDGMENTS

We thank the Tagkopoulos lab for helpful discussions and the reviewers for their feedback.

B.M.P.P. and I.T. were supported by award 1743101 from the National Science Foundation.

REFERENCES

1. European Commission EURL-SRM. 2012. Analysis of quaternary ammonium compounds (QACs) in fruits and vegetables using QuEChERS and LC-MS/MS. EU Reference Laboratories, Fellbach, Germany.
2. Price PB. 1950. Benzalkonium chloride (zephiran chloride) as a skin disinfectant. Arch Surg 61:23–33. https://doi.org/10.1001/archsurg.1950.01250020026004.
3. U.S. Environmental Protection Agency (EPA). 2006. Reregistration eligibility decision for alkyl dimethyl benzyl ammonium chloride (ADBAC). EPA739-R-06-009. U.S. Environmental Protection Agency, Washington, DC.
4. Tezel U, Pavlostathis S, Tezel U, Pavlostathis S. 2012. The role of quaternary ammonium compounds on antimicrobial resistance in the environment, p 349–386. *In* Keen PL, Montforts MHMM (ed), Antimicrobial resistance in the environment. John Wiley & Sons, Inc., Hoboken, NJ.
5. Choi SM, Roh TH, Lim DS, Kacew S, Kim HS, Lee B-M. 2018. Risk assessment of benzalkonium chloride in cosmetic products. J Toxicol Environ Health B Crit Rev 21:8–23. https://doi.org/10.1080/10937404.2017.1408552.
6. Committee for Human Medicinal Products (CHMP). 2017. Questions and answers on benzalkonium chloride used as an excipient in medicinal products for human use. EMA/CHMP/495737/2013. European Medicines Agency, Amsterdam, The Netherlands.

7. Condell O, Iversen C, Cooney S, Power KA, Walsh C, Burgess C, Fanning S. 2012. Efficacy of biocides used in the modern food industry to control *Salmonella enterica*, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. Appl Environ Microbiol 78:3087–3097. https://doi.org/10.1128/AEM.07534-11.
8. Zion Market Research. 2016. Antiseptics and disinfectants market by type (alcohol and aldehyde, phenols and derivatives, biguanides and amides, quaternary ammonium compounds, iodine compounds and others) for domestics, institutional and other end-users–global industry perspective, comprehensive analysis and forecast, 2015–2021. Zion Market Research, New York, NY.
9. European Commission. 2014. Commission regulation (EU) no. 1119/2014 of 16 October 2014 amending annex III to regulation (EC) no. 396/2005 of the European Parliament and of the Council as regards maximum residue levels for benzalkonium chloride and didecyldimethylammonium chloride in or on certain products. European Commission, Brussels, Belgium.
10. European Parliament, Council of the European Union. 2012. Biocidal products regulation. European Union, Brussels, Belgium.
11. European Commission. 2016. Commission implementing decision (EU) 2016/1950 of 4 November 2016 on the non-approval of certain biocidal active substances pursuant to regulation (EU) no. 528/2012 of the European Parliament and of the Council. European Commission, Brussels, Belgium.
12. Food and Drug Administration, HHS. 2016. Safety and effectiveness of consumer antiseptics; topical antimicrobial drug products for over-the-counter human use. Fed Regist 81:61106–61130.
13. Food and Drug Administration, HHS. 2017. Safety and effectiveness of health care antiseptics; topical antimicrobial drug products for over-the-counter human use. Fed Regist 82:60474–60503.
14. Food and Drug Administration. 2016. Safety and effectiveness of consumer antiseptics; topical antimicrobial drug products for over-the-counter human use; proposed amendment of the tentative final monograph; reopening of administrative record. U.S. Food and Drug Administration, Silver Spring, MD.
15. American Cleaning Institute. 19 December 2017. Letter from American Cleaning Institute. American Cleaning Institute, Washington, DC. https://www.regulations.gov/document?D=FDA-2016-N-0124-0267.
