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FORBES, Sarah <http://orcid.org/0000-0002-8361-6390>, DOBSON, Curtis B., HUMPHREYS, Gavin J. and MCBAIN, Andrew J.

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Transient and Sustained Bacterial Adaptation following Repeated Sublethal Exposure to Microbicides and a Novel Human Antimicrobial Peptide

Sarah Forbes,a Curtis B. Dobson,b Gavin J. Humphreys,a Andrew J. McBaina

Manchester Pharmacy Schoola and Faculty of Life Sciences,b The University of Manchester, Manchester, United Kingdom

Microbicides (biocides) play an important role in the prevention and treatment of infections. While there is currently little evidence for in-use treatment failures attributable to acquired reductions in microbicide susceptibility, the susceptibility of some bacteria can be reduced by sublethal laboratory exposure to certain agents. In this investigation, a range of environmental bacterial isolates (11 genera, 18 species) were repeatedly exposed to four microbicides (cetrimide, chlorhexidine, polyhexamethylene biguanide [PHMB], and triclosan) and a cationic apolipoprotein E-derived antimicrobial peptide (apoEdpL-W) using a previously validated exposure system.

Susceptibilities (MICs and minimum bactericidal concentrations [MBCs]) were determined before and after 10 passages (P10) in the presence of an antimicrobial and then after a further 10 passages without an antimicrobial to determine the stability of any adaptations. Bacteria exhibiting >4-fold increases in MBCs were further examined for alterations in biofilm-forming ability. Following microbicide exposure, ≥4-fold decreases in susceptibility (MIC or MBC) occurred for cetrimide (5/18 bacteria), apoEdpL-W (7/18), chlorhexidine (8/18), PHMB (8/18), and triclosan (11/18). Of the 34 ≥4-fold increases in the MICs, 15 were fully reversible, 13 were partially reversible, and 6 were nonreversible. Of the 26 ≥4-fold increases in the MBCs, 7 were fully reversible, 14 were partially reversible, and 5 were nonreversible. Significant decreases in biofilm formation in P10 strains occurred for apoEdpL-W (1/18 bacteria), chlorhexidine (1/18), and triclosan (2/18), while significant increases occurred for apoEdpL-W (1/18), triclosan (1/18), and chlorhexidine (2/18). These data indicate that the stability of induced changes in microbicide susceptibility varies but may be sustained for some combinations of a bacterium and a microbicide.

Microbicides have been used for more than a century to control microbial growth in the domiciliary, clinical, and industrial environments (1–4). The modes of action of cationic agents, such as quaternary ammonium compounds (QACs) (e.g., cetrimide) and biguanides (e.g., chlorhexidine and polyhexamethylene biguanide), are believed to rely largely on interactions with the bacterial cell envelope, leading to membrane disruption and the leakage of cytoplasmic components (5). Microbicides may also interact with specific pharmacological targets, such as the enoyl-acyl carrier protein reductase FabI, which is a major target of triclosan (1, 6).

Microbial compounds are used for a range of applications, including clinical antisepsis and disinfection (2, 4) and the control of biofouling and contamination in industry (7, 8) and in food production (9, 10), while in the domestic setting, they have been incorporated as hygienic adjuncts into various products, including hand washes (11) and hard surface disinfectants (12). There is also increasing interest in their incorporation into medical devices such as urinary catheters (2) and surgical dressings (4) with the intention of inhibiting bacterial colonization and biofilm formation (13–16).

Despite the demonstrable benefits of microbicides in some applications (2, 17–20), concerns have been raised that their extensive use may select for bacteria with reduced susceptibility (21–23). This could occur through the selection of target site-adapted mutants (6) or reversibly through induced phenotypic adaptation (24). While bacterial insusceptibility to in-use concentrations of microbicides is apparently uncommon, there is some evidence of bacteria surviving an antimicrobial challenge, for instance, in microbicide-containing solutions (25), leading to product contamination-related outbreaks (26). It is, however, important to note when considering such reports that microbicides exhibit a spectrum of activity and that some microorganisms may be nonsusceptible without prior microbicide exposure (27). It is therefore possible that in some cases sublethal microbicide exposure could result in the clonal expansion of preexisting bacterial populations with comparatively lower intrinsic susceptibilities, rather than the clonal selection of resistance (28).

While induced changes in antimicrobial susceptibility have been reported in laboratory studies through the exposure of bacteria to sublethal concentrations of microbicides (24, 29, 30), there are very few reports in the literature that document the stability of such changes or that compare the selective potential of multiple microbicides against a range of taxonomically diverse bacteria. We have therefore assessed the potential changes in susceptibility for multiple bacteria after repeated exposure to the microbicides cetrimide, chlorhexidine, polyhexamethylene biguanide (PHMB), and triclosan and a novel human apolipoprotein E-derived antimicrobial peptide (apoEdpL-W). Additionally, since previous investigations have indicated that microbicide (30) and antibiotic (31) adaptations may result in alterations in biofilm-forming ability, the influence of sublethal microbicide exposure on bacterial biofilm formation was also evaluated.
MATERIALS AND METHODS

Bacteria. Pseudomonas aeruginosa ATCC 9027, Staphylococcus aureus ATCC 6538, and Serratia marcescens ATCC 13880 were obtained from Oxoid (Basingstoke, United Kingdom). Burkholderia cepacia ATCC BAA-245, Escherichia coli ATCC 25922, and Klebsiella pneumoniae ATCC 13883 were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Micrococcus luteus MRBG 9.25, Staphylococcus caprae MRBG 9.3, Staphylococcus capitis MRBG 9.34, Staphylococcus lugdunensis MRBG 9.36, Staphylococcus warneri MRBG 9.27, Staphylococcus epidermidis MRBG 9.33, and Staphylococcus haemolyticus MRBG 9.35 were previously isolated from the axillae of three healthy male volunteers (23). Bacillus cereus MRBG 4.21, Stenotrophomonas malophilia MRBG 4.17, and Chryseobacterium indolgenes MRBG 4.29 were isolated from a domestic kitchen drain biofilm (28). Enterococcus faecalis WIBG 1.1 and Corynebacterium xerosis WIBG 1.2 were wild-type wound isolates provided by Angela Oates, The University of Manchester.

