Cetylpyridinium Chloride: Mechanism of Action, Antimicrobial Efficacy in Biofilms, and Potential Risks of Resistance

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ABSTRACT Antimicrobial resistance is a serious issue for public health care all over the world. While resistance toward antibiotics has attracted strong interest among researchers and the general public over the last 2 decades, the directly related problem of resistance toward antiseptics and biocides has been somewhat left untended. In the field of dentistry, antiseptics are routinely used in professional care, but they are also included in lots of oral care products such as mouthwashes or dentifrices, which are easily available for consumers over-the-counter. Despite this fact, there is little awareness among the dental community about potential risks of the widespread, unreflected, and potentially even needless use of antiseptics in oral care. Cetylpyridinium chloride (CPC), a quaternary ammonium compound, which was first described in 1939, is one of the most commonly used antiseptics in oral care products and included in a wide range of over-the-counter products such as mouthwashes and dentifrices. The aim of the present review is to summarize the current literature on CPC, particularly focusing on its mechanism of action, its antimicrobial efficacy toward biofilms, and on potential risks of resistance toward this antiseptic as well as underlying mechanisms. Furthermore, this work aims to raise awareness among the dental community about the risk of resistance toward antiseptics in general.

KEYWORDS CPC, adaptation, antiseptic, biocide, cetylpyridinium chloride, oral, resistance

The World Health Organization postulates a postantibiotic era “in which common infections could once again kill” unless an immediate effort is made to prevent the pervasion of antimicrobial resistance (1). To be precise, the 2016 Review on Antimicrobial Resistance predicts a worrying scenario in which the annual global death toll from antimicrobial resistance will rise from 700,000 today to 10 million by 2050 (2).

Antimicrobial resistance is a growing issue worldwide, but regrettably, it is unlikely that many new classes of antibiotics will be developed in the near future (1). This is a severe burden on health care, the economy, and the food industry (1). The search for new antimicrobial therapies like cold atmospheric plasma or antimicrobial photodynamic therapy has since increased in its importance (3–6).

Contradictory findings describing a correlation between antibiotic resistance and biocide resistance seem to attract little public interest (7). Increasing evidence exists that long-term use of antiseptics may result in increased MICs and resistance *in vivo* due to the exposure to sublethal concentrations that has arisen over the last century (8–12). However, there seems to be awareness and action from government bodies. A prominent example is the ban of triclosan from household washing products by the Federal

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FIG 1 Chemical structure of CPC. Atom colors: gray, carbon; white, hydrogen; blue, nitrogen; green, chlorine (generated by MolView v2.4; molview.org).

Drug Administration (FDA) in 2016 due to the discovery of resistance development to this biocide and cross-resistance to antibiotics in anaerobic digesters (13).

Recently, we have summarized the evidence on the potential risk of resistance to the oral gold-standard antiseptic chlorhexidine (CHX) in oral bacteria (14). Russell et al. mentioned that frequent use of the quaternary ammonium compound cetylpyridinium chloride (CPC) could likewise result in bacterial drug resistance (15).

Besides other medical fields, CPC is also frequently used in dental practice and is also included in a wide range of consumer products like mouthwashes and dentifrices (16, 17). Therefore, the aim of this study was to review the mechanism of action and the antimicrobial efficacy of CPC toward biofilms as well as to summarize the evidence for the risk of resistance toward CPC with a special focus on the oral cavity. As our previous work (14), the present review further aims to raise awareness among the dental community that wide and unconscious use of antiseptics may lead to drug resistance and, potentially, to concomitant cross-resistance toward antibiotics.

History, chemistry, and fields of application. Cetylpyridinium chloride (CPC; IUPAC name, 1-hexadecylpyridinium chloride) is a monocationic quaternary ammonium compound (QAC) which consists of quaternary nitrogen connected with one or more hydrophobic side chains (Fig. 1) (18). The antimicrobial activity of QACs correlates with hydrophobicity of the side chain and shows a maximum effect if the alkyl chain contains 12 to 16 carbon atoms (19). Gilbert and Moore further specified that maximum antimicrobial effect can be achieved with alkyl chain lengths of 12 to 14 carbon atoms in Gram-positive and 14 to 16 carbon atoms in Gram-negative bacteria (19).

CPC appears as a beige-colored salt and shows good solubility in water (20). It is assembled by a positively charged pyridine as a hydrophilic headgroup in combination with a hexadecane chain as a lipophilic side chain (21). Due to this molecular structure, CPC is characterized as an amphoteric surfactant (18). Depending on the respective manufacturer, the hexadecane side chain is often derived from different natural oils, which can result in variations concerning alkyl chain length and saturation (18).