16. American Cleaning Institute. 6 May 2016. Response from American Cleaning Institute to FDA CDER (re: benzalkonium chloride). American Cleaning Institute, Washington, DC. https://www.regulations.gov/document?D=FDA-1975-N-0012-0646.
17. Food and Drug Administration. 19 January 2017. FDA letter regarding deferral of benzalkonium chloride. U.S. Food and Drug Administration, Silver Spring, MD. https://www.regulations.gov/document?D=FDA-2015-N-0101-1321.
18. Food and Drug Administration (FDA) CDER. 24 March 2017. Letter from CDER FDA to American Cleaning Institute. U.S. Food and Drug Administration, Silver Spring, MD. https://www.regulations.gov/document?D=FDA-2015-N-0101-1336.
19. Food and Drug Administration (FDA) CDER. 10 March 2016. Letter from FDA CDER to Lonza America, Inc. American Cleaning Institute and Henkel North America regarding review of benzalkonium chloride. U.S. Food and Drug Administration, Silver Spring, MD. https://www.regulations.gov/document?D=FDA-1975-N-0012-0639.
20. Basketter DA, Marriott M, Gilmour NJ, White IR. 2004. Strong irritants masquerading as skin allergens: the case of benzalkonium chloride. Contact Dermatitis 50:213–217. https://doi.org/10.1111/j.0105-1873.2004.00331.x.
21. Ferk F, Misik M, Hoelzl C, Uhl M, Fuerhacker M, Grillitsch B, Parzefall W, Nersesyan A, Micieta K, Grummt T, Ehrlich V, Knasmuller S. 2007. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. Mutagenesis 22:363–370. https://doi.org/10.1093/mutage/gem027.
22. De Saint Jean M, Brignole F, Bringuier AF, Bauchet A, Feldmann G, Baudouin C. 1999. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. Invest Ophthalmol Vis Sci 40:619–630.
23. Marple B, Roland P, Benninger M. 2004. Safety review of benzalkonium chloride used as a preservative in intranasal solutions: an overview of conflicting data and opinions. Otolaryngol Head Neck Surg 130:131–141. https://doi.org/10.1016/j.otohns.2003.07.005.
24. Kitazawa Y, Smith P, Sasaki N, Kotake S, Bae K, Iwamoto Y. 2011. Travoprost 0.004%/timolol 0.5%-fixed combination with and without benzalkonium chloride: a prospective, randomized, doubled-masked comparison of safety and efficacy. Eye (Lond) 25:1161–1169. https://doi.org/10.1038/eye.2011.134.
25. Whitson JT, Cavanagh HD, Lakshman N, Petroll WM. 2006. Assessment of corneal epithelial integrity after acute exposure to ocular hypotensive agents preserved with and without benzalkonium chloride. Adv Ther 23:663–671. https://doi.org/10.1007/BF02850305.
26. Baudouin C, de Lunardo C. 1998. Short-term comparative study of topical 2% carteolol with and without benzalkonium chloride in healthy volunteers. Br J Ophthalmol 82:39–42. https://doi.org/10.1136/bjo.82.1.39.
27. Rosin LM, Bell NP. 2013. Preservative toxicity in glaucoma medication: clinical evaluation of benzalkonium chloride-free 0.5% timolol eye drops. Clin Ophthalmol 7:2131–2135. https://doi.org/10.2147/OPTH.S41358.
28. European Commission. 2017. Annex to the European Commission guideline on 'Excipients in the labelling and package leaflet of medicinal products for human use' (SANTE-2017-11668). European Commission, Brussels, Belgium. https://www.ema.europa.eu/en/documents/scientific-guideline/annex-european-commission-guideline-excipients-labelling-package-leaflet-medicinal-products-human_en.pdf.
29. Aguayo Bonniard A, Yeung JY, Chan CC, Birt CM. 2016. Ocular surface toxicity from glaucoma topical medications and associated preservatives such as benzalkonium chloride (BAK). Expert Opin Drug Metab Toxicol 12:1279–1289. https://doi.org/10.1080/17425255.2016.1209481.