Chemical reagents and bacterial growth media. Tricosan, cetrimide, and chlorhexidine were purchased from Sigma-Aldrich (Dorset, United Kingdom). Vantocil (a 20% aqeous solution of PHMB) was obtained from Arch Chemicals, Inc. (Manchester, United Kingdom). Peptides were purchased from Alta Bioscience (West Midlands, United Kingdom), having been synthesized using 9-fluorenylmethyl carbamate chemistry and purified by high-performance liquid chromatography. Bacteriological media were purchased from Oxoid. All other chemical reagents were purchased from Sigma-Aldrich unless otherwise stated. Bacterial growth media were sterilized by filtration (0.22 μM), and all microbicide stock solutions were prepared at 5 times the highest test concentration in water and filter sterilized (0.22 μM). All microbicide stock solutions were prepared at 5 times the highest test concentration in water and filter sterilized (0.22 μM). The plates were incubated at 37°C (24 h) with agitation (100 rpm). The MIC was defined as the lowest concentration for which bacterial growth did not occur. Growth was viewed as turbidity (600 nm) in comparison to that of a sterile negative control (33).

RESULTS

Cetrimide. The majority of the test bacteria in this study underwent comparatively minor reductions (≤2-fold) in susceptibility following repeated exposure to cetrimide (Table 1). From those that underwent a ≥4-fold decrease, three of the five strains were staphylococci, with the MICs increasing by in excess of 18-fold for S. haemolyticus. Following the cessation of microbiocide exposure, full reversions in the MICs occurred for E. coli, K. pneumoniae, and S. epidermidis, while no reversion in the MICs was apparent for S. haemolyticus and S. lugdunensis. E. coli was the only bacterium to display a ≥4-fold increase in the MIC, which completely reverted when grown in the absence of cetrimide.

Chlorhexidine. Data in Table 2 indicate that ≥4-fold increases in the MICs occurred for B. cepacia, E. faecalis, K. pneumoniae, S. marcescens, S. lugdunensis, and S. maltophilia following 10 passages in the presence of chlorhexidine. E. faecalis demonstrated a complete reversion in the MIC after the cessation of microbiocide exposure. Partial reversions (MICs) were observed for B. cepacia and S. marcescens, while K. pneumoniae, S. maltophilia, and S. lugdunensis values failed to revert when passaged in the absence of chlorhexidine. Partial reversions in MICs were observed in B. cepacia, B. cereus, S. marcescens, and S. aureus, while S. lugdunensis and S. maltophilia did not revert significantly after growth in a microbiocide-free environment.

PHMB. After 10 passages in the presence of PHMB, ≥4-fold increases in the MICs were observed for C. indolgenes, E. faecalis, K. pneumoniae, M. luteus, S. capitis, and S. caprae (Table 3). C. indolgenes, E. faecalis, and S. capitis also showed ≥4-fold increases in the MICs, as did S. lugdunensis. Following 10 passages in the presence of PHMB, the MICs partially reverted to within a ≤2-fold difference from preexposure levels, with the exception of E. faecalis, which only partially reverted. Similarly, all test bacteria yielded X10 MIC values within a 2-fold difference from preexposure values, with the exception of C. indolgenes (a 4-fold difference). Of note, B. cepacia, B. cereus, C. xerosis, and S. marcescens exhibited X10 MIC/MBC values that were moderately lower (≤3-fold) than those determined prior to microbiocide exposure.

Tricosan. Eleven out of 18 test bacteria underwent a ≥4-fold increase in the MICs, and 9 out of 18 exhibited a ≥4-fold increase in the MICs following 10 passages in the presence of tricosan (Table 4). The bacterium most susceptible to tricosan (MIC) was S. aureus. However, this bacterium also underwent the greatest reduction in susceptibility (MIC) following repeated sublethal microbiocide exposure. In terms of the MICs, E. coli was the most susceptible bacterium, followed by S. aureus and E. faecalis. Interestingly, these bacteria also exhibited the most pronounced

100-fold antimicrobial concentration gradients on Mueller-Hinton agar plates using a spiral plater (Wellcome automated spiral plater; Don Whitley Scientific, Shipley, United Kingdom). The initial antimicrobial stock solutions (50 μl) were deposited on the agar surface. Plates were dried for 1 h at room temperature prior to radial deposition of bacterial pure cultures and then incubated (4 days at 37°C) in a static aerobic incubator. After incubation, growth observed at the highest microbiocide concentration was aseptically removed and radially streaked onto a fresh plate containing the same antimicrobial concentration gradient. If growth was observed across the whole antimicrobial gradient, a new plate containing a 5× higher stock solution concentration was used (30). This process was repeated until 10 passages had occurred. Bacteria were then passaged a further 10 times in the absence of any antimicrobial (X10). Bacteria at P0, P10, and X10 were archived for subsequent MBC and MIC testing.
changes in the MBCs during the investigation, with a 58-fold increase observed in the case of *E. coli*. *P. aeruginosa* was shown to be intrinsically nonsusceptible to all test concentrations of triclosan (27). After repeated cycles of growth in a triclosan-free medium, the MICs of the majority (8/11) of test bacteria reverted to preexposure levels with the exception of those for *E. coli*, *K. pneumoniae*, and *S. aureus*, which remained elevated. With regard to the MBCs, *E. faecalis*, *E. coli*, and *S. aureus* only partial reverted to preexposure values. The remaining test bacteria yielded MBCs comparable to preexposure levels following the cessation of triclosan dosing (X10) (Table 4).

