Long-Term Exposure to Octenidine in a Simulated Sink Trap Environment Results in Selection of *Pseudomonas aeruginosa*, *Citrobacter*, and *Enterobacter* Isolates with Mutations in Efflux Pump Regulators

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ABSTRACT Octenidine-based disinfection products are becoming increasingly popular for infection control of multidrug-resistant (MDR) Gram-negative isolates. When a waste trap was removed from a hospital and allowed to acclimatize in a standard tap rig in our laboratory, it was shown that *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Citrobacter* and *Enterobacter* spp. were readily isolated. This study aimed to understand the potential impact of prolonged exposure to low doses of a commercial product containing octenidine on these bacteria. Phenotypic and genotypic analyses showed that *P. aeruginosa* strains had increased tolerance to octenidine, which was characterized by mutations in the Tet repressor *SmvR*. *Enterobacter* species demonstrated increased tolerance to many other cationic biocides, although not octenidine, as well as the antibiotics ciprofloxacin, chloramphenicol, and ceftazidime, through mutations in another Tet repressor, *RamR*. *Citrobacter* species with mutations in *RamR* and *MarR* were identified following octenidine exposure, and this is linked to development of resistance to ampicillin, piperacillin, and chloramphenicol, as well as an increased MIC for ciprofloxacin. Isolates were able to retain fitness, as characterized by growth, biofilm formation, and virulence in *Galleria mellonella*, after prolonged contact with octenidine, although there were strain-to-strain differences. These results demonstrate that continued low-level octenidine exposure in a simulated sink trap environment selects for mutations that affect *smvR*. It may also promote microbial adaptation to other cationic biocides and cross-resistance to antibiotics, while not incurring a fitness cost. This suggests that hospital sink traps may act as a reservoir for more biocide-tolerant organisms.

IMPORTANCE Multidrug-resistant (MDR) strains of bacteria are a major clinical problem, and several reports have linked outbreaks of MDR bacteria with bacterial populations in hospital sinks. Biocides such as octenidine are used clinically in body washes and other products, such as wound dressings for infection control. Therefore, increased tolerance to these biocides would be detrimental to infection control processes. Here, we exposed bacterial populations originally from hospital sink traps to repeated dosing with an octenidine-containing product over several weeks and observed how particular species adapted. We found mutations in genes related to biocide and antibiotic susceptibility, which resulted in increased tolerance, although this was species dependent. Bacteria that became more tolerant to octenidine also showed no loss of fitness. This shows that prolonged octenidine exposure has the potential to promote microbial adaptation in the environment and that hospital sink traps may act as a reservoir for increased biocide- and antibiotic-tolerant organisms.

KEYWORDS octenidine, *Citrobacter*, *Enterobacter*, *Pseudomonas aeruginosa*, sink waste trap, *smvR*

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The rise in bacterial infections that are resistant to one or more frontline antibiotics has led to a greater reliance on prevention through effective infection control practices (IPCs). This is essential for reducing the burden of nosocomial infections and includes the use of antiseptics and disinfectants—e.g., the use of body washes to help reduce nosocomial infections in high-risk areas such as intensive care units (ICUs) (1, 2). The antiseptic octenidine has showed promising efficacy against a wide variety of multidrug-resistant (MDR) pathogens (3, 4), as well as reducing the rate of infection with Gram-negative agents through daily body washing (5). Octenidine is a cationic biocide in which the two cationic centers will readily bind to the negatively charged bacterial cell membrane. This leads to disassociation of cations and neutralization of the bacterial cell surface charge (6), which at high concentrations, causes a decrease in membrane fluidity with eventual membrane fracture (7–10). Previous research has shown that *Staphylococcus aureus* isolates have increased their tolerance to octenidine through repeated exposure (11), and *Klebsiella pneumoniae* strains isolated in the 1920s and 1930s (i.e., before the introduction of octenidine) have lower octenidine MIC values than those isolated more recently (12). Isolates of *Pseudomonas aeruginosa* can become more tolerant to octenidine (13), with mutations shown in the TetR efflux pump regulator SmvR and PssA/PgsA, which are enzymes involved in phospholipid biosynthesis (L. J. Bock, P. M. Ferguson, M. Clarke, V. Pumpitakul, M. E. Wand, P. E. Fady, L. Allison, R. A. Fleck, M. J. Shepherd, A. J. Mason, and J. M. Sutton, unpublished data). *K. pneumoniae* is also able to survive increased exposure through mutations in the major facilitator superfamily (MFS) efflux pump SmvA, which increased the strength of interactions between the pump and octenidine (12).

Hospital drains and sink waste traps have been well documented as harboring MDR bacteria, with them acting either as a source for potential outbreaks through droplet dispersal (14) or as a reservoir between outbreaks (15–21). MDR bacteria from sinks can reenter the clinical environment during flushing and contribute to onward transmission (22). Upon washing of hands and when biocidal products are used as body washes for decolonization purposes, biocides are regularly discharged into the hospital waste pipes. Therefore, bacteria existing in these waste traps will come into regular long-term exposure to many biocides included in hand and body washes. Constant exposure to the active components of these body washes may either select for “more intrinsically tolerant” Gram-negative bacterial populations and/or force the species present to develop increased tolerance. The aim of this study was to evaluate changes in the culturable Gram-negative bacterial population in sink trap water from traps taken from a hospital upon long-term exposure to octenidine. We looked at whether select bacterial species from this mixed-species environment can adapt phenotypically, leading to decreased susceptibility to octenidine as well as potential cross-resistance to other antimicrobial agents. The genomes of several isolates were also sequenced to understand if mutations following exposure to octenidine were similar to those already seen in monospecies adaptation experiments described previously (12, 13).