30. Lavorgna M, Russo C, D'Abrosca B, Parrella A, Isidori M. 2016. Toxicity and genotoxicity of the quaternary ammonium compound benzalkonium chloride (BAC) using *Daphnia magna* and *Ceriodaphnia dubia* as model systems. Environ Pollut 210:34–39. https://doi.org/10.1016/j.envpol.2015.11.042.
31. Elersek T, Zenko M, Filipic M. 2018. Ecotoxicity of disinfectant benzalkonium chloride and its mixture with antineoplastic drug 5-fluorouracil towards alga P*seudokirchneriella subcapitata*. PeerJ 6:e4986. https://doi.org/10.7717/peerj.4986.
32. Martínez-Carballo E, Gonzalez-Barreiro C, Sitka A, Kreuzinger N, Scharf S, Gans O. 2007. Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. Environ Pollut 146:543–547. https://doi.org/10.1016/j.envpol.2006.07.016.
33. Kreuzinger N, Fuerhacker M, Scharf S, Uhl M, Gans O, Grillitsch B. 2007. Methodological approach towards the environmental significance of uncharacterized substances—quaternary ammonium compounds as an example. Desalination 215:209–222. https://doi.org/10.1016/j.desal.2006.10.036.
34. Kümmerer K, Eitel A, Braun U, Hubner P, Daschner F, Mascart G, Milandri M, Reinthaler F, Verhoef J. 1997. Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. J Chromatogr A 774:281–286. https://doi.org/10.1016/S0021-9673(97)00242-2.
35. Tezel U, Pavlostathis SG. 2015. Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. Curr Opin Biotechnol 33:296–304. https://doi.org/10.1016/j.copbio.2015.03.018.
36. Estévez E, del Carmen Cabrera M, Molina-Díaz A, Robles-Molina J, del Pino Palacios-Díaz M. 2012. Screening of emerging contaminants and priority substances (2008/105/EC) in reclaimed water for irrigation and groundwater in a volcanic aquifer (Gran Canaria, Canary Islands, Spain). Sci Total Environ 433:538–546. https://doi.org/10.1016/j.scitotenv.2012.06.031.
37. Li X, Brownawell BJ. 2010. Quaternary ammonium compounds in urban estuarine sediment environments-a class of contaminants in need of increased attention? Environ Sci Technol 44:7561–7568. https://doi.org/10.1021/es1011669.
38. European Food Safety Authority (EFSA). 2013. Evaluation of monitoring data on residues of didecyldimethylammonium chloride (DDAC) and benzalkonium chloride (BAC). European Food Safety Authority, Parma, Italy. https://www.efsa.europa.eu/en/supporting/pub/en-483.
39. Weber DJ, Rutala WA, Sickbert-Bennett EE. 2007. Outbreaks associated with contaminated antiseptics and disinfectants. Antimicrob Agents Chemother 51:4217–4224. https://doi.org/10.1128/AAC.00138-07.
40. Frank MJ, Schaffner W. 1976. Contaminated aqueous benzalkonium

chloride. An unnecessary hospital infection hazard. JAMA 236:2418–2419. https://doi.org/10.1001/jama.1976.03270220038032.
41. Tiwari TSP, Ray B, Jost KCJ, Rathod MK, Zhang Y, Brown-Elliott BA, Hendricks K, Wallace R. 2003. Forty years of disinfectant failure: outbreak of postinjection *Mycobacterium abscessus* infection caused by contamination of benzalkonium chloride. Clin Infect Dis 36:954–962. https://doi.org/10.1086/368192.
42. Malizia WF, Gangarosa EJ, Goley AF. 1960. Benzalkonium chloride as a source of infection. N Engl J Med 263:800–802. https://doi.org/10.1056/NEJM196010202631608.
43. Adair FW, Geftic SG, Gelzer J. 1969. Resistance of *Pseudomonas* to quaternary ammonium compounds. I. Growth in benzalkonium chloride solution. Appl Microbiol 18:299–302.