**ApoEdpL-W.** ApoEdpL-W was most potent against *S. caprae* and *S. epidermidis*, followed by *S. warneri* and *C. indologenes*. *S. marcescens* was the least susceptible of the test bacteria to the peptide (Table 5). With respect to changes in susceptibility following antimicrobial exposure, *S. caprae* exhibited the largest decrease in apoEdpL-W susceptibility after 10 passages, with the MIC value increasing by up to 21-fold (P10). After growth in peptide-free medium (X10), markedly increased MIC/MBC values (≥4-fold) partially reverted for a number of test bacteria, including *C. indologenes*, *S. caprae*, and *K. pneumoniae*. Changes in sensitivities (MBCs) for *S. epidermidis* and *S. haemolyticus* were stable, with no reversion in susceptibility being detected following 10 passages in the absence of the peptide.

### Table 1

| Test bacterium                          | MIC (µg/ml) | MBC (µg/ml) |
|----------------------------------------|-------------|-------------|
|                                         | Before exposure | P10  | X10  | Before exposure | P10  | X10  |
| *Bacillus cereus*                      | 7.3         | 14.5       | 7.3  | 14.5           | 48.3 (8) | 29  |
| *Burkholderia cepacia*                 | 38.7 (17)   | 38.6 (8)   | 29   | 116            | 232   | 232  |
| *Chryseobacterium indologenes*         | 12.1 (4)    | 14.5       | 14.5 | 29             | 29    | 29   |
| *Corynebacterium xerosis*              | 3.6         | 3.6        | 3.6  | 14.5           | 9.7 (4) | 14.5 |
| *Enterococcus faecalis*                | 12.1 (4)    | 14.5       | 14.5 | 29             | 38.7 (16) | 58  |
| *Escherichia coli*                     | 29.17 (8)   | 116        | 29   | 116            | 464   | 116  |
| *Klebsiella pneumoniae*                | 29.3 (8)    | 116        | 29   | 29             | 58    | 58   |
| *Micrococcus luteus*                   | 14.5        | 7.3 (33)   | 14.5 | 58             | 19.3 (8) | 29  |
| *Pseudomonas aeruginosa*               | 232         | 232        | 232  | 246            | 464   | 464  |
| *Serratia marcescens*                  | 24.2 (8)    | 37.3 (14)  | 29   | 37.3 (14)      | 116   | 58   |
| *Staphylococcus aureus*                | 4.8 (2)     | 6 (2)      | 7.3  | 14.5           | 7.3   | 7.3  |
| *Staphylococcus capitis*               | 3.6         | 7.3        | 7.3  | 14.5           | 7.3   | 7.3  |
| *Staphylococcus caprae*                | 0.9         | 1.8        | 1.8  | 14.5           | 14.5  | 14.5 |
| *Staphylococcus epidermidis*           | 1.8         | 7.3        | 7.3  | 14.5           | 7.3   | 7.3  |
| *Staphylococcus haemolyticus*          | 0.4         | 3.6        | 3.6  | 14.5           | 14.5  | 14.5 |
| *Staphylococcus lugdunensis*           | 0.4         | 3.6        | 3.6  | 29             | 14.5  | 29   |
| *Staphylococcus warneri*               | 4.8 (2)     | 6.1 (2)    | 7.3  | 193.3          | 232   | 116  |
| *Stenotrophomonas maltophilia*         | 19.3 (8)    | 29         | 29   | 58             | 24.2 (8) | 58  |

* Data show the mean MICs and minimum bactericidal concentrations of bacteria before and after microbiocide exposure in µg/ml and represent samples taken from two separate experiments each with three technical replicates. For data that varied between replicates, SDs are given in parentheses. Bold type indicates a ≥4-fold change when comparing P0 to P10 and X10 values.

### Table 2

| Test bacterium                          | MIC (µg/ml) | MBC (µg/ml) |
|----------------------------------------|-------------|-------------|
|                                         | Before exposure | P10  | X10  | Before exposure | P10  | X10  |
| *Bacillus cereus*                      | 14.5        | 14.5       | 14.5 | 29             | 232   | 116  |
| *Burkholderia cepacia*                 | 3.6         | 36         | 7.3  | 26.6 (6)       | 232   | 116  |
| *Chryseobacterium indologenes*         | 7.3         | 7.3        | 7.3  | 7.3            | 14.5  | 7.3  |
| *Corynebacterium xerosis*              | 3.3 (1)     | 3.6        | 3.6  | 218.8          | 14.5  | 14.5 |
| *Enterococcus faecalis*                | 3.6         | 24.2 (8)   | 3.6  | 26.6 (6)       | 58    | 29   |
| *Escherichia coli*                     | 6.7 (1)     | 7.3        | 7.3  | 133.3          | 29    | 29   |
| *Klebsiella pneumoniae*                | 2.1 (1)     | 14.5       | 14.5 | 16.3 (5)       | 58    | 116  |
| *Micrococcus luteus*                   | 3.6         | 3.6        | 3.6  | 14.5           | 7.3   | 14.5 |
| *Pseudomonas aeruginosa*               | 7.3         | 14.5       | 7.3  | 14.5           | 29    | 14.5 |
| *Serratia marcescens*                  | 12.1 (4)    | 116        | 58   | 24.2 (7)       | 232   | 116  |
| *Staphylococcus aureus*                | 8.5 (4)     | 3.6        | 3.6  | 133.3          | 58    | 29   |
| *Staphylococcus capitis*               | 3.6         | 6 (2)      | 7.3  | 14.5           | 14.5  | 29   |
| *Staphylococcus caprae*                | 3.6         | 3.6        | 7.3  | 29             | 29    | 29   |
| *Staphylococcus epidermidis*           | 13.3 (3)    | 9.7 (4)    | 14.5 | 33.8 (12)      | 24.2 (8) | 29  |
| *Staphylococcus haemolyticus*          | 1.4 (0.4)   | 3 (1)      | 1.8  | 4.2(1)         | 14.5  | 7.3  |
| *Staphylococcus lugdunensis*           | 0.9         | 3.6        | 4.8 (2) | 1.7 (0.3) | 48.3 (17) | 58  |
| *Staphylococcus warneri*               | 29          | 29         | 29   | 58             | 58    | 58   |
| *Stenotrophomonas maltophilia*         | 4.8 (2)     | 29         | 29   | 14.5           | 58    | 58   |

* See Table 1 footnote for explanation of data.
Changes in biofilm formation in antimicrobial-insusceptible microorganisms. Bacteria that had undergone >4-fold changes in the MBCs during the training procedure were further assessed for changes in their ability to form biofilms. The following bacteria exhibited a significant decrease in biofilm formation in a microtiter plate-based system: *E. faecalis* following exposure to apoEdpL-W, *B. cepacia* following exposure to chlorhexidine, and *S. aureus* and *S. lugdunensis* following exposure to triclosan.

