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Mechanisms of Increased Resistance to Chlorhexidine and Cross-Resistance to Colistin following Exposure of *Klebsiella pneumoniae* Clinical Isolates to Chlorhexidine

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ABSTRACT *Klebsiella pneumoniae* is an opportunistic pathogen that is often difficult to treat due to its multidrug resistance (MDR). We have previously shown that *K. pneumoniae* strains are able to "adapt" (become more resistant) to the widely used bisbiguanide antiseptic chlorhexidine. Here, we investigated the mechanisms responsible for and the phenotypic consequences of chlorhexidine adaptation, with particular reference to antibiotic cross-resistance. In five of six strains, adaptation to chlorhexidine also led to resistance to the last-resort antibiotic colistin. Here, we show that chlorhexidine adaptation is associated with mutations in the two-component regulator *phoPQ* and a putative Tet repressor gene (*smvR*) adjacent to the major facilitator superfamily (MFS) efflux pump gene, *smvA*. Upregulation of *smvA* (10- to 27-fold) was confirmed in *smvR* mutant strains, and this effect and the associated phenotype were suppressed when a wild-type copy of *smvR* was introduced on plasmid pACYC. Upregulation of *phoPQ* (5- to 15-fold) and *phoPQ*-regulated genes, *pmrD* (6- to 19-fold) and *pmrK* (18- to 64-fold), was confirmed in *phoPQ* mutant strains. In contrast, adaptation of *K. pneumoniae* to colistin did not result in increased chlorhexidine resistance despite the presence of mutations in *phoQ* and elevated *phoPQ*, *pmrD*, and *pmrK* transcript levels. Insertion of a plasmid containing *phoPQ* from chlorhexidine-adapted strains into wild-type *K. pneumoniae* resulted in elevated expression levels of *phoPQ*, *pmrD*, and *pmrK* and increased resistance to colistin, but not chlorhexidine. The potential risk of colistin resistance emerging in *K. pneumoniae* as a consequence of exposure to chlorhexidine has important clinical implications for infection prevention procedures.

KEYWORDS *Klebsiella pneumoniae*, PhoPQ, chlorhexidine, colistin, *smvA*, *smvR*

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The emergence of multidrug-resistant (MDR) pathogens poses a significant challenge to health care delivery. Gram-negative pathogens, including *Klebsiella pneumoniae*, from the so-called "ESKAPEE" group are of particular concern due to their high levels of antibiotic resistance. The emergence and rapid dissemination of particular strains of carbapenemase-producing *Enterobacteriacae* (1, 2) has caused an overreliance on last-resort antibiotics, e.g., colistin (CST). It has also highlighted the need for infection control to support clinical practice by reducing the potential for establishment of antibiotic-resistant bacteria.

A range of disinfectants and antiseptics are commonly used in clinical practice and underpin current health care. There is ongoing debate about the presence or emergence of resistance to biocides in clinical populations and the potential for this to translate into cross-resistance to antibiotics. There are relatively few examples where this has been identified and fewer still where there is strong evidence for its happening

TABLE 1 MIC values of various antibiotics and disinfectants for chlorhexidine-adapted strains

Strain	MIC (mg/liter)[a]										
	CHD	CHD + CCCP	BCl	Oct	HDPCM	EtOH (%)	CST	CST + CCCP	AZM	FEP	TEC
M109 WT	8	0.5–1	16	4	4–8	3.125	2	2	8–16	0.06–0.125	>64
M109 CA	32–64[b]	0.5–1	8–16	2–4	4–8	6.25	2–4	0.5–1	8–16	0.06–0.125	>64
NCTC 13439 WT	8–16	2–4	16	2–4	16	6.25	4	2	32	>64	>64
NCTC 13439 CA	256[b]	1–2	16	2–4	8–16	6.25	>64[b]	1	32	>64	>64
M3 WT	8–16	1–2	8–16	2–4	8	6.25	2–4	2	16–32	>64	>64
M3 CA	32–64[b]	0.5–2	8–16	2–4	8–16	3.125	>64[b]	1–2	8–16	>64	>64
NCTC 13443 WT	8–16	1–2	8–16	4	8–16	3.125	2	2	64	>64	>64
NCTC 13443 CA	256–512[b]	1–2	8–16	2	8–16	3.125	>64[b]	2	16–32	>64	>64
NCTC 13368 WT	32	2–4	32	4–8	32–64	6.25	2–4	2–4	64	64	>64
NCTC 13368 CA	256[b]	1–2	16	4–8	16	6.25	>64[b]	2–4	64	64	>64
MGH 78578 WT	8–16	1–2	8–16	4	8–16	6.25	2–4	2–4	32	>64	>64
MGH 78578 CA	256–512[b]	0.5–2	8–16	4	8	3.125	>64[b]	1–2	32–64	0.5[b]	>64