44. Zorraquino V, Kim M, Rai N, Tagkopoulos I. 2017. The genetic and transcriptional basis of short and long term adaptation across multiple stresses in *Escherichia coli*. Mol Biol Evol 34:707–717. https://doi.org/10.1093/molbev/msw269.
45. Dragosits M, Mozhayskiy V, Quinones-Soto S, Park J, Tagkopoulos I. 2013. Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*. Mol Syst Biol 9:643. https://doi.org/10.1038/msb.2012.76.
46. Mavri A, Mozina SS. 2012. Involvement of efflux mechanisms in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. J Med Microbiol 61:800–808. https://doi.org/10.1099/jmm.0.041467-0.
47. Gilbert P, McBain AJ. 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin Microbiol Rev 16:189–208. https://doi.org/10.1128/CMR.16.2.189-208.2003.
48. Gerba CP. 2015. Quaternary ammonium biocides: efficacy in application. Appl Environ Microbiol 81:464–469. https://doi.org/10.1128/AEM.02633-14.
49. Mereghetti L, Quentin R, Marquet-Van Der Mee N, Audurier A. 2000. Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. Appl Environ Microbiol 66:5083–5086. https://doi.org/10.1128/AEM.66.11.5083-5086.2000.
50. Aase B, Sundheim G, Langsrud S, Rorvik LM. 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. Int J Food Microbiol 62:57–63. https://doi.org/10.1016/S0168-1605(00)00357-3.
51. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830. https://doi.org/10.1126/science.aaj2191.
52. AKimitsu N, Hamamoto H, Inoue R, Shoji M, Akamine A, Takemori K, Hamasaki N, Sekimizu K. 1999. Increase in resistance of methicillin-resistant *Staphylococcus aureus* to beta-lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals. Antimicrob Agents Chemother 43:3042–3043. https://doi.org/10.1128/AAC.43.12.3042.
53. Bore E, Hebraud M, Chafsey I, Chambon C, Skjaeret C, Moen B, Moretro T, Langsrud O, Rudi K, Langsrud S. 2007. Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. Microbiol Read Engl 153:935–946. https://doi.org/10.1099/mic.0.29288-0.
54. Braoudaki M, Hilton AC. 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol 42:73–78. https://doi.org/10.1128/JCM.42.1.73-78.2004.
55. Rakic-Martinez M, Drevets DA, Dutta V, Katic V, Kathariou S. 2011. Listeria monocytogenes strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. Appl Environ Microbiol 77:8714–8721. https://doi.org/10.1128/AEM.05941-11.
56. Kim M, Hatt JK, Weigand MR, Krishnan R, Pavlostathis SG, Konstantinidis KT. 2018. Genomic and transcriptomic insights into how bacteria withstand high concentrations of benzalkonium chloride biocides. Appl Environ Microbiol 84:e00197-18. https://doi.org/10.1128/AEM.00197-18.
57. Ertekin E, Hatt JK, Konstantinidis KT, Tezel U. 2016. Similar microbial consortia and genes are involved in the biodegradation of benzalkonium chlorides in different environments. Environ Sci Technol 50:4304–4313. https://doi.org/10.1021/acs.est.5b05959.
58. Tandukar M, Oh S, Tezel U, Konstantinidis KT, Pavlostathis SG. 2013. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. Environ Sci Technol 47:9730–9738. https://doi.org/10.1021/es401507k.
59. Forbes S, Cowley N, Humphreys G, Mistry H, Amezquita A, McBain AJ. 2017. Formulation of biocides increases antimicrobial potency and mitigates the enrichment of nonsusceptible bacteria in multispecies biofilms. Appl Environ Microbiol 83:e03054-16. https://doi.org/10.1128/AEM.03054-16.
60. Mc Cay PH, Ocampo-Sosa AA, Fleming G. 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. Microbiol Read Engl 156:30–38. https://doi.org/10.1099/mic.0.029751-0.