QACs have been used since the 1930s and are widely used for the disinfection of skin, mucous membranes, hard surface cleaning, deodorization, and cosmetic formulations today (20–22). The antimicrobial activity of CPC was first described in a set of studies by the laboratories of the Wm. S. Merrell Company in Cincinnati, Ohio, in 1939 (22). C. Lee Huyck was the first to demonstrate bacteriostatic or bactericidal effects of CPC to bacteria in the oral cavity by measuring the pH drop in saliva after chewing sweetened gum (23). In today's clinical dental practice, CPC is mainly used as an antimicrobial ingredient in over-the-counter products such as mouthwashes and dentifrices, which are marketed for reducing plaque accumulation and gingival inflammation (16, 17, 24). Furthermore, the combination of CPC with other antiseptics like CHX or the mixture of multiple QACs with various side chain lengths has been proposed in

recent years for a potential increase of antimicrobial activity when applied in mouthwashes (18, 25).

Mechanism of action. The bacterial membrane carries a natural negative charge due to its composition of lipoteichoic acid (LTA; Gram-positive) or lipopolysaccharides (LPS; Gram-negative), respectively, and the phospholipids of the lipid bilayer membrane itself, neutralized by counterions like Mg^{2+} and Ca^{2+} . This poses a possible point of interaction of the positively charged QACs with the bacteria by initially substituting these ions—in the case of CPC—with a positively charged pyridine ion. The hexadecane tail integrates into the lipid membrane and disorganizes it (18, 21). At low concentrations, CPC affects the cell by interfering with its osmoregulation and its homeostasis, measurably proven by K^+ and pentose leakage in *Saccharomyces cerevisiae*, which might initiate autolysis by activation of intracellular latent ribonucleases (18, 21, 26). At high concentrations, CPC leads to disintegration of the membranes with subsequent leakage of cytoplasmic contents (Fig. 2) (18). Damage of proteins and nucleic acids as well as cell wall lysis by autolytic enzymes are the consequences (21). In a previous study, we found vesicle-like structures on bacterial cell surfaces after treatment with CPC that may be indicative of membrane damage when visualizing bacteria in polymicrobial biofilms comprising *Streptococcus mutans*, *Actinomyces naeslundii*, and *Actinomyces odontolyticus* by means of scanning electron microscopy (Fig. 3) (27). In contrast to Gram-positive bacteria with their rather simple composition of the cell wall, the more complex cell wall composition of Gram-negative bacteria with an outer membrane and a periplasm usually represents a hindrance to penetration of compounds with molecular weight higher than 600 Da (26). Since the molecular mass of CPC is 339 Da, it is also active against Gram-negative bacteria. Additionally, QACs in general improve their antimicrobial efficacy in Gram-negative bacteria by self-enhancing their influx rate through the damaged cell wall (21). Thereby, susceptibility to CPC is independent of the amount of CPC bound by bacteria, as shown already in 1975 for *Escherichia coli* (28). The surfactant properties of QACs like CPC further enhance their efficacy at a microbiological level, as they can cover irregular surfaces evenly (21, 22).

Antimicrobial efficacy in biofilms. The antimicrobial efficacy of CPC has been investigated in numerous *in vitro* studies. While the vast majority of these studies have been conducted on planktonic, i.e., free-floating, microorganisms, bacteria embedded in biofilms exhibit utterly distinct properties compared to their planktonic counterparts, e.g., an up to 50- to 1,000-fold higher tolerance toward antimicrobial agents (29). For instance, when screening 80 oral streptococcal isolates for MICs measured in planktonic cultures and minimum biofilm inhibitory concentrations (MBICs) toward CPC, the researchers found median MICs of 0.12 or 0.24 $\mu\text{g/ml}$, while they found median MBICs of 7.81 to 15.63 $\mu\text{g/ml}$, depending on the respective species (30). The following section summarizes only studies on the antimicrobial efficacy of CPC toward biofilms.

Luppens et al. cultured single-species biofilms of *S. mutans* and *Veillonella parvula* and dual-species biofilms of both bacteria in 96-well polystyrene microtiter plates for 48 h. Biofilms were treated with 0.2 mmol/liter (0.0068%) CPC for 5 min. Treatment with CPC led to higher killing efficacy toward *S. mutans* when grown in single-species biofilms ($\geq 2 \log_{10}$ steps) than dual-species biofilms ($\geq 1 \log_{10}$ steps). Therefore, it was concluded that *S. mutans* showed decreased susceptibility to CPC when grown in biofilms with *V. parvula* (31).