In contrast, repeated exposures of *E. coli* and *S. epidermidis* to triclosan and apoEdpL-W appeared to promote biofilm formation (Fig. 1), while chlorhexidine exposure was associated with increases in biofilm formation in *K. pneumoniae* and *S. marcescens*. For PHMB, no significant difference in biofilm formation was observed between the unexposed and exposed counterparts.

In addition, none of the microorganisms investigated showed a >4-fold change in the MBC toward cetrimide, and they were therefore not examined for changes in biofilm formation.

**DISCUSSION**

In agreement with previous in vitro studies (23, 24, 29, 34), repeated laboratory exposure of certain bacteria to microbicides resulted in decreases in bacterial susceptibility. Of the 34 >4-fold decreases in susceptibility (MICs) observed in this study, 15 were fully reversible, 13 were partially reversible, and 6 were nonrevers-

| Test bacterium                | MIC (μg/ml) Before exposure P10 X10 | MBC (μg/ml) Before exposure P10 X10 |
|------------------------------|------------------------------------|-------------------------------------|
| *Bacillus cereus*            | 58 29 58                           | 58 58 58                            |
| *Burkholderia cepacia*       | 58 58 29                           | 116 58 58                           |
| *Chryseobacterium indologenes* | 0.9 3.6 1.8                      | 1.8 14.5 7.3                        |
| *Corynebacterium xerosis*    | 2.7 (1) 7.3 2.2 (0.4)             | 21.8 (8) 7.3 14.5                  |
| *Enterococcus faecalis*      | 1.8 14.5 9.7                       | 7.3 29 7.3                          |
| *Escherichia coli*           | 13.3 (3) 24.2 (8) 7.3             | 26.6 (6) 58 14.5                    |
| *Klebsiella pneumoniae*      | 7.3 29 9.7 (4)                    | 29 96.7 (34) 58                     |
| *Micrococcus luteus*         | 1.8 7.3 1.8                       | 7.3 14.5 7.3                        |
| *Pseudomonas aeruginosa*     | 31.3 (6) 38.7 (15) 29             | 116 232 116                         |
| *Serratia marcescens*        | 38.7 (15) 29 29                   | 38.7 (15) 29 29                     |
| *Staphylococcus aureus*      | 7.3 7.3 7.3                       | 52 (11) 58 58                       |
| *Staphylococcus capsici*     | 1.1 (0.3) 6 (2) 1.8               | 7.3 48.3 (17) 7.3                   |
| *Staphylococcus caprae*      | 6.7 (2) 4.9 (2) 7.3               | 29 38.7 (17) 29                     |
| *Staphylococcus epidermidis* | 3 (1) 14.5 3.6                    | 26.6 (6) 38.7 (17) 29               |
| *Staphylococcus haemolyticus* | 1.8 7.3 1.8                      | 29 58 29                            |
| *Staphylococcus lugdunensis* | 3.6 7.3 1.8                       | 5.4 (2) 48.3 (17) 7.3               |
| *Staphylococcus warneri*     | 3.6 6 (2) 3.6                     | 29 58 29                            |
| *Stenotrophomonas maltophilia* | 3 (1) 3.6 3.6                  | 29 29 29                            |

*See Table 1 footnote for explanation of data.*

**DISCUSSION**

In agreement with previous in vitro studies (23, 24, 29, 34), repeated laboratory exposure of certain bacteria to microbicides resulted in decreases in bacterial susceptibility. Of the 34 >4-fold decreases in susceptibility (MICs) observed in this study, 15 were fully reversible, 13 were partially reversible, and 6 were nonrevers-

| Test bacterium                | MIC (μg/ml) Before exposure P10 X10 | MBC (μg/ml) Before exposure P10 X10 |
|------------------------------|------------------------------------|-------------------------------------|
| *Bacillus cereus*            | 7.3 29 7.3                         | 58 116 58                           |
| *Burkholderia cepacia*       | 232 116 232                        | 464 464 464                         |
| *Chryseobacterium indologenes* | 0.9 3.6 1.8                      | 3.6 7.3 3.6                         |
| *Corynebacterium xerosis*    | 7.3 58 7.3                         | 7.3 7.3 7.3                         |
| *Enterococcus faecalis*      | 3.3 (1) 58 3.3 (1)                 | 3.3 (1) 96.7 (34) 14.5              |
| *Escherichia coli*           | 0.5 29 4.82                       | 0.5 29 14.5                         |
| *Klebsiella pneumoniae*      | 0.9 116 14.5                      | 29 116 14.5                         |
| *Micrococcus luteus*         | 7.3 12.1 (4) 3.63                  | 7.3 14.5 7.3                        |
| *Pseudomonas aeruginosa*     | NS NS NS                           | NS NS NS                            |
| *Serratia marcescens*        | 232 116 232                        | 232 464 232                         |
| *Staphylococcus aureus*      | 0.2 29 2.4                         | 1.8 58 12.1 (4)                     |
| *Staphylococcus capsici*     | 24.2 (8) 29 14.5                  | 29 77.3 (33) 29                     |
| *Staphylococcus caprae*      | 12.3 (4) 29 14.5                  | 24.2 (8) 58 29                     |
| *Staphylococcus epidermidis* | 13.3 (3) 38.7 (17) 14.5            | 53.2 (12) 116 58                   |
| *Staphylococcus haemolyticus* | 0.4 29 0.4                        | 7.3 58 7.3                          |
| *Staphylococcus lugdunensis* | 0.9 29 0.9                        | 7.3 58 7.3                          |
| *Staphylococcus warneri*     | 0.9 24.2 (8) 0.9                   | 14.5 38.7 (17) 14.5                |
| *Stenotrophomonas maltophilia* | 14.5 232 14.5                  | 58 463 48.3                          |