61. Soumet C, Ragimbeau C, Maris P. 2005. Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production. Lett Appl Microbiol 41:291–296. https://doi.org/10.1111/j.1472-765X.2005.01763.x.
62. Mullapudi S, Siletzky RM, Kathariou S. 2008. Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. Appl Environ Microbiol 74:1464–1468. https://doi.org/10.1128/AEM.02426-07.
63. Wessels S, Ingmer H. 2013. Modes of action of three disinfectant active substances: a review. Regul Toxicol Pharmacol 67:456–467. https://doi.org/10.1016/j.yrtph.2013.09.006.
64. Sakagami Y, Yokoyama H, Nishimura H, Ose Y, Tashima T. 1989. Mechanism of resistance to benzalkonium chloride by *Pseudomonas aeruginosa*. Appl Environ Microbiol 55:2036–2040.
65. Ishikawa S, Matsumura Y, Yoshizako F, Tsuchido T. 2002. Characterization of a cationic surfactant-resistant mutant isolated spontaneously from *Escherichia coli*. J Appl Microbiol 92:261–268. https://doi.org/10.1046/j.1365-2672.2002.01526.x.
66. Ceragioli M, Mols M, Moezelaar R, Ghelardi E, Senesi S, Abee T. 2010. Comparative transcriptomic and phenotypic analysis of the responses of *Bacillus cereus* to various disinfectant treatments. Appl Environ Microbiol 76:3352–3360. https://doi.org/10.1128/AEM.03003-09.
67. Machado I, Coquet L, Jouenne T, Pereira MO. 2013. Proteomic approach to *Pseudomonas aeruginosa* adaptive resistance to benzalkonium chloride. J Proteomics 89:273–279. https://doi.org/10.1016/j.jprot.2013.04.030.
68. Frenzel E, Schmidt S, Niederweis M, Steinhauer K. 2011. Importance of porins for biocide efficacy against *Mycobacterium smegmatis*. Appl Environ Microbiol 77:3068–3073. https://doi.org/10.1128/AEM.02492-10.
69. Jaglic Z, Cervinkova D. 2012. Genetic basis of resistance to quaternary ammonium compounds–the qac genes and their role: a review. Vet Med (Praha) 57:275–281. https://doi.org/10.17221/6013-VETMED.
70. Romanova NA, Wolffs PFG, Brovko LY, Griffiths MW. 2006. Role of efflux pumps in adaptation and resistance of *Listeria monocytogenes* to benzalkonium chloride. Appl Environ Microbiol 72:3498–3503. https://doi.org/10.1128/AEM.72.5.3498-3503.2006.
71. Kovacevic J, Ziegler J, Wałecka-Zacharska E, Reimer A, Kitts DD, Gilmour MW. 2016. Tolerance of *Listeria monocytogenes* to quaternary ammonium sanitizers is mediated by a novel efflux pump encoded by emrE. Appl Environ Microbiol 82:939–953. https://doi.org/10.1128/AEM.03741-15.
72. Edgar R, Bibi E. 1997. MdfA, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. J Bacteriol 179:2274–2280. https://doi.org/10.1128/jb.179.7.2274-2280.1997.
73. Stoitsova SO, Braun Y, Ullrich MS, Weingart H. 2008. Characterization of the RND-type multidrug efflux pump MexAB-OprM of the plant pathogen *Pseudomonas syringae*. Appl Environ Microbiol 74:3387–3393. https://doi.org/10.1128/AEM.02866-07.
74. He G-X, Kuroda T, Mima T, Morita Y, Mizushima T, Tsuchiya T. 2004. An H^+-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. J Bacteriol 186:262–265. https://doi.org/10.1128/JB.186.1.262-265.2004.
75. Müller A, Rychli K, Muhterem-Uyar M, Zaiser A, Stessl B, Guinane CM, Cotter PD, Wagner M, Schmitz-Esser S. 2013. Tn6188–a novel transposon in *Listeria monocytogenes* responsible for tolerance to benzalkonium chloride. PLoS One 8:e76835. https://doi.org/10.1371/journal.pone.0076835.