Smith et al. cultured biofilms from 10 oral and 18 bloodstream isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in 96-peg plates for 48 h and investigated the antimicrobial efficacy of over-the-counter mouthwashes after treatment of 0.5, 1, or 2 min by employing a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. Mouthwashes containing CPC reduced bacterial viability by not more than 60%, and it was concluded that these products are ineffective at eradicating MRSA biofilms (32).

FIG 2 Schematic depicting the mechanism of action of CPC toward bacterial membranes. (A) The bacterial cytoplasmic membrane, composed of proteins embedded into a phospholipid bilayer, carries a negative charge neutralized by Ca^{2+} . This hydrophobic environment is vital for an unimpeded protein function. (B) CPC substitutes the Ca^{2+} ions with its pyridine and integrates its hexadecane tail into the phospholipid bilayer. (C) The membrane starts to derange, and hydrophilic domains develop. (D, E) Decreased fluidity of the membrane induces growth of the hydrophilic vacancies and impaired protein function. (F) Finally, CPC induces cell lysis and solubilization of the phospholipid bilayer and proteins into CPC-phospholipid micelles. This schematic was adapted from reference 18.

FIG 3 Scanning electron microscopic (SEM) visualization of an *in vitro* polymicrobial biofilm comprising *A. naeslundii*, *A. odontolyticus*, and *S. mutans* (culture conditions and SEM specifications described in detail in reference 27) following treatment with 0.1% CPC for 10 min. (A) Magnification, $\times 12,000$. (B) Magnification, $\times 24,000$. (C) Magnification, $\times 50,000$. Vesicle-like structures indicate membrane-disruptive effects due to CPC.

Latimer et al. formed saliva-derived biofilms in a hydroxyapatite disc biofilm reactor and treated them with mouthwash formulations with or without 0.075% CPC. A one-time treatment of 24-h biofilms led to higher proportions of red fluorescence among the biofilms treated with CPC as visualized by live/dead staining and confocal microscopy. Multiple treatments of the biofilms twice daily for 4 days were assessed by differential CFU counting and showed slight reductions of CFU by $<1 \log_{10}$ step compared to the CPC-free control. However, when the hydroxyapatite discs were pretreated by soaking in the mouthwash formulations, biofilm formation was inhibited by $>3 \log_{10}$ steps compared to the control without CPC (33).

In our previous research, we cultured *S. mutans* biofilms for 24 or 72 h and polymicrobial biofilms comprising *S. mutans*, *A. naeslundii*, and *A. odontolyticus* for 72 h and treated them with CPC. Treatment with 0.05% CPC for 10 min showed a pronounced antimicrobial efficacy reducing CFU by $\geq 5 \log_{10}$ in 24-h initial *S. mutans* biofilms, while 0.1% CPC reduced CFU of 72-h mature *S. mutans* biofilms by 3 $\log_{10}$ after treatment of 1 min, by 2.6 $\log_{10}$ after 3 min and, by $\geq 5 \log_{10}$ after 10 min. In polymicrobial biofilms, 0.1% CPC reduced CFU of *S. mutans* by 4 $\log_{10}$, of *A. naeslundii* by 6 $\log_{10}$, and of *A. odontolyticus* by 5.2 $\log_{10}$ after 10 min exposure (27).

Given the different antimicrobial efficacy rates found in the studies described above, it must be considered that the respective biofilm culture protocols (e.g., in terms of culture periods) as well as the respective CPC treatment modalities (concentrations, treatment periods) vastly differed among the studies. In general, it seems rational that the biofilm matrix, the so-called extracellular polymeric substances (EPS), may retard penetration of CPC during its diffusion throughout the biofilm structure (34, 35). Accordingly, Xiang et al. found that treatment with a mouthwash containing 0.074% CPC had only effects on the outer and middle layers of biofilms formed for 48 h on plaque accumulators *in situ*, as shown by live/dead staining and confocal microscopy (36). This retarded penetration may either be attributed to reactions or sorption of CPC with matrix components, e.g., by interaction of the positively charged CPC with negatively charged EPS residues (37) or by hydrophobic interactions involving alkyl chains (38).