RESULTS

Characterization of trap water isolates before treatment with octenidine. Hospital traps were removed from working hospital wards as part of routine or preventative maintenance and installed in a standard tap rig at Public Health England-Porton Down (PHE-Porton) as described previously (22). As far as the authors were aware, the sink traps had not come into contact with any octenidine-containing products prior to removal. Since we were interested in Gram-negative bacterial populations that could be most readily transmitted by splashing within the hospital environment, we sampled bacterial isolates from the trap water and cultured them on selective agar, as detailed in the Materials and Methods section; this was designated “time original” (T_{original}). Colonies that displayed different morphologies were selected, and individual organisms were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) to gain an understanding of the bacterial species

present. There was a mixture of *Enterobacteriaceae*, including *Enterobacter cloacae* complex, *Kluyvera ascorbata*, *Klebsiella pneumoniae*, and *Raoultella* and *Hafnia* spp. present. Several isolates were identified as *Pseudomonas nitroreducens* or could not be reliably identified (MALDI-TOF MS result of “no ID”). A subset of isolates was sent for whole-genome sequencing (WGS) to confirm/identify species. Many of the no ID isolates were found to be closely related to either *Phytobacter* spp. (48%) or *Kluyvera intestini* (70%), with one isolate identified as having high homology to *Azospira oryzae* (94%). ResFinder identified antibiotic resistance genes, including *act-3* and *qnrE* in *Enterobacter asburiae* and *bla*_{CTX-M-15} in *K. intestini*. Antimicrobial MIC testing revealed ampicillin and piperacillin resistance (MICs of >512 mg/liter) in many isolates, as well as resistance to colistin in *Enterobacter asburiae* (MICs of >64 mg/liter) and *Klebsiella pneumoniae* (MICs of 8 to 16 mg/liter) isolates. Subsequent sequence analysis on these *K. pneumoniae* isolates showed a unique variation (E57G) in *PmrA*, although its role in colistin resistance was not confirmed. However, all identified isolates were susceptible to several antibiotics tested, including carbapenems and cephalosporins, and there was no evidence for carriage of MDR plasmids (see Table S1 in the supplemental material). The isolates also had MIC values of between 2 and 32 mg/liter and 1 to 4 mg/liter for the antiseptics chlorhexidine and octenidine, respectively. *K. intestini*-related isolates showed a degree of lysis when grown in tryptic soy broth (TSB), and we were unable to grow *A. oryzae* in TSB; therefore, these were left off the MIC analysis.

Upon installation of the trap at PHE-Porton, the Gram-negative bacterial population was allowed to stabilize for 28 days before challenge with octenidine. This was because water conditions such as water hardness are different from those of the hospital from which the traps were acquired. After 28 days, the population was reanalyzed in the same manner as T^{original} (designated time 0 [T⁰]). *Citrobacter* spp. and *Pseudomonas aeruginosa* were now identified, and *K. pneumoniae* and *P. nitroreducens* were present at counts of over 1×10^6 CFU/ml. There was also infrequent identification of several species, such as *Klebsiella michigenensis*, *Stenotrophomonas* spp., *Aeromonas caviae*, and *Delftia tsuruhatensis*. We did not detect any *Kluyvera* spp., and there were only sporadic isolates of *Phytobacter* spp.

Challenge with a variety of antimicrobial agents showed again that the majority of isolates studied were susceptible, with the exception of ampicillin and piperacillin (MICs of 256 to >512 mg/liter), and that all isolates showed low levels of tolerance to octenidine (MICs of 0.5 to 8 mg/liter) (see Table S2 in the supplemental material).

Change in specific Gram-negative organisms present over the time course of challenge with Octenisan. Following the stabilization period, at T⁰ dosing of the sink trap with Octenisan (Schülke & Mayr, Sheffield, United Kingdom) was initiated (see the Materials and Methods section for the dosing regimen). Since it was reasoned that *K. pneumoniae*, *Citrobacter* spp., *E. asburiae*, and *P. aeruginosa* were of the greatest clinical significance, analysis was concentrated on these organisms. Samples of trap water were taken at various time points over the course of the experiment and plated on selective agar for identification of these organisms. We sporadically identified other organisms, including *D. tsuruhatensis*, *Hafnia paralvei*, and *Klebsiella michigenensis* at various time points (see Fig. S1 in the supplemental material), but *P. aeruginosa*, *E. asburiae*, and *Citrobacter* spp. were consistently isolated across all time points (Fig. 1). *K. pneumoniae* was only identified when the Octenisan challenge was removed later in the experiment (time points 76 and 84 days [T⁷⁶ and T⁸⁴, respectively]), indicating that it might be present in the biofilm but was not present in the trap water in large numbers.