76. Gilmour MW, Graham M, Van Domselaar G, Tyler S, Kent H, Trout-Yakel KM, Larios O, Allen V, Lee B, Nadon C. 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a

large foodborne outbreak. BMC Genomics 11:120. https://doi.org/10.1186/1471-2164-11-120.
77. Katharios-Lanwermeyer S, Rakic-Martinez M, Elhanafi D, Ratani S, Tiedje JM, Kathariou S. 2012. Coselection of cadmium and benzalkonium chloride resistance in conjugative transfers from nonpathogenic *Listeria* spp. to other listeriae. Appl Environ Microbiol 78:7549–7556. https://doi.org/10.1128/AEM.02245-12.
78. Giaouris E, Chorianopoulos N, Doulgeraki A, Nychas G-J. 2013. Co-culture with *Listeria monocytogenes* within a dual-species biofilm community strongly increases resistance of P*seudomonas putida* to benzalkonium chloride. PLoS One 8:e77276. https://doi.org/10.1371/journal.pone.0077276.
79. Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34–39. https://doi.org/10.1016/S0966-842X(00)01913-2.
80. Corcoran M, Morris D, De Lappe N, O'Connor J, Lalor P, Dockery P, Cormican M. 2014. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilms from food contact surface materials. Appl Environ Microbiol 80:1507–1514. https://doi.org/10.1128/AEM.03109-13.
81. Ibusquiza PS, Herrera JJ, Vázquez-Sánchez D, Cabo ML. 2012. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*. Food Control 25:202–210. https://doi.org/10.1016/j.foodcont.2011.10.002.
82. Pagedar A, Singh J, Batish VK. 2012. Adaptation to benzalkonium chloride and ciprofloxacin affects biofilm formation potential, efflux pump and haemolysin activity of Escherichia coli of dairy origin. J Dairy Res 79:383–389. https://doi.org/10.1017/S0022029912000295.
83. Houari A, Di Martino P. 2007. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. Lett Appl Microbiol 45:652–656. https://doi.org/10.1111/j.1472-765X.2007.02249.x.
84. Machado I, Lopes SP, Sousa AM, Pereira MO. 2012. Adaptive response of single and binary *Pseudomonas aeruginosa* and *Escherichia coli* biofilms to benzalkonium chloride. J Basic Microbiol 52:43–52. https://doi.org/10.1002/jobm.201100137.
85. Zhang C, Tezel U, Li K, Liu D, Ren R, Du J, Pavlostathis SG. 2011. Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. Water Res 45:1238–1246. https://doi.org/10.1016/j.watres.2010.09.037.
86. Oh S, Kurt Z, Tsementzi D, Weigand MR, Kim M, Hatt JK, Tandukar M, Pavlostathis SG, Spain JC, Konstantinidis KT. 2014. Microbial community degradation of widely used quaternary ammonium disinfectants. Appl Environ Microbiol 80:5892–5900. https://doi.org/10.1128/AEM.01255-14.
87. Tezel U, Pavlostathis SG. 2009. Transformation of benzalkonium chloride under nitrate reducing conditions. Environ Sci Technol 43:1342–1348. https://doi.org/10.1021/es802177f.
88. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2019. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0. European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf.
89. Langsrud S, Sundheim G, Holck AL. 2004. Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol 96:201–208. https://doi.org/10.1046/j.1365-2672.2003.02140.x.
90. Dean CR, Visalli MA, Projan SJ, Sum P-E, Bradford PA. 2003. Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. Antimicrob Agents Chemother 47:972–978. https://doi.org/10.1128/AAC.47.3.972-978.2003.
91. Sidhu MS, Heir E, Leegaard T, Wiger K, Holck A. 2002. Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci. Antimicrob Agents Chemother 46:2797–2803. https://doi.org/10.1128/AAC.46.9.2797-2803.2002.