*See Table 1 footnote for explanation of data.*

*NS, nonsusceptible (MIC/MBC ratio of >1,000 μg/ml).
Adaptation to Microbicides

While there are multiple reports in the literature of the laboratory generation of bacteria with decreased susceptibilities toward microbicides, adapted bacteria may remain effectively susceptible to in-use concentrations of the agent. For example, in the current investigation, the exposure of some bacteria to concentrations of microbicides, such as PHMB and chlorhexidine, reportedly target the bacterial cytoplasmic membrane (1,45, 47, 55) and the expression of efflux pumps (41–43). For example, reduced triclosan susceptibility in E. coli has been generated in the laboratory by the selection of bacteria with mutations in fabI or through the upregulation of the multidrug efflux pump AcrAB or its positive regulators, MarA and SoxS (51–53). Similarly, in another laboratory-based investigation, the exposure of S. maltophilia to triclosan selected for insusceptible variants that overexpress the SmelDEF multidrug efflux pump (39), while mutations in fabI in S. aureus have also been shown to reduce triclosan susceptibility (54).

The quaternary ammonium compound cetrimide and biguanides, such as PHMB and chlorhexidine, reportedly target the bacterial cell envelope which affect cell permeability (44–46). Barriers to microbicidal penetration, such as the additional outer membrane in Gram-negative bacteria (47) or the presence of a spore coat in bacterial endospores (48), can confer protection against microbicides and possibly account for some of the low microbicidal susceptibilities observed in these respective groups of bacteria in the current study. In Gram-positive bacteria, compounds such as QACs and biguanides may readily transverse the cell wall, making the bacteria relatively susceptible to these compounds (49, 50). However, as is apparent in the current investigation, susceptibility can range widely within each bacterial group. When challenged with a microbicide, a reduction in microbicide accumulation in the bacterial cell is a common survival mechanism and may be partly achieved by decreased cell permeability. The effectiveness of this strategy depends on several factors relating to the particular bacterium and the microbicide.

While triclosan induced the highest frequency and largest magnitude of changes in bacterial susceptibility and cetrimide exposure resulted in the lowest, all changes in the MICs and MBCs toward triclosan were either partially or reversibly. The laboratory selection of bacteria with reduced susceptibility toward triclosan has been previously documented and has been attributed to mutations in the enoyl-acyl carrier reductase encoded by fabI or the overexpression of efflux pumps (6, 51). For example, reduced triclosan susceptibility in E. coli has been generated in the laboratory by the selection of bacteria with mutations in fabI or through the upregulation of the multidrug efflux pump AcrAB or its positive regulators, MarA and SoxS (51–53). Similarly, in another laboratory-based investigation, the exposure of S. maltophilia to triclosan selected for insusceptible variants that overexpress the SmelDEF multidrug efflux pump (39), while mutations in fabI in S. aureus have also been shown to reduce triclosan susceptibility (54). The quaternary ammonium compound cetrimide and biguanides, such as PHMB and chlorhexidine, reportedly target the bacterial cytoplasmic membrane (1,45, 47, 55) and the expression of multidrug resistance efflux pumps can influence bacterial susceptibility towards these agents (38; previously reviewed in reference 56). The plasmid-encoded OqxAB multidrug resistance pump reportedly conferred a decrease in cetrimide susceptibility in E. coli (57), and overexpression of the major facilitator superfamily efflux pump NorA in S. aureus has also been linked to reduced cetrimide susceptibility (58). In the present study, tran-
sient upregulation of efflux pumps may explain the decreases in the susceptibilities of *E. coli*, *S. epidermidis*, *S. haemolyticus*, and *S. lugdunensis* following cetrimide treatment. Furthermore, reductions in the permeability of the outer membrane have also been related to reduced microbicide susceptibility in many Gram-negative bacteria, particularly toward QACs (45) and biguanides (44). The mechanisms responsible include changes in lipopolysaccharide expression or structure (44), loss of porin proteins (59), and alterations in outer membrane fatty acid composition (45).

Efflux pump expression also apparently contributes to changes in biguanide susceptibility in bacteria (60). Fang and colleagues previously documented the isolation of chlorhexidine-nonsusceptible *K. pneumoniae*, which expressed a novel locus with a sequence compatible to that of a cation efflux pump, designated *cepA* (60). *S. marcescens* isolated from a chlorhexidine-containing contact lens solution exhibited alterations in outer membrane protein composition, which was linked to chlorhexidine nonsusceptibility (61). It is also possible that the induction of efflux mechanisms may have contributed to the reductions in biguanide susceptibility observed in the current study. Moore and colleagues previously examined the effect of sublethal PHMB exposure on a selection of bacteria isolated from the human skin and a domestic drain. Similar to our findings, they observed changes in the susceptibilities of various staphylococcal strains after PHMB exposure (23).

Four species of staphylococci exhibited decreases in apoEdpL-W susceptibility, three of which were nonreversible. It has been documented that staphylococci produce extracellular "V8" proteases that play a role in their pathogenesis (62). Certain cationic peptides are substrates for such proteases and, therefore, when expressed, confer stable cationic antimicrobial peptide (CAMP) resistance to the bacteria (62). Expression of efflux systems, such as the *qacA*-mediated efflux system in *S. aureus*, has also been associated with CAMP resistance in staphylococci. Furthermore, it has been shown that CAMP exposure in certain Gram-negative bacteria may induce protein, phospholipid, and lipopolysaccharide (LPS) modifications due to activation of the PhoP/PhoQ regulon (63), decreasing the attracting force between the positively charged peptide and negatively charged bacterial cell wall. In *K. pneumoniae*, a bacterial capsular polysaccharide (CPS) is thought to mediate CAMP resistance (64). *K. pneumoniae* was one of the few organisms that showed a widespread decrease in susceptibility to all the antimicrobials tested in this study. It is therefore plausible that upregulation of capsule synthesis in *K. pneumoniae* may confer a broad-range defense mechanism when antimicrobial stress is experienced.