Decreased susceptibility to octenidine in *P. aeruginosa* linked to mutations in the TetR family repressor *smvR*. At time points T⁰, T²⁸, T⁶², T⁹⁷, and T¹¹⁸, at least two isolates that were identified as either *P. aeruginosa*, *Enterobacter* spp., or *Citrobacter* spp. were cultured, and the MIC/minimum bactericidal concentration (MBC) was tested against octenidine to determine if there has been an increase in tolerance (Fig. 2 and Table 1). For *P. aeruginosa* isolates, there was an increase in octenidine tolerance following challenge, with MIC and MBC values increasing around 16-fold (from 4 mg/liter

FIG 1 Analysis of population changes of *P. aeruginosa*, *Citrobacter* spp., *Enterobacter* spp., and *K. pneumoniae* in a drain trap after installation in an automated sink drainage system with prolonged exposure to Octenisan. Drain trap water was sampled at regular intervals across the experimental timeline and selectively cultured for Gram-negative organisms. The number of CFU was calculated, and an average of all isolates at each time point sampled is shown. Error bars indicate standard deviation (SD). Shaded areas indicate the time frame when the sink was dosed with Octenisan, and nonshaded areas indicate when there was no dosing with Octenisan. T⁰ (where the dosing with Octenisan begins) is indicated as 0 days. Time points prior to this represent the establishment of the sink trap in the laboratory.

to >64 mg/liter) after 28 days. This level of tolerance was maintained in all isolates tested throughout the remaining time points, even when the Octenisan selection was removed (between time points T⁶² and T⁹⁷). For *Citrobacter* spp., there was a consistent 2-fold increase in the modal MIC and MBC values at the later time points (T⁶² onwards) compared to T⁰. For *Enterobacter* spp., from T²⁸ onwards, a different sequence type was isolated (ST997), which was identified as *Enterobacter roggenkampii* rather than *E. asburiae*. The *E. roggenkampii* isolates had comparable octenidine tolerance to *E. asburiae* (MICs of 4 to 8 mg/liter compared to 4 mg/liter), and these MIC/MBC values did not change during repeated challenge with octenidine. Since Octenisan contains other components that have antibacterial properties namely, lactic acid, MIC/MBC analyses were performed against this. Throughout the time course, there was little change in the levels of tolerance to lactic acid in all three species tested (Table 1).

At least two isolates for *P. aeruginosa*, *Citrobacter* spp., and *Enterobacter* spp. from each time point were sent for whole-genome sequencing (WGS) to understand genetic changes following exposure to Octenisan (see Table S3 in the supplemental material). All *P. aeruginosa* isolates from T²⁸ onwards contained changes in the TetR family regulator, which we have termed SmvR, which functions to regulate the divergently transcribed MFS pump SmvA. The appearance of mutations in *smvR* coincides with the increase in tolerance levels to octenidine. Subsequent analysis of transposon mutants of this gene and *smvA* in strain PAO1 indicated that there was little change in the MIC values between PAO1 and PAO1Δ*smvR* (2 mg/liter for both), but there was an increase in the MBC value from 2 mg/liter to 4 to 8 mg/liter in PAO1Δ*smvR*. For PAO1Δ*smvA*,

FIG 2 Octenidine susceptibility over time for organisms isolated from sink trap water. The MIC value (mg/liter) for octenidine was tested for a range of *P. aeruginosa*, *Enterobacter*, *Citrobacter*, and *K. pneumoniae* isolates at the time points indicated. The values shown are the mode at each time point. Error bars represent the upper and lower limits. Shaded areas indicate the time frame when the sink was dosed with Octenisan, and nonshaded areas indicate when there was no dosing with Octenisan.

TABLE 1 Biocide MIC/MBC values during time course

		MIC/MBC (mg/liter) value or % of active ingredient for ^a :									
Time point (days)	Octenisan	OCT	CET	CPC	HDPCM	BAC	CTAB	TRC	CHX	L acid	
<i>P. aeruginosa</i>											
0	25 (25)	4 (8–16)	6.25–12.5 (25)	512 (>512)	512 (>512)	32–64 (64–128)	512 (>512)	>64 (>64)	16–32 (32)	0.16 (0.16)	
28	25–50 (50)	32–64 (64–>64)	6.25–12.5 (12.5–25)	512 (>512)	512 (>512)	64 (128)	512 (>512)	>64 (>64)	32–64 (>128)	0.16 (0.16)	
62	25–50 (50)	32–64 (64)	6.25–25 (12.5–25)	512 (>512)	512 (>512)	64 (128–256)	512 (>512)	>64 (>64)	32 (>128)	0.16 (0.16)	
97	25–50 (50)	32–64 (64–>64)	6.25–12.5 (12.5–50)	512 (>512)	512 (>512)	64 (128–256)	512 (>512)	>64 (>64)	32 (>128)	0.16 (0.16)	
118	25–50 (50)	32–64 (64–>64)	6.25–12.5 (25)	512 (>512)	512 (>512)	64 (128–256)	512 (>512)	>64 (>64)	32 (128–>128)	0.16 (0.16)	
<i>E. asburiae/E. roggenskampi</i>											
0	12.5 (12.5–25)	4 (4–8)	0.19–0.39 (0.39)	8–16 (32)	16 (16–32)	32 (32)	16–32 (32–64)	1 (1–2)	16–32 (32)	0.16–0.33 (0.33)	
28	12.5–25 (25)	4 (4–8)	0.78 (0.78–1.56)	16–32 (64)	16–32 (16–32)	32–64 (64)	32–128 (128)	2 (4–8)	32 (32)	0.33 (0.33)	
62	12.5–25 (25)	4–8 (8–16)	3.125–6.25 (6.25)	128 (128–256)	128 (128–256)	32–64 (64)	128–256 (256)	4–8 (8)	64–128 (64–128)	0.16–0.33 (0.33)	
97	25 (25)	4 (4–16)	1.56–3.125 (3.125–6.25)	64–256 (128–256)	128–256 (256)	32–64 (64)	128–256 (128–256)	4–8 (8)	128–256 (256–512)	0.16–0.33 (0.33)	
118	25 (25)	4 (8)	1.56–3.125 (3.125)	128–256 (128–256)	128–256 (256)	32–64 (64)	128–256 (128–256)	4–8 (8)	128–256 (128–256)	0.16–0.33 (0.33)	
<i>Citrobacter</i> spp.											
0	1.56–3.125 (3.125)	2 (2)	0.09–0.19 (0.19–0.39)	8 (8–16)	4–8 (4–8)	8–16 (16–32)	8–16 (16–32)	0.25 (0.25)	8–16 (16)	0.16–0.33 (0.33)	
28	3.125 (3.125)	2 (2)	0.09–0.39 (0.19–0.39)	8–16 (16)	8–16 (8–16)	8–16 (16)	16–32 (16–32)	0.125–0.25 (1)	8–16 (32)	0.16–0.33 (0.33)	
62	1.56–3.125 (3.125–6.25)	2 (2)	0.19–0.39 (0.19–0.39)	8–16 (16)	8–16 (8–16)	8–16 (16–32)	16–32 (16–32)	0.25–0.5 (>4)	8–16 (16–32)	0.16–0.33 (0.33)	
97	3.125–6.25 (12.5)	2 (2–4)	0.09–0.39 (0.09–0.39)	8–16 (16)	8–16 (16)	16–32 (32)	16–32 (16–32)	0.25–0.5 (>4)	16 (16–32)	0.16–0.33 (0.33)	
118	3.125–6.25 (6.25–12.5)	2 (2–4)	0.09–0.19 (0.19–0.39)	8–16 (16)	8–16 (8–16)	16–32 (32)	16–32 (16–32)	0.25–0.5 (>4)	16–32 (16–32)	0.16–0.33 (0.33)	