Furthermore, specific EPS components like poly-*N*-acetylglucosamine (PNAG) may play important roles in biofilm tolerance toward biocides like CPC. Accordingly, two studies have shown increased antimicrobial efficacy rates of CPC after pretreatment with dispersin B (DspB), an enzyme capable of hydrolyzing PNAG (39, 40). Izano et al. cultured *Aggregatibacter actinomycetemcomitans* biofilms in polystyrene tubes for 24 h and treated them with 0.02% CPC for 5 min with or without a 30-min pretreatment with 20 $\mu\text{g}/\text{ml}$ DspB. While biofilms treated with DspB or CPC alone exhibited little or no reduction of CFU, biofilms that were pretreated with DspB showed a 3- $\log_{10}$ -step

decrease in the number of CFU compared to biofilms treated with DspB or CPC alone. Therefore, the authors concluded that the degradation of PNAG with DspB made *A. actinomycetemcomitans* biofilm bacteria more susceptible to CPC (39). Ganeshnarayan et al. cultured *Staphylococcus epidermidis* and *Actinobacillus pleuropneumoniae* biofilms in Microcon centrifugal filter devices for 24 h. Then, they measured the volumetric flow rate of liquids and solute transport through the biofilms. Perfusion of biofilms with 0.03% CPC resulted in no detectable CPC in the flowthrough volume. However, when biofilms were cultured in the presence of 20 μ g/ml DspB or when biofilms were perfused with 20 μ g/ml DspB before the CPC perfusion, CPC could be detected in the flowthrough volume. Therefore, PNAG may specifically impede the penetration of CPC throughout the biofilms (40).

The protective effects provided by the EPS toward treatment with antimicrobials like CPC may also be overcome by adjunct mechanical stress factors. For instance, Fabbri et al. cultured *S. mutans* biofilms on glass microscope slides for 72 h and treated them with 0.085% CPC or 0.2% CHX by static immersion or by using a Philips Sonicare AirFloss to generate high-velocity water microsprays. By means of live/dead staining and confocal laser scanning microscopic visualization, they found that the bacterial killing depth of a static 30-s immersion with CPC was approximately 20% compared to about 5% for CHX, which both may be attributed to sticky glucans in the *S. mutans* biofilm matrix. The bacterial killing depth could be increased to about 80% for both antiseptics by using the high-velocity water microsprays (41).

Evidence for resistance toward CPC and concomitant cross-resistances. In order to approach the subject of antiseptic resistance adequately, it is crucial to clearly distinguish between the definitions of antimicrobial resistance, tolerance, and susceptibility. Antimicrobial resistance (AMR) can, in general, be subdivided into three different categories. Multidrug resistance (MDR) is defined as nonsusceptibility to one antimicrobial from three or more antimicrobial classes, whereas extensive drug resistance (XDR) is the nonsusceptibility to one or more agents from all classes with the exception of one or two classes. Finally, pan-drug resistance (PDR) means nonsusceptibility to all classes of antimicrobials and agents (42). Resistance usually originates either from the natural and inherent characteristics of the respective microorganism or is genetically acquired via mutation or horizontal gene transfer (21, 43). On the contrary, tolerance is the ability of microorganisms to withstand high concentrations of an antimicrobial due to a decrease in metabolic activity (43, 44).

Although clear frameworks exist for determining resistance toward antibiotics, the term of biocide or antiseptic resistance still seems to cause some confusion (43). For antibiotics, susceptibility and resistance are separated by a breakpoint defined by parameters like the MIC (43). On the contrary, such breakpoint MICs do not exist for antiseptics and biocides, wherefore biocide resistance is usually defined as a measurable increase of the MIC by a factor of 4 to 16 upon repeated exposure (i.e., adaptation) (43). Adaptation of microorganisms to given antiseptics or biocides is usually investigated *in vitro* by the broth microdilution method where MICs are determined and bacterial cultures from the so-called sub-MIC populations are recultured for further MIC evaluations (Fig. 4). This procedure is usually repeated at least 10 times. Afterward, the MIC measured in the 10th passage can be compared to the MIC from the first passage. In case of an increase by a factor of at least 4, this can be defined as clinically relevant adaptation (43). If this MIC increase is also stable after a few passages of culture without selection pressure (i.e., without the antiseptic or biocide), the respective isolate may be defined as “resistant” (43) or as showing “decreased susceptibility” (45). However, it must be kept in mind that the clinical in-use concentrations of antiseptics usually are much higher than the measured MICs in that 10th passage (46, 47). However, as it is well-known that the EPS limit and retard the penetration of antiseptics throughout the biofilm structure (37), it seems reasonable that bacteria in deeper strata of biofilms will be exposed to antiseptic concentrations in the range of these MICs (14). Consequently, these rather low MICs may definitely still have some clinical relevance, and investigation