As well as showing decreases in competitive fitness (65), bacteria adapted to grow in the presence of microbicides can display further phenotypic alterations such as decreases in growth rate, pigmentation, and biofilm formation, which could lead to altered

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**FIG 1** Bacterial biofilm formation before (■) and after (□) long-term exposure to ApoEdpL-W (A), chlorhexidine (B), PHMB (C), and triclosan (D). Data represent changes in biofilm formation in selected bacteria that underwent >4-fold changes in the MBCs following microbicide exposure. *, significant change in result (*P* < 0.001). Data show the mean levels of biofilm-bound crystal violet for P0 and P10 samples taken from two separate experiments each with three technical replicates.
pathogenic capability (30, 65, 66). After exposure to antimicrobials, several bacteria in the current study demonstrated significant alterations (increases and decreases) in their ability to form biofilms in a microtiter plate assay. The mechanisms responsible for such changes and their implications are currently poorly understood, but they may be due to the selection of mutants with alterations in factors directly involved in bacterial adhesion and biofilm maturation or the selection of isolates with altered growth rates and fitness, which can indirectly affect biofilm formation (30, 65). Any adaptation that renders a bacterium less susceptible to an antimicrobial may therefore also result in reduced or increased fitness (65, 67), which may influence pathogenic ability. An increased capacity to form biofilms was observed after apoEdpL-W adaptation in *S. epidermidis*. While the mechanisms underlying this change have not been elucidated, a similar effect observed for *S. epidermidis* after exposure to alcohol-containing skin disinfectants was explained on the basis of increased polysaccharide intracellular adhesin (PIA) synthesis (68).

In the current study, *S. marcescens* and *K. pneumoniae* also exhibited increased biofilm-forming abilities after chlorhexidine exposure, which could potentially be mediated through altered capsule formation in *K. pneumoniae* (64, 69) or the upregulation of efflux pumps (60).

Decreases in bacterial specific growth rates have been reported following sublethal exposure to antimicrobials, and such changes may have influenced biofilm formation in our bacterial isolates (30, 70). The apparent decreases in biofilm formation observed for *E. faecalis* after apoEdpL-W exposure and *B. cepacia* after exposure to chlorhexidine may result from a lower density of cells within a slower growing culture, which could influence the expression of cell density-dependent genes involved in the process of biofilm formation (71). *S. aureus* and *S. lugdunensis* showed a decrease in biofilm formation after triclosan exposure. A decrease in staphylococcal biofilm production has previously been attributed to alterations in PIA and *Agrobacterium* aerugina peroxidase (Aap) production or to changes in sarA, a regulatory gene which controls the expression of virulence determinants involved in biofilm development, such as DNase (72).

In conclusion, repeated exposure of bacteria to certain microbicides in *vitro* can result in decreases in antimicrobial susceptibility that may be transient or sustained, probably resulting from temporary phenotypic adaptations or the selection of stable genetic mutations, respectively. In adapting to antimicrobial stress, bacteria can exhibit alterations in other physiological characteristics, such as increases or decreases in biofilm-forming ability.

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REFERENCES

1. Gilbert P, Moore LE. 2005. Cationic antiseptics: diversity of action under a common epithet. J. Appl. Microbiol. 99:703–715. http://dx.doi.org/10.1111/j.1365-2672.2005.02664.x.

2. Gaonkar TAP, Sampath LABA, Modak SMP. 2003. Evaluation of the antimicrobial efficacy of urinary catheters impregnated with antiseptics in an *in vitro* urinary tract model. Infect. Control Hosp. Epidemiol. 24:506–513. http://dx.doi.org/10.1086/502241.

3. Sampath LABA, Tambe SMP, Modak SMP. 2001. *In vitro* and *in vivo* efficacy of catheters impregnated with antiseptics or antibacterials: evaluation of the risk of bacterial resistance to the antimicrobials in the catheters. Infect. Control Hosp. Epidemiol. 22:640–646. http://dx.doi.org/10.1086/501836.

4. Silver S, Phung Le T, Silver G. 2006. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. J. Ind. Microbiol. Biotechnol. 33:627–634. http://dx.doi.org/10.1007/s10399-006-0139-7.

5. Broxton P, Woodcock PM, Heafley F, Gilbert P. 1984. Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Pseudomonas aeruginosa*. J. Appl. Bacteriol. 57:115–124. http://dx.doi.org/10.1111/j.1365-2672.1984.tb02363.x.

6. McMurry LM. 1998. Triclosan targets lipid synthesis. Nature 394:531. http://dx.doi.org/10.1038/28970.

7. Blanco MA, Negro C, Gaspar I, Tijero J. 1996. Sible problems in the paper and board industry. Appl. Microbiol. Biotechnol. 46:203–208. http://dx.doi.org/10.1007/s002530050806.

8. Pereira MO, Vieira VM, Beleza VM, Melo LF. 2001. Comparison of two biocides—carbamate and glutaraldehyde—in the control of fouling in pulp and paper industry. Environ. Technol. 22:781–790. http://dx.doi.org/10.1080/095933322086180318.

9. Condell O, Condell I, Iversen S, Cooney KA, Power C, Walsh C, Burgess S, Fanning. 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. http://dx.doi.org/10.1128/AEM.07534-11.

10. Quintavalla S, Quintavalla I, Vicini. 2002. Antimicrobial food packaging in meat industry. Meat Sci. 62:373–380. http://dx.doi.org/10.1016/S0309-1740(02)00121-3.