^aMIC/MBC values are shown for the biocides cetylpyridinium chloride (CPC), hexadecylpyridinium chloride monohydrate (HDPCM), benzalkonium chloride (BAC), cetyltrimethylammonium bromide (CTAB), triclosan (TRC), and chlorhexidine digluconate (CHX), as well as the octenidine-containing body wash Octenisan. Cetrimide (CET) and lactic acid (L acid) values are expressed as a percentage of the active ingredient. MBC values are shown in parentheses.

the MIC and MBC values for octenidine were 1 mg/liter. Since SmvA is an efflux pump, the effect of the efflux pump inhibitors CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and Pa β N (phenylalanine-arginine β -naphthylamide) was tested. The addition of Pa β N reduced the MIC/MBC values slightly (2- to 4-fold), while CCCP increased the MIC/MBC values of T⁰ isolates 8- to 16-fold (see Table S4 in the supplemental material). Mutations were also observed in the catalase KatA, the motility protein MorA, and the promoter regions of *cheZ* and *arcD* (Table S3).

For *E. roggenkampii*, when isolates were mapped to MPT87 (from time point T²⁸), there were two distinct lineages identified: those that contained deletions or deleterious single nucleotide polymorphisms (SNPs) in the regulator of the maltose regulon, *malT*, and the anaerobically synthesized molybdoenzyme *torA*, and those isolates that contained a conservative change (D21E) in SmvA (see Table S5 in the supplemental material). *Citrobacter* strains isolated at later time points showed mutations in the transcriptional regulator *marR* and the gene coding for the two-component sensor kinase involved in porin regulation, *envZ* (see Table S6 in the supplemental material).

Enterobacter spp. acquired increased tolerance to several other cationic biocides, while Citrobacter spp. developed increased resistance to β -lactam and fluoroquinolone antibiotics. To understand if adaptation to octenidine led to cross-tolerance to other biocides and antibiotics, susceptibility of isolates to several antimicrobial agents was tested at all time points. There was little change in MIC/MBC levels against biocides for *P. aeruginosa*, except for chlorhexidine, for which the MBC values increased from 32 mg/liter to >128 mg/liter (Table 1). For *Citrobacter*, there was a 2-fold increase for most biocides tested: up to 4-fold for Octenisan and triclosan (Table 1). In *Enterobacter*, there was an increase in MIC values (>4-fold) for the following biocides tested: cetrimide (CET), cetylpyridinium chloride (CPC), hexadecylpyridinium chloride monohydrate (HDPCM), cetyltrimethylammonium bromide (CTAB), triclosan (TRC), and chlorhexidine digluconate (CHX). There was not an increase in MIC values for Octenisan and benzalkonium chloride (BAC). The increase was stable and was not lost when the octenidine challenge was removed (between time points T⁶² and T⁹⁷).

A range of antibiotics were tested, which included frontline therapies for treatment of *P. aeruginosa*, *Citrobacter*, and *Enterobacter* infections. Little cross-resistance was seen, except for *Citrobacter*, for which there was an increase (4-fold) in resistance to ampicillin, piperacillin, and ceftazidime, with a 2- to 4-fold increase in resistance to ciprofloxacin and meropenem (Table 2).

In *P. aeruginosa*, acquisition of increased octenidine tolerance leads to retention of preexposure fitness levels. To understand if continued exposure and adaption to low levels of octenidine had affected the growth rate, *P. aeruginosa*, *Enterobacter*, and *Citrobacter* isolates from all time points were examined in both R2A broth, to stimulate low-nutrient conditions encountered in the sink trap, but also in a rich medium, TSB. There was no significant difference in the growth rates of all *P. aeruginosa* isolates in both TSB and R2A (Fig. 3). For *Enterobacter*, there was no difference in the growth rates of *E. roggenkampii* isolates from time points T²⁸ through to T¹¹⁸. There was, however, a difference in growth rates between the *E. asburiae* isolates from T⁰ and the *E. roggenkampii* isolates, with the latter isolates being slower growing in both TSB and, more extremely, in R2A broth, although this is likely to reflect differences between *E. asburiae* and *E. roggenkampii* rather than differences caused by exposure to Octenisan. *Citrobacter* isolates from the later time points had a growth defect compared to isolates from the T⁰ and T²⁸ time points; this effect was more pronounced in TSB, suggesting that prolonged exposure to Octenisan resulted in growth retardation.