FIG 4 Schematic illustration of the broth microdilution method for investigating phenotypic adaptation of bacteria upon repeated exposure to antiseptics like CPC. (A) Forty-eight-well plates with planktonic bacterial cultures in nutrient broth or in serial 2-fold dilutions of antiseptic in nutrient broth, respectively. (B) After incubation for at least 24 h, turbidity as a measure of growth is examined, and MICs are recorded. Bacteria from the sub-MIC well are used for inoculating another passage of MIC determination (see panel A). This whole procedure is repeated for at least 10 passages.

of MICs upon repeated exposure of bacteria to subinhibitory concentrations may be a worthwhile tool for studying resistance mechanisms *in vitro* (14, 46). Mechanisms inducing resistance to antiseptics and biocides, as shown phenotypically by MIC increases, may also lead to cross-adaptation or cross-resistance toward other antimicrobials (48), which was already found in several studies for *Klebsiella* spp., *Proteus* spp., and *Staphylococcus* spp. (11, 21, 49). The following paragraphs shall summarize studies investigating the potential emergence of CPC resistance and concomitant cross-resistance toward other antimicrobials.

In 1996, Irizarry et al. measured susceptibilities of 120 *S. aureus* isolates toward CPC using the agar dilution technique. The concentrations of CPC used in this study were 1, 2, 2.5, 5, and 10 $\mu\text{g/ml}$. The MICs for CPC were 10 $\mu\text{g/ml}$ in MRSA strains and 2 $\mu\text{g/ml}$ in the methicillin-sensitive *S. aureus* (MSSA) strains (50). Heir et al. found one QAC-resistant strain ST2H6 from a poultry processing plant in Norway in 1998. They evaluated it by comparative 16S-rRNA gene sequencing and identified it as *Staphylococcus saprophyticus*. They concluded that excessive use of QACs will induce selective pressure that, in turn, will lead to the development of QAC-resistant microbes (51). Likewise, Suller et al. compared the susceptibilities of MRSA and MSSA strains to CPC.

FIG 5 Phenotypic adaptation. (A) *P. stutzeri* (strains: ●, NCIMB 568; Δ, NCIMB 10783; ■, NCIMB 11358; ◆, NCIMB 11359; ●, JM302; ■, JM375). (B) *P. aeruginosa* toward CPC. MICs of CPC were investigated for all strains, and bacteria from the sub-MIC populations were subcultured. Suchlike stepwise serial subcultures were made in increasing concentrations of CPC for a period of 6 weeks (for more details, please see reference 53). This figure is reprinted from reference 53 with kind permission from the publisher.

The authors used the stepwise broth microdilution method and repeated exposure and recovery of survivors to develop potential adaptation in those two strains. They found that MRSA showed “low-level resistance” to CPC, with MICs of 2 or 4 $\mu\text{g/ml}$ compared to 1 $\mu\text{g/ml}$ for the MSSA strain. At the same time, they found that this adaptation was unstable (52).

Tattawasart et al. evaluated adaptation of *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* toward CPC upon serial repeated exposure for 6 weeks. For strains of *P. stutzeri*, MICs of CPC increased 24- to 60-fold to final concentrations of 150 to 400 $\mu\text{g/ml}$ (Fig. 5A). MICs of CPC for *P. aeruginosa* increased 8-fold from 250 to 2,000 $\mu\text{g/ml}$ (Fig. 5B). These MICs are in the same range or even higher than CPC in-use concentrations, which typically are around 0.05% (i.e., 500 $\mu\text{g/ml}$). CPC resistance in *P. stutzeri* was retained after 10 passages culture without CPC biocide but was partially lost after cultivation for 15 passages in biocide-free medium. Two- to 10-fold MIC increases to triclosan and CHX diacetate were found in CPC-adapted isolates of *P. stutzeri* (53).

Mavri and Smole Možina determined MICs of CPC according to the broth microdilution method. *Campylobacter jejuni* and *Campylobacter coli* strains were cultured with CPC for 15 passages, and 20% of those strains showed phenotypic adaptation to CPC after repeated exposure. The MICs of *C. jejuni* (NCTC 11168) increased from 2 $\mu\text{g/ml}$ to 4 to 8 $\mu\text{g/ml}$. They further found that adaption in *C. jejuni* and *C. coli* toward CPC was retained for up to 10 passages in biocide-free nutrient broth. CPC-adapted *C. jejuni* and *C. coli* also showed cross-resistance toward erythromycin (54).