11. John MG, Goddard PA, Iliffe C, Timmins B, Rickard AH, Robson G, Handley PS. 2002. Comparative susceptibility of resident and transient hand bacteria to *para*-chloro-meta-xylene and triclosan. J. Appl. Microbiol. 93:336–344. http://dx.doi.org/10.1046/j.1365-2672.2002.01691.x.

12. Tanner RS. 1989. Comparative testing and evaluation of hard-surface disinfectants. J. Ind. Microbiol. 4:145–154. http://dx.doi.org/10.1007/BF01697999.

13. Campapanac C, Pineau L, Payard A, Baziard-Myousset G, Roques C. 2002. Interactions between biocidal cationic agents and bacterial biofilms. Antimicrob. Agents Chemother. 46:1469–1474. http://dx.doi.org/10.1128/AAC.46.5.1469-1474.2002.

14. Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15:167–193. http://dx.doi.org/10.1128/CMR.15.2.167-193.2002.

15. Mah TFC, O’Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9:34–39. http://dx.doi.org/10.1016/S0966-842X(00)01913-2.

16. Bjarnsholt T. 2008. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen. 16. http://dx.doi.org/10.1111/j.1527-495X.2007.00283.x.

17. Zabranski JM, Whiting D, Darouiche RO, Horgan TG, Olson J, Robertson C, Hamilton AI. 2003. Efficacy of antimicrobial-impregnated external ventricular drain catheters: a prospective, randomized, controlled trial. J. Neurosurg. 98:725–730. http://dx.doi.org/10.3171/jns.2003.98.07.225.

18. Edmiston CE, Seabrook GR, Goheen MP, Krepel CJ, Johnson CP, Lewis BD, Brown KR, Towne JB. 2006. Bacterial adherence to surgical sutures: can bacterial-coated sutures reduce the risk of microbial contamination? J. Am. Coll. Surg. 203:481–489. http://dx.doi.org/10.1016/j.jamcollsurg.2006.06.026.

19. Pitten FA, Kramer A. 1999. Antimicrobial efficacy of antiseptic mouthrinse solutions. Eur. J. Clin. Pharmacol. 55:95–100. http://dx.doi.org/10.1007/s002280050601.

20. Darouiche RO, Mansouri MD, Zakarevich D, AlShairf A, Landon GC. 2007. In vivo efficacy of antimicrobial-coated devices. J. Bone Joint Surg. 89:792–797. http://dx.doi.org/10.1021/IBS.F000414.

21. Tattawasart U, Henn AC, Maillard J-Y, Furr JR, Russell AD. 2000. Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*. Antimicrob. Agents Chemother. 45:145–152. http://dx.doi.org/10.1128/JAC.45.2.145.

22. Furi I, Ciusa ML, Knight D, Di Lorenzo V, Tocci N, Cirasola D, Aragonese L, Coelho JR, Freitas AT, Marchi E, Mosci L, Visa P, Northwood JB, Viti C, Borghi E, Orefici G, Consortium TB, Morrissy J, Oggioni MR. 2013. Evaluation of reduced susceptibility to quaternary ammonium compounds and bisbiguanides in clinical isolates and laboratory-generated mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. 57:3488–3497. http://dx.doi.org/10.1128/AAC.00498-13.

23. Moore LE, Lederer RG, Gilbert P, McBain AJ. 2008. In *vitro* study of the
effect of cationic biocides on bacterial population dynamics and susceptibility. Appl. Environ. Microbiol. 74:4825. http://dx.doi.org/10.1128/AEM.00573-08.

24. Joyon J A, Forbes B, Lambert RJ. 2002. Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. J. Appl. Microbiol. 95:96. http://dx.doi.org/10.1046/j.1365-2672.2002.01667.x.

25. Marrie TJ, Costerton JW. 1981. Prolonged survival of Serratia marcescens in chlorhexidine. Appl. Environ. Microbiol. 42:1093–1102.

26. Weber DJ, Weber WA, Rutala EA, Sickbert B. 2007. Outbreaks associated with contaminated antiseptics and disinfectants. Antimicrob. Agents Chemother. 51:4217–4224. http://dx.doi.org/10.1128/AAC.00138-07.

27. Chuanchuen R, Karkhoff-Schweizer RR, Schweizer HP. 2003. High-level triclosan resistance in Pseudomonas aeruginosa is solely a result of efflux. Am. J. Infect. Control 31:124–127. http://dx.doi.org/10.1016/S0196-6553(03)00203.11.

28. Maseda JM, Upton M. 2009. Low concentrations of vancomycin stimulate biofilm formation in some clinical isolates of Staphylococcus epidermidis. J. Clin. Pathol. 62:1112–1116. http://dx.doi.org/10.1136/jcp.2009.069021.

29. Joukhadar C, Butler RC, Reese DJ, Gaydos LA, Mennonna PA. 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 48:5–16. http://dx.doi.org/10.1093/jac/dkm314.

30. Latimer J, Forbes S, McBain AJ. 2012. Attenuated virulence and biofilm formation in Staphylococcus aureus following sublethal exposure to triclosan. J. Antimicrob. Chemother. 56:3902–3903. http://dx.doi.org/10.1093/jac/dks090.

31. Charbonneau DL, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Price BB, Gilbert P. 2003. Exposure of sink drain microcosms to triclosan: population dynamics and antimicrobial susceptibility. Appl. Environ. Microbiol. 69:5433–5442. http://dx.doi.org/10.1128/AEM.04212-03.

32. Karatzas KA, Webber MA, Jorgensen F, Woodward MJ, Piddock LJ, Humphrey TJ. 2007. Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J. Antimicrob. Chemother. 60:947–955. http://dx.doi.org/10.1093/jac/dkm314.

33. Djordjevic D, Wiedmann M, McLeansborough LA. 2002. Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl. Environ. Microbiol. 68:2950–2958. http://dx.doi.org/10.1128/AEM.68.6.2950-2958.2002.