Since biofilms are often the source of bacterial contamination in wastewater/sink water, the ability of the isolates to form biofilms was examined in both TSB and R2A broth. There was no statistical significance between any of the time points within the same species for biofilm formation in both TSB and R2A broth. Typically, *P. aeruginosa* was able to form increased biofilm in TSB, but surprisingly, *Enterobacter* appeared to be a better biofilm former in R2A medium rather than TSB (see Fig. S2 in the supplemental material).

To further understand the effect on fitness, virulence of isolates was examined in

TABLE 2 Antibiotic MICs during the time course of this study

Time point (days)	MIC (mg/liter) for ^a :								
	AMK	GEN	AMP	PIP	CAZ	MEM	CIP	CHL	CST
<i>P. aeruginosa</i>									
0	4–8	2–4	>512	8	1–2	2–4	0.125–0.25	128	0.5–1
28	4–8	2–4	>512	8	2	2–4	0.06–0.25	NT	0.5–1
62	4–8	2–8	>512	8	2	2–4	0.125–1	NT	0.5–1
97	4–8	4–8	>512	8	2	1–4	0.06–1	NT	0.5–2
118	4–8	4–8	>512	8–16	2	1–2	0.06–0.125	NT	0.5–1
<i>E. asburiae/E. roggenkampii</i>									
0	2–4	2–4	512–>512	2–4	1–2	0.15–0.25	0.06–0.125	4–8	>64
28	16–32	8	4–16	4–8	0.5–1	≤0.06	≤0.06	8–16	16–32
62	8–16	4–8	16–32	4–16	1–2	≤0.06	0.125	8–16	16–32
97	16–32	8–16	8–16	8–16	1–2	≤0.06	0.125–0.5	16	16–64
118	16–32	8–16	8–16	8	1–2	≤0.06	0.125–0.5	8–16	32–64
<i>Citrobacter</i> spp.									
0	2–4	4	4–8	2–4	0.5–1	0.015	0.015	4–8	0.06–0.125
28	2–4	4–8	8–16	2–4	0.5–1	0.015	0.015	4–8	0.125–0.25
62	2–8	4–8	8–16	8–16	1–2 (16–32) ^b	0.015–0.03	0.03	8	0.125–0.25
97	4–8	2–4	16–32	8	2–4	0.03	0.03	8–16	0.125–0.25
118	4–8	2–4	16–32	8–16	1–4	0.03–0.06	0.03	16–32	0.125

^aMIC values are shown for the antibiotics amikacin (AMK), gentamicin (GEN), ampicillin (AMP), piperacillin (PIP), ceftazidime (CAZ), meropenem (MEM), ciprofloxacin (CIP), chloramphenicol (CHL), and colistin (CST). NT, not tested.

^bValue in parentheses represents one isolate which had consistently higher CAZ MIC values.

the invertebrate model *Galleria mellonella*. Isolates from all time points were injected into *Galleria* at 10⁵ and 10⁶ CFU for *Enterobacter* and *Citrobacter* spp., respectively, and 10² CFU for *P. aeruginosa*. The results showed that for *Enterobacter* and *P. aeruginosa*, there was no difference in any of the time points investigated. *Citrobacter* isolates for later time points showed a significant loss of virulence when compared to T⁰ ($P = 0.0005$) (Fig. 4).

A second independent sink trap replicates the study's findings. A second sink trap, which had been left to stabilize at PHE-Porton for several weeks, was used to understand if the findings from the first sink were reproducible. Strains of *P. aeruginosa*, *Enterobacter* spp. (a mixture of *E. roggenkampii* and strains more closely related to *E. asburiae*), *Citrobacter* spp. (*C. freundii*), and *K. pneumoniae* were readily isolated from the initial sink trap population (see Fig. S3 in the supplemental material). These were different sequence types from those found in the previous sink waste trap and included strains of *Enterobacter* spp. and *K. pneumoniae* that were carbapenem resistant due to the presence of plasmids containing *bla*_{KPC-2}. Following the same dosing and sampling regimen, isolates of *P. aeruginosa*, *Enterobacter* spp., and *C. freundii* were obtained at all time points. Again, *K. pneumoniae* was only sporadically isolated, and at the later time points, a different sequence type (ST915) was identified from those of isolates at T⁰ (ST11). The sink population also contained multiple sequence types of *Enterobacter* spp. and *P. aeruginosa*, which made comparison of the susceptibility profiles of isolates between time points problematic. However, regardless of sequence type, all *P. aeruginosa* isolates at the later time points after exposure to Octenisan contained changes in *smvR*, and again the appearance of these changes corresponded with an increase in tolerance to octenidine (see Table S7 in the supplemental material). Other interesting changes observed in *P. aeruginosa* include changes in an *marR*-type regulator (PAO1 equivalent PA3458) and a divergently transcribed gene (PA3459) encoding an *N*-acetylglutaminylglutamine amidotransferase. Changes in PA3458 were observed in multiple isolates, but were not universal, and similar mutations were also found in isolates from the previous sink trap analysis. For *Enterobacter* spp., mutations were seen in the TetR family regulator RamR and the chemotaxis protein CheA as well as select isolates showing a conservative mutation in SmvA, D31E (see Table S8 in the