Zhang et al. investigated the susceptibility of 255 *E. coli* isolates from retail meats toward CPC and benzalkonium chloride. They determined the MICs to CPC using the agar dilution method and found MICs ranging from 8 to 512 $\mu\text{g/ml}$, while the MIC of the *E. coli* type strain (ATCC 10536) was 16 $\mu\text{g/ml}$. The *E. coli* type strain showed higher susceptibility to CPC than 67.5% of the *E. coli* isolates. One hundred seventy-five out of

225 benzalkonium chloride-resistant strains also showed cross-adaptation to CPC at the same time. The authors concluded that there may be cross-resistance between QACs in *E. coli* (55). Yang et al. evaluated the susceptibility of 111 *Salmonella* isolates from egg production chains compared to an *E. coli* type strain (ATCC 10536) toward CPC. The MICs of CPC against those *Salmonella* strains ranged from 8 to 256 $\mu\text{g/ml}$, wherefore the authors concluded that *Salmonella* isolates had resistances to CPC compared to the *E. coli* type strain (56). Wu et al. evaluated agar dilution and broth microdilution methods to determine the susceptibility of foodborne and zoonotic isolates (*Salmonella* spp., *E. coli*, *K. pneumoniae*, and *S. aureus*) toward different QACs. For CPC, there was a 94.55% agreement between MICs obtained by agar dilution and broth microdilution methods. The MICs for *Salmonella* spp., *E. coli*, and *S. aureus* were 256, 128, and 256 mg/liter in both agar dilution and broth microdilution, while the MIC of *K. pneumoniae* was 512 mg/liter in agar dilution and 256 mg/liter in broth microdilution. All in all, *K. pneumoniae* and *Salmonella* spp. were the least susceptible isolates toward CPC (57).

Humayoun et al. determined the susceptibility of *Salmonella* isolates to commercial and household biocides by the microdilution method. One *Salmonella Heidelberg* isolate from turkey carcass was not inhibited by 160 $\mu\text{g/ml}$ CPC and was considered to be resistant to CPC (58). Resistance to CPC of 510 *E. coli* isolates from retail chicken was investigated by Sun et al. using the agar dilution method. The MICs of CPC to these isolates ranged from 32 $\mu\text{g/ml}$ to 256 $\mu\text{g/ml}$, while the MIC of the *E. coli* type strain was 64 $\mu\text{g/ml}$. Thirty percent of the *E. coli* isolates showed higher MICs toward CPC than the type strain (59).

Considering oral bacteria, Kitagawa et al. measured the MICs of CPC, CHX, and 12-methacryloyloxydodecylpyridinium bromide (MDPB) against *S. mutans* and *Enterococcus faecalis* by modified microdilution methods after repeated exposure. The MICs of CPC against both *E. faecalis* and *S. mutans* did not increase during 10 passages of CPC challenge (60). Likewise, Verspecht et al. investigated adaptation as well as cross-adaptation of oral bacteria (*Prevotella intermedia*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *S. mutans*, *Streptococcus sobrinus*, and *A. actinomycetemcomitans*) upon repeated exposure to CHX or CPC (61). *S. sobrinus* increased its MIC for CPC almost 6-fold after exposure to CPC for 10 serial passages, while *P. intermedia* increased its MICs for CPC 4-fold. *S. mutans* increased its MICs for CPC approximately 2.5-fold (61), while *S. mutans* exhibited no MIC increase in the study by Kitagawa et al. (60). Adaptation toward CPC was partially stable in most of the exposed strains after regrowth in the absence of CPC for 10 passages. Interestingly, one obvious increase in MIC was found in CPC-adapted *P. gingivalis* after regrowth in the absence of CPC. Furthermore, there was a 1.2-, 3.9-, or 2.1-fold increase in CHX-MICs for CPC-adapted *P. gingivalis*, *P. intermedia*, and *S. sobrinus*, respectively. Also, 1.7-, 1.6-, 3.7-, and 3-fold increases in CPC-MICs were found for CHX-adapted *F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *S. sobrinus*, respectively (61).