92. Liu WJ, Fu L, Huang M, Zhang JP, Wu Y, Zhou YS, Zeng J, Wang GX. 2017. Frequency of antiseptic resistance genes and reduced susceptibility to biocides in carbapenem-resistant *Acinetobacter baumannii*. J Med Microbiol 66:13–17. https://doi.org/10.1099/jmm.0.000403.
93. Lambert RJ, Joynson J, Forbes B. 2001. The relationships and susceptibilities of some industrial, laboratory and clinical isolates of *Pseudomonas aeruginosa* to some antibiotics and biocides. J Appl Microbiol 91:972–984. https://doi.org/10.1046/j.1365-2672.2001.01460.x.
94. Ortiz S, López P, López V, Martínez-Suárez JV. 2014. Antibiotic susceptibility in benzalkonium chloride-resistant and -susceptible *Listeria monocytogenes* strains. Foodborne Pathog Dis 11:517–519. https://doi.org/10.1089/fpd.2013.1724.
95. Loughlin MF, Jones MV, Lambert PA. 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrob Chemother 49:631–639. https://doi.org/10.1093/jac/49.4.631.
96. Clinical and Laboratory Standards Institute (CLSI). 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
97. Centers for Disease Control and Prevention (CDC). 2018. Show me the science - how to wash your hands. Centers for Disease Control and Prevention, Atlanta, GA. https://www.cdc.gov/handwashing/show-me-the-science-handwashing.html.
98. Food and Drug Administration (FDA). 2016. Antibacterial soap? You can skip it, use plain soap and water. U.S. Food and Drug Administration, Silver Spring, MD. https://www.fda.gov/consumers/consumer-updates/antibacterial-soap-you-can-skip-it-use-plain-soap-and-water.
99. Shreiner AB, Kao JY, Young VB. 2015. The gut microbiome in health and in disease. Curr Opin Gastroenterol 31:69–75. https://doi.org/10.1097/MOG.0000000000000139.
100. Byrd AL, Belkaid Y, Segre JA. 2018. The human skin microbiome. Nat Rev Microbiol 16:143. https://doi.org/10.1038/nrmicro.2017.157.
101. Carbajo JB, Perdigón-Melón JA, Petre AL, Rosal R, Letón P, García-Calvo E. 2015. Personal care product preservatives: risk assessment and mixture toxicities with an industrial wastewater. Water Res 72:174–185. https://doi.org/10.1016/j.watres.2014.12.040.
102. Ferrer I, Furlong ET. 2001. Identification of alkyl dimethylbenzylammonium surfactants in water samples by solid-phase extraction followed by ion trap LC/MS and LC/MS/MS. Environ Sci Technol 35:2583–2588. https://doi.org/10.1021/es001742v.
103. Ruan T, Song S, Wang T, Liu R, Lin Y, Jiang G. 2014. Identification and composition of emerging quaternary ammonium compounds in municipal sewage sludge in China. Environ Sci Technol 48:4289–4297. https://doi.org/10.1021/es4050314.
104. EPA. 1976. Zephiran chloride reg. no. 944·15·IA. U.S. Environmental Protection Agency, Washington, DC.
105. European Commission. 2009. Regulation (EC) no. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. European Commission, Brussels, Belgium. https://ec.europa.eu/health/sites/health/files/endocrine_disruptors/docs/cosmetic_1223_2009_regulation_en.pdf.
106. European Commission. 2008. Annex 1. Summary description of product types 1–23. European Commission, Brussels, Belgium. http://ec.europa.eu/environment/archives/ppps/pdf/annex1.pdf.
107. Dutta V, Elhanafi D, Kathariou S. 2013. Conservation and distribution of the benzalkonium chloride resistance cassette bcrABC in *Listeria monocytogenes*. Appl Environ Microbiol 79:6067–6074. https://doi.org/10.1128/AEM.01751-13.
108. Worthing KA, Marcus A, Abraham S, Trott DJ, Norris JM. 2018. Qac genes and biocide tolerance in clinical veterinary methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* and *Staphylococcus pseudintermedius*. Vet Microbiol 216:153–158. https://doi.org/10.1016/j.vetmic.2018.02.004.