FIG 3 Growth rates of *P. aeruginosa* (a and b), *Enterobacter* spp. (c and d), and *Citrobacter* spp. (e and f) in R2A medium (a, c, and e) and TSB (b, d, and f) at different time points during the time course. Time points are indicated by the number of days into the time course. T²⁸, T⁶², and T¹¹⁸ are the time points (days) after a prolonged period of Octenisan dosing, whereas T⁹⁷ is the day after a period of 35 days when Octenisan dosing was stopped.

supplemental material). *C. freundii* isolates from the later time points also contained mutations in RamR, as well as SNPs in the trimethylamine *N*-oxide (TMAO) reductase system sensor kinase TorS. Specific isolates also had mutations in the promoter region of *smvAR* (see Table S9 in the supplemental material).

No cross-tolerance to biocides was observed in *P. aeruginosa*, but again, *Enterobacter* isolates showed a significant decrease in susceptibility to several cationic biocides (>4-fold), but not for octenidine (see Table S10 in the supplemental material). *C. freundii* isolates did not show any biocidal cross-tolerance, but there was a decrease in susceptibility to ampicillin, piperacillin, and ciprofloxacin (4-fold) and development of resistance to chloramphenicol. *Enterobacter* isolates at the later time points also showed a decrease in susceptibility to ciprofloxacin and chloramphenicol. These isolates are already resistant to ampicillin, piperacillin, and ceftazidime (see Table S11 in the supplemental material).

DISCUSSION

This study focused on the impact of disposal of the cationic antiseptic octenidine on Gram-negative populations of *K. pneumoniae*, *Citrobacter*, *Enterobacter*, and *P. aeruginosa* in a mixed-species environment. The study showed that the latter three organisms were able to survive continuous exposure to Octenisan, and for *P. aeruginosa*, this led to the culture of isolates that had become increasingly tolerant to octenidine. Why *K. pneumoniae* numbers were greatly reduced in the water samples is unknown. *K. pneumoniae* is typically

FIG 4 Virulence in *Galleria* of *P. aeruginosa*, *Enterobacter* spp., and *Citrobacter* spp. following prolonged exposure to octenidine. Groups of 30 larvae were challenged with 1×10^6 CFU of *Citrobacter*, 1×10^5 CFU of *Enterobacter*, and 1×10^2 CFU of *P. aeruginosa*. The number of live versus dead larvae was determined every 24 h postinfection up to 120 h. Significance as determined by the Mantel-Cox test is shown next to the species name. ***, $P = 0.0004$. Time points are indicated by the number of days into the time course. T²⁸, T⁶², and T¹¹⁸ are time points (days) after a prolonged period of Octenisan dosing, whereas T⁹⁷ is after a period of 35 days when Octenisan dosing was stopped.

not more susceptible to octenidine than the other species, and its growth in R2A broth is similar to that of *Citrobacter* and *Enterobacter*. We have evidence of maintenance of *K. pneumoniae* in non-octenidine-treated sink traps installed in the same tap rig (data not shown), which suggests that the observed reduction in *K. pneumoniae* in the sink trap water is a result of the Octenisan treatment. A summary of the results for *P. aeruginosa* and *Citrobacter* and *Enterobacter* spp. from both sinks analyzed is shown in Table 3.

Octenisan selection in multiple sequence types of *P. aeruginosa*, including the international MDR clone readily isolated from wastewater (ST235) (23), resulted in deletion of a functional *smvR*, which in *Klebsiella* leads to derepression of the *smvA* promoter and increased expression of the SmvA efflux pump (40). Laboratory-adapted *P. aeruginosa* to octenidine also showed deletions in *smvR* (Bock et al., unpublished), some of which are replicated here (notably a 12-bp deletion of bases 318 to 329 in *smvR*). Therefore, it is likely that in *P. aeruginosa* SmvA acts as an efflux pump that ameliorates the effects of octenidine, probably through direct efflux of the biocide. Mutations in SmvA and the *smvAR* promoter region were also observed in *Enterobacter* and *Citrobacter* spp., respectively, including identification of *Enterobacter* isolates with the D21E mutation from both sinks. This amino acid is present in a highly conserved predicted transmembrane region of *Enterobacteriaceae* SmvA. Increased efflux is a recognized mechanism of increased tolerance to biocides (24–26).

TABLE 3 Summary of findings from both sink traps for *P. aeruginosa*, *Citrobacter* spp., and *Enterobacter* spp.

Organism	Decrease in octenidine susceptibility	Biocide cross-tolerance	Antibiotic cross-resistance	Growth	Virulence	Important genes
<i>P. aeruginosa</i>	Yes (>4-fold)	No	No	WT ^a	WT	<i>smvA</i> , PA3458–PA3461
<i>Enterobacter</i> spp.	Slight (2-fold)	Yes (multiple biocides)	Yes (ciprofloxacin)	WT	WT	<i>smvA</i> , <i>malT</i> , <i>ramR</i> , <i>torA</i>
<i>Citrobacter</i> spp.	Slight (2-fold)	No	Yes (select β -lactams, ciprofloxacin)	Retardation	Loss of virulence	<i>marR</i> , <i>ramR</i> , <i>torS</i> , <i>envZ</i>

^aWT, wild type.