Several studies reported cross-resistance in QAC-adapted bacteria. For instance, QAC-adapted *Pseudomonas fluorescens* showed resistance to several antibacterial agents, such as cocoamine acetate, benzalkonium chloride, and an amphoteric tenside after 5 min exposure (62). Back in 1994, Leelaporn et al. tested 164 clinical isolates of coagulase-negative staphylococci to investigate the occurrence of resistance to antiseptics. They found 64 of them resistant to QACs, and they performed isolation of plasmid DNA, digestion with restriction endonucleases, agarose gel electrophoresis, and DNA-DNA hybridization analysis. The authors finally found that reduced susceptibility to CPC is encoded by the same MDR plasmids that induce resistance to penicillins and aminoglycosides (63). Cadena et al. challenged *S. Heidelberg* with CPC at a concentration of 62.5 ppm for 8 s and evaluated changes in gene expression by RNA sequencing. Among the 90 genes associated with virulence, pathogenicity, and resistance (VPR) in wild-type *S. Heidelberg*, 10.0% (9 of 90 genes) or 23.3% (21 of 90 genes) of VPR genes were upregulated after exposure to CPC in 2014 and 1992 serovar *S. Heidelberg* field strains. They concluded that genes which can make multiple antibiotic-

resistant proteins and multidrug-resistant proteins were mainly upregulated in CPC-treated *S. Heidelberg* (64).

Mechanisms conveying resistance toward CPC: cell surface alterations and efflux pumps. It seems consequential that phenotypic adaption or resistance toward membrane-disrupting agents such as CPC may be caused by changes in membrane properties, such as thickness, structure, and permeability (53, 65). In the prementioned study by Tattawasart et al., sodium dodecyl sulfate (SDS)-induced lysis was carried out in wild-type and CPC-adapted *P. stutzeri* strains. CPC-adapted strains were less sensitive to the lytic effects of SDS than their respective parental strains. Using the bacterial adherence to hydrocarbon (BATH) method for determining cell surface hydrophobicity of the adapted and wild-type strains, they found that the adapted strains were more hydrophobic than the parental strains (53). In another study of this group, they isolated outer membrane proteins of *P. stutzeri* and analyzed protein profiles by SDS-polyacrylamide gel electrophoresis (PAGE). When *P. stutzeri* acquired resistance to CPC, its outer membrane protein profiles exhibited alterations compared to the CPC-sensitive parental strains (66).

Mavri and Smole Možina showed by transmission electron microscopic analyses that CPC-adapted *C. jejuni* and *C. coli* had a thicker cell envelope than the respective wild-type strains (54). Likewise, García et al. reported significant thicker cell walls in MRSA than in MSSA strains, which they explained by concomitant decreased susceptibility to cell wall synthesis inhibitors, such as vancomycin, which may lead to increased production of wall teichoic acid (67). In the study by Kitagawa et al., cell surface hydrophobicity of CPC-adapted *E. faecalis* was measured by microbial adherence to *n*-hexadecane. After exposure to CPC, the surface hydrophobicity of *E. faecalis* was significantly increased. No changes in *E. faecalis* protein expression profile were found after CPC exposure in this study (60).

In the research done by Verspecht et al. (61), cell surface hydrophobicity measurements were performed by measuring adherence to *n*-hexadecane, and proteomic analysis of antiseptic-adapted strains was performed by means of mass spectrometry. Higher cell surface hydrophobicity was found in all antiseptic-adapted strains than in the wild-type controls. Compared to exposure to CHX, exposure to CPC commonly resulted in higher numbers of upregulated or unique proteins that were involved in bacterial metabolism, membrane transport, cell wall modifications, bacterial virulence, and oxidative stress protection. For example, the gingipain proteins RgpA and Kgp were only found in CHX- and CPC-adapted *P. gingivalis* but not in the wild-type strain (61). These gingipains are proteinases produced by *P. gingivalis* that play a major role in the degradation of host tissues and deregulation of the immune response in periodontal inflammation (68).

Active efflux is another well-established mechanism for reduced susceptibility toward QACs like CPC. Efflux pumps are membrane proteins comprising transmembrane domains that form channels for actively removing substances from the cytoplasm or the membrane (69, 70). In Gram-positive bacteria (particularly staphylococci), plasmid-borne *qac* genes (e.g., *qacA/B*, *qacC/D* [also called *smr*], *qacH*, and *qacJ*) encode Qac efflux proteins that either belong to the major facilitator superfamily (MFS; e.g., QacA/B) or to the small multidrug resistance (SMR) family (e.g., Smr, QacH, and QacJ) and have various cationic antiseptics as their substrates (70–74). Efflux proteins from the SMR family (e.g., QacE, QacEΔ1, QacF, and QacG) and also from the resistance-nodulation-division (RND) superfamily have also been reported in Gram-negative bacteria (71). It is not entirely clear yet whether *qac* genes can directly confer resistance to antibiotics (72), although it has been reported that the *qacC* gene found on a plasmid pSepCH isolated from a heavy metal-resistant *S. epidermidis* strain mediates resistance to β-lactam antibiotics and ethidium bromide in *S. epidermidis* and Gram-negative hosts (75). Likewise, the MFS efflux pump NorA, which is encoded by the chromosomal gene *norA* in *S. aureus*, also confers resistance not only to QACs but also to fluoroquinolones like norfloxacin and ciprofloxacin and to ethidium bromide (76).