34. Thomas L, Maillard J-Y, Lambert RJW, Russell AD. 2000. Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a "residual" concentration. J. Hosp. Infect. 46:297–303. http://dx.doi.org/10.1016/S0195-6701(00)00322-8.

35. Webber MA, Coldham NG, Woodward MJ, Piddock LJ. 2008. Protoen analysis of triclosan resistance in Salmonella enterica serovar Typhimurium. J. Antimicrob. Chemother. 62:92–97. http://dx.doi.org/10.1093/jac/dkm138.

36. Allen MJ, White GF, Morby AP. 2006. The response of Escherichia coli to exposure to the biocide polycyhexamethylene biguanide. Microbiology 152:989–1000. http://dx.doi.org/10.1099/mic.0.28463-0.

37. Briand F, Wendum C, Malack SM, Boulietns TE, Horrocks AJ, Piffl P. 2000. Selection for high-level resistance by chronic triclosan exposure is not universal. J. Antimicrob. Chemother. 55:73–77. http://dx.doi.org/10.1093/jac/dkh168.

38. Yu BJ, Kim JA, Pan JG. 2010. Signature gene expression profile of triclosan-resistant Escherichia coli. J. Antimicrob. Chemother. 65:1171–1177. http://dx.doi.org/10.1093/jac/dkq114.

39. Jiang HJ, Chang MW, Toghril F, Bentley WE. 2008. Microarray analysis of cytotoxic effects of triclosan on Staphylococcus aureus. Appl. Microbiol. Biotechnol. 78:695–707. http://dx.doi.org/10.1007/s00253-008-1349-9.

40. Poole K. 2004. Eflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. 10:12–26. http://dx.doi.org/10.1111/j.1469-0691.20000763.x.

41. Hansen LH, Jensen LB, Sorensen HI, Sorensen SJ. 2007. Substrate specificity of the OprxAB multidrug resistance pump in Escherichia coli and selected enteric bacteria. J. Antimicrob. Chemother. 60:145–147. http://dx.doi.org/10.1093/jac/dkm167.

42. Kaatzy GW, Seo SM. 1995. Inducible NorA-mediated multidrug resistance in Staphylococcus aureus. Antimicrob. Agents Chemother. 39:2650–2655. http://dx.doi.org/10.1128/AAC.39.12.2650.

43. Winder CJ, Al-Adhami W, Vasiljev MS, Boulriens TE, Horrocks AJ, Collier PJ. 2002. Outer membrane protein shifts in biocide resistant Pseudomonas aeruginosa PAO1. J. Appl. Microbiol. 89:289–295. http://dx.doi.org/10.1046/j.1365-2672.2000.01119.x.

44. Fang CT, Chen HC, Chuang YP, Chang SC, Wang JT. 2002. Cloning of a cation efflux pump gene associated with chlorhexidine resistance in Klebsiella pneumoniae. Antimicrob. Agents Chemother. 46:2024–2028. http://dx.doi.org/10.1128/AAC.46.6.2024-2028.2002.

45. Gandhi PA. 1993. Adaptation and growth of Serratia marcescens in contact lens disinfectant solutions containing chlorhexidine gluconate. Appl. Environ. Microbiol. 59:183–188.

46. Siepawska-Lupa M, Mydel P, Krawczyk K, Wokjic K, Puklo M, Buda S, Buder P, Silbering J, Reid M, Pohl J, Shafer W, McAleave F, Foster T, Travis J, Potempa J. 2004. Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteases. Antimicrob. Agents Chemother. 58:158.
63. Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SL. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of \textit{Salmonella enterica} serovar Typhimurium. Infect. Immun. 68:6139–6146. http://dx.doi.org/10.1128/IAI.68.11.6139-6146.2000.

64. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albert S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect. Immun. 72:7107–7114. http://dx.doi.org/10.1128/IAI.72.12.7107-7114.2004.

65. Rozen DE, McGee L, Levin BR, Klugman KP. 2007. Fitness costs of fluoroquinolone resistance in \textit{Streptococcus pneumoniae}. Antimicrob. Agents Chemother. 51:412–416. http://dx.doi.org/10.1128/AAC.01161-06.

66. Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V. 2005. \textit{Staphylococcus aureus} golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J. Exp. Med. 202:209–215. http://dx.doi.org/10.1084/jem.20050846.

67. Kunz AN, Begum AA, Jerse AE. 2012. Impact of fluoroquinolone resistance mutations on gonococcal fitness and \textit{in vivo} selection for compensatory mutations. J. Infect. Dis. 205:1821–1829. http://dx.doi.org/10.1093/infdis/jis277.

68. Knobloch JK-M, Horstkotte MA, Rohde H, Kaufers PM, Mack D. 2002. Alcoholic ingredients in skin disinfectants increase biofilm expression of \textit{Staphylococcus epidermidis}. J. Antimicrob. Chemother. 49:683–687. http://dx.doi.org/10.1093/jac/49.4.683.

69. Hernández-Allés S, Hernandez A. 2000. Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of \textit{Klebsiella pneumoniae}. J. Antimicrob. Chemother. 46:273–277. http://dx.doi.org/10.1093/jac/46.2.273.

70. Bayston R, Ashraf W, Smith T. 2007. Triclosan resistance in methicillin-resistant \textit{Staphylococcus aureus} expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. J. Antimicrob. Chemother. 59:848–853. http://dx.doi.org/10.1093/jac/dkm031.

71. Kong KF, Vuong C, Otto M. 2006. \textit{Staphylococcus} quorum sensing in biofilm formation and infection. Int. J. Med. Microbiol. 296:133–139. http://dx.doi.org/10.1016/j.ijmm.2006.01.042.

72. Kiedrowski MR, Kavanaugh JS, Malone CL, Mootz JM, Voyich JM, Smeltzer MS, Bayles KW, Horswill AR. 2011. Nuclease modulates biofilm formation in community-associated methicillin-resistant \textit{Staphylococcus aureus}. PLoS One 6:e26714. http://dx.doi.org/10.1371/journal.pone.0026714.