The transcriptional regulators MarR and RamR are important in the regulation of the MDR efflux pump AcrAB-TolC and function to repress the activators MarA and RamA, respectively (27). Mutations in MarR lead to increased expression of the *marRAB* operon, increased expression of oxidative stress genes either directly or through SoxRS (28, 29), and increased antibiotic resistance through overexpression of the efflux pump AcrAB-TolC, which is also activated through mutations in RamR (30). Mutations were seen in both *marR* and *ramR* in *Citrobacter* spp. and in *ramR* in *Enterobacter* spp., and these are linked to the decrease in susceptibility to antibiotics such as ampicillin, ciprofloxacin, piperacillin, ceftazidime, and chloramphenicol, all antibiotics known to be substrates for AcrAB-TolC (31). Mutations in RamR have also been described before in *Salmonella enterica* following exposure to specific biocides (32) as well as being implicated in increased tolerance to the biocides benzalkonium chloride and triclosan (33, 34). Although there have been limited studies on the role of RamR and MarR in *Citrobacter* and *Enterobacter*, it is predicted that they act in a similar manner to other members of the *Enterobacteriaceae*. Therefore, the potential for continuous exposure to octenidine to repeatedly select for mutations leading to increased antibiotic resistance, through mutations in important regulators of efflux pumps, is a cause for concern. Discussion of other mutations is continued in the supplemental material.

A limitation of the current study is that the sink trap biofilm was not studied, and a detailed analysis of the sink trap population was not undertaken. While we have generated evidence that there was a shift in the Gram-negative population following challenge with Octenisan (i.e., the *P. aeruginosa* and *Citrobacter* and *Enterobacter* spp. appear to be more prevalent), the study did not look at changes in the Gram-positive population and those viable but nonculturable organisms. It is plausible that by only sampling the trap water, there could be species such as *K. pneumoniae*, which are underrepresented in the water samples but are present within the deeper layers of the biofilm, at least during Octenisan challenge. This multispecies biofilm-mediated tolerance to biofilms is something we have observed previously (35), but it would require a different study protocol to evidence clearly in the sink trap biofilm model. We equally recognize that the biofilm populations of different sink traps are not likely to be entirely consistent with each other, as evidenced by a recent publication (36), especially given different experimental conditions, but we found that there was consistent isolation in the trap water of *P. aeruginosa* and *Enterobacter* and *Citrobacter* spp. that allowed a degree of comparison between both sinks analyzed.

As well as selecting for specific mutations or species, particular phenotypic characteristics were also selected for such as decreased biocide susceptibility in *Enterobacter* spp. that were not necessarily defined by individual mutations: e.g., isolates with *SmvA* mutations did not appear to be more biocide tolerant than those with mutations in *MalT*. In both sinks, *E. roggenkampii* became the dominant *Enterobacter* sp. at the later time points, with *E. asburiae* only cultured from isolates at the earlier time points. This suggests that *E. roggenkampii* either is intrinsically more tolerant to octenidine, is more genetically adaptable, or is better able to exist synergistically with other bacteria such as *P. aeruginosa*. There is also little evidence to suggest that particular *P. aeruginosa* sequence types are more able to adapt to octenidine since we isolated multiple sequence types (ST27, ST282, and ST319) with high MIC values against octenidine; what was common were mutations in *SmvR*. In previous studies, isolates from multiple sequence types were able to adapt to octenidine (13), again suggesting that, at least for *P. aeruginosa*, octenidine does not select for particular sequence types. *P. aeruginosa* and *Enterobacter* isolates that had adapted to octenidine also displayed no loss of fitness or virulence compared to preexposure strains. There is precedence for strains of *Enterobacteriaceae* with mutations, particularly in *smvR*, to persist in the environment with no obvious selective disadvantage (37).

This study has shown that repeated exposure to low concentrations of octenidine, in a simulated clinical environment, enhanced selection of individual *P. aeruginosa* mutants with elevated tolerance (≥ 8 -fold) to octenidine. These tolerance levels are still

below the in-use concentrations of octenidine, and therefore octenidine remains an effective product for decontamination. However, the *SmvA* efflux pump-mediated increased-tolerance phenotype in *P. aeruginosa* could be an intermediate step toward development of resistance, through secondary mutations such as membrane modification. This is evidenced by a stepwise development of fluoroquinolone resistance, with efflux-mediated resistance first, followed by mutations in *griA* (38) or elevation of expression of the *NorA* expression pump potentiating a fitness benefit for DNA topoisomerase mutations in *S. aureus* (39). Our own research showed that *smvR* mutations predated *PhoPQ* mutations in the development of increased chlorhexidine tolerance in *K. pneumoniae* (40), and *smvR* mutations were first observed before secondary high-level tolerance to octenidine in *P. aeruginosa* (13; Bock et al., unpublished). Mutations in regulators of efflux pumps (e.g., *MexZ* in *P. aeruginosa*), while not actually increasing the tolerance to antibiotics, may enable increased fitness in the presence of antibiotic (41). Taken together with the evidence of long-lasting colonization of hospital wastewater systems with MDR pathogens, notably carbapenem-resistant *Enterobacteriaceae* (CRE) (42–44), it is perhaps timely to reevaluate whether it is sensible to discharge unneutralized cationic biocides into an environmental reservoir that might be colonized with complex biofilms of MDR Gram-negative pathogens and that could pose an elevated infection risk. Residues of antimicrobial substances have been detected in the drainpipes from sinks (45) and in wastewater (46), and therefore it is plausible that octenidine discharged down the sink would remain at very low concentrations. Exposure to low concentrations of biocides remains a poorly understood risk factor for the development of decreased antibiotic and biocide susceptibility. Previous studies have shown that long-term exposure to biocides in drains resulted in little alteration in antimicrobial susceptibility (47–49), but other experiments have indicated that low-level biocide exposure leads to cross-tolerance to other disinfectants or antibiotics (20, 40, 50). Therefore, more studies are required to fully understand the role of environmental biocide exposure on bacterial adaptation and potential progression toward tolerance. However, biocides remain an effective weapon against the spread of MDR pathogens, and their continued use is essential for infection control.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *P. aeruginosa*, *K. pneumoniae*, *Citrobacter*, and *Enterobacter* strains in this study are described in Table S12 (original sink waste trap) and Table S13 (second sink waste trap) in the supplemental material. All strains were grown in tryptic soy broth (TSB) with aeration or on tryptic soy agar (TSA) plates at 37°C unless otherwise stated.