Currently, there is still poor understanding of whether transcription of these genes can be affected by antiseptic exposure and, in turn, confer antibiotic resistance (72, 77). In a recent study, LaBreck et al. showed that exposure to the QAC benzalkonium chloride induced a sustained 10-fold increase in *qacA* expression as well as a sustained 2-fold increase in *norA* expression in isogenic strains of *S. aureus* (77). Although suchlike effects have not been studied for CPC so far, it must be kept in mind that genetic determinants conferring resistance to antibiotics and antiseptics are often linked to each other and located on the same plasmids (72). For example, Weigel et al. described a multiresistance conjugative plasmid pLW1043 isolated from a clinical *S. aureus* isolate with high-level vancomycin resistance. This plasmid comprised determinants encoding resistance toward vancomycin (*vanA*), β -lactams antibiotics (*blaZ*), trimethoprim (*dhfrA*), aminoglycosides (*aacA-aphD*), and antiseptics (*qacC*) (78). Since *qac* genes are often located on such multiresistance plasmids with multiple genes conferring resistance to various antibiotics and other antimicrobials (72), low-level exposure to QACs like CPC may foster transfer of these plasmids among bacteria in biofilms and thus, in turn, contribute to spreading the antibiotic resistance genes on these plasmids. For instance, when reporting the spread of a unique community-acquired MRSA USA 300 clone in a Jewish community in Brooklyn, Copin et al. found that acquisition and evolution of the plasmid pBSRC1 carrying genes mediating resistance to topical antimicrobials chlorhexidine (*qacA/B*) and mupirocin (*mupA*) drove expansion of a dominant clone (79). This strongly suggests that acquisition of resistance toward antimicrobials that were used for decolonization therapy were crucial factors for the further spread of this clone (79).

Is there evidence for resistance toward CPC in the oral cavity? As discussed above, CPC is widely used in dental practice and included in lots of over-the-counter products like mouthwashes or dentifrices (16, 17, 24). Therefore, it seems rational to assess evidence for resistance toward CPC in the oral cavity. To the best knowledge of the authors, there is just one study investigating the development of resistance in oral bacteria as well as one on dermal bacteria in dental students, as follows. Radford et al. investigated 129 pharmacy students attending Brighton University whether there were qualitative changes in their oral microbiota after CPC mouthwash was used twice per day for 6 weeks. They found neither colonization of the oral cavity by nonnative microorganisms nor an increase in the number of Gram-negative bacteria (80). Millns et al. examined *Staphylococcus* isolates from the hands of dental students who used CPC-coated gloves. They evaluated the vital capacity of the staphylococcal isolates and the control *S. aureus* type strain (NCTC 6571) after various periods of exposure to CPC at the MICs, which was found to be 0.6 $\mu\text{g}/\text{ml}$ for the control strain. They found that no bacterial strains survived upon CPC exposure for 30 min. Consequently, CPC-coated gloves did not appear to have led to resistance problems in the dermal microbiota on the hands of dental students (81).

Although the findings of these studies indicate no long-term effects on oral or dermal microbiota due to exposure to CPC over given periods of time, published evidence is too scarce in order to conclude that long-term exposure at low concentrations, as may typically occur in deeper layers of biofilms after using a mouthwash or a dentifrice (14), may not have side effects at all. Furthermore, the risk of emergence of CPC-resistant bacteria in the oral cavity has not been examined systematically so far. Given the reports on CPC resistance in nonoral bacteria outlined above, it should be a major goal to investigate whether oral bacteria can also phenotypically adapt toward CPC and what are the underlying molecular mechanisms behind such adaptations. This is even more important, as the oral microbiota has recently been highlighted as a potential reservoir for resistance genes that can be potentially transferred among bacteria via horizontal gene transfer (82–84).

Conclusions. Although CPC is included in a wide range of oral care products that are easily available for consumers as over-the-counter products, there is little awareness among the dental community about potential risks of inducing resistance toward CPC

due to its widespread use. Given the available evidence on potential emergence of phenotypic adaptation or resistance in nonoral bacteria summarized in this review, it should be a future goal to systematically address the topic of resistance toward CPC in oral bacteria in the future and reconsider its unreflected use.

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