Automated model sink drain system. Water from a United Kingdom hospital sink waste trap known to be contaminated with a mixed-species population biofilm was sampled on site for analysis. The sink waste trap was transported at room temperature and attached to an automated model sink drain system at PHE-Porton within 48 h, where over the duration of the time course, the water temperature was kept constant at 41°C. Details of the sink drain system have been described previously (22). After installation, the waste trap was allowed to “acclimatize” for 21 days, during which it was dosed daily with 5 ml of TSB to maintain the original levels of *Enterobacteriaceae*. Throughout the time course, the associated tap was operated four times a day for 40 s at a flow rate of 4 liters/min. Ten seconds into the flush, the sink was dosed with 2 ml of a commercial octenidine formulation body wash (Octenisan; Schülke & Mayr) containing 0.3% octenidine. The Octenisan dosing was sustained for 62 days, paused for 35 days, and then resumed for another 21 days. This is shown diagrammatically in Fig. S4 in the supplemental material. Water from the waste trap was sampled throughout the acclimatization period, immediately and again prior to commencement of challenge with octenidine (T^0), as well as at various time points across the experimental timeline. Again, this process is demonstrated diagrammatically in Fig. S5 in the supplemental material. Each sample was serially diluted (10-fold) and selectively cultured for Gram-negative organisms by plating out 100 μ l of select dilutions on ChromID CARBA agar (bioMérieux, Basingstoke, United Kingdom), Brilliance *E. coli*/coliform selective agar, MacConkey no. 3 agar, cefrimide agar, and, to obtain a heterotrophic plate count, R2A agar (all from Oxoid, Ltd., Basingstoke, United Kingdom). Before plating out, Octenisan was neutralized using Dey-Engley (D/E) neutralizing broth. Different Gram-negative morphotypes were subcultured and identified via MALDI-TOF MS (MALDI Biotyper; Bruker Daltonics, Germany) and stored at -80°C . Up to three isolates that had been identified as either *P. aeruginosa*, *Citrobacter* spp., or *Enterobacter* spp. were phenotypically analyzed and sent for whole-genome sequencing.

Determination of MIC/MBC and evaluation of growth and biofilm formation. The MICs for antibiotics and biocides were determined by a standard broth microdilution method at a starting inoculum of 5×10^5 CFU/ml following guidelines detailed by EUCAST, except that 96-well polypropylene plates

(Griener Bio-One, Ltd., Stonehouse, United Kingdom) were used instead of polystyrene plates to test colistin (51). Antibiotic susceptibility was measured utilizing EUCAST breakpoints for *Pseudomonas* spp. and *Enterobacterales*. MBCs were measured by plating out onto TSA plates 10 μ l of MIC dilutions from and including the MIC level and the subsequent three further higher biocide concentrations (where applicable). The efflux pump inhibitors carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and phenyl-arginine β -naphthylamide (Pa β N) were added at concentrations of 10 and 25 mg/liter, respectively. Bacterial growth in both TSB and R2A broth was measured by taking an optical density at 600 nm (OD₆₀₀) reading every hour for 20 h using a FLUOstar Omega plate reader (BMG Labtech, GmbH, Ortenberg, Germany). Biofilm formation was carried out in both TSB and R2A broth using a modification of the Calgary method already described (52).

Virulence in *Galleria mellonella*. Wax moth (*G. mellonella*) larvae were purchased from Livefood UK, Ltd. (Rooks Bridge, Somerset, United Kingdom), and were maintained on wood chips in the dark at 14°C. They were stored for not longer than 2 weeks. Bacterial infection of *G. mellonella* was performed as essentially described previously (53). Briefly, three groups of 10 larvae were challenged with isolates from all time points at starting inocula in phosphate-buffered saline (PBS) of 1×10^5 and 1×10^6 CFU for *Enterobacter* and *Citrobacter*, respectively, and 1×10^2 CFU for *P. aeruginosa*. The number of live versus dead larvae was determined every 24 h postinfection up to 120 h. *Galleria* larvae injected with sterile PBS were used as a control, and results were only validated if there were no deaths in the control group. Data were analyzed by the Mantel-Cox method using Prism Software version 8 (GraphPad, San Diego, CA, USA).

Whole-genome sequencing. Whole-genome sequencing (WGS) was carried out as previously described (40), and PHE Galaxy was used to analyze genetic changes (54). Species identification from the genome sequences was carried out using ribosomal multilocus sequence typing (rMLST) (55), and antibiotic resistome analysis was carried out using ResFinder (56).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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