[a]The disinfectants used were chlorhexidine digluconate (CHD), benzalkonium chloride (BCl), octenidine dihydrochloride (Oct), hexadecylpyridinium chloride monohydrate (HDPCM), and ethanol (EtOH). The antibiotics used were CST, AZM, FEP, and TEC. All the MICs are shown as ranges of the results of at least three independent experiments. "+ CCCP" indicates the addition of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone. Additional antibiotics are shown in Table S2 in the supplemental material.

[b]There was a ≥4-fold increase or decrease in the MIC for chlorhexidine-adapted strains (CA) relative to nonadapted strains (WT).

in the clinic (3, 4). While resistance to many biocides has been reported for multiple organisms, there is a distinct lack of understanding of the mechanisms responsible for the resistance.

Chlorhexidine is a bisbiguanide antiseptic that is cationic in nature and functions by membrane disruption (5). It forms a bridge between pairs of adjacent phospholipid headgroups and displaces the associated divalent cations (Mg^{2+} and Ca^{2+}) (6). This results in a reduction of membrane fluidity and osmoregulation, as well as changes in the metabolic capability of the cell membrane-associated enzymes (7). At higher concentrations, the interaction between chlorhexidine and the cellular membrane causes the membrane to lose its structural integrity and adopt a liquid crystalline state, which leads to a rapid loss of cellular contents (8).

A review of products containing chlorhexidine that are commonly sold to the National Health Service (NHS) in England, identified a wide range of active concentrations of chlorhexidine, ranging from 0.02% in catheter maintenance solutions through 0.2% for mouthwash, 0.5% in chlorhexidine-impregnated wound dressings, and 2 and 4% (often in alcohol) solutions for skin antisepsis. Therefore, the potential for exposed bacteria to develop resistance to chlorhexidine is increased due to the variable selection pressure. Increased bacterial chlorhexidine resistance has implications for a wide range of applications, including treatment of wounds.

In this study, we investigated whether adaptation of clinical *K. pneumoniae* isolates to chlorhexidine caused cross-resistance to other biocides and antibiotics and whether the adapted strains maintained fitness and virulence. The underlying mechanisms of increased resistance to chlorhexidine in *K. pneumoniae* were also investigated, particularly in connection with the observed cross-resistance to colistin.

RESULTS

Phenotypic assessment of *K. pneumoniae* isolates following chlorhexidine adaptation. Previous work has shown that *K. pneumoniae* strains are able to adapt to increasing concentrations of chlorhexidine, and this leads to increased MIC/MBC (minimum bactericidal concentration) levels of chlorhexidine digluconate (CHD) and chlorhexidine-containing clinical products (9). To determine whether adaptation to chlorhexidine also led to development of increased resistance to other antiseptics and cross-resistance to frontline antibiotics, the MICs of a range of biocides for wild-type (WT) and chlorhexidine-adapted (CA) strains were determined (Table 1). Following chlorhexidine adaptation, MIC values for colistin increased from 2 to 4 mg/liter to >64 mg/liter in five out of the six strains. The only strain that did not show increased resistance to colistin following chlorhexidine adaptation was M109 CA. The strain also

had the lowest MIC for chlorhexidine (32 mg/liter compared to a range between 128 and 512 mg/liter for the other strains). Resistance to colistin is often accompanied by changes in membrane composition, which can be detected by altered MIC values of azithromycin (AZM), teicoplanin (TEC), and cefepime (FEP). Apart from MGH 78578 CA, where the MIC of cefepime changed from >64 mg/liter to 0.5 mg/liter following chlorhexidine exposure, no significant differences were observed. For other antibiotics tested, there was also no general change in susceptibility, although there were individual strain differences, e.g., for strain NCTC 13443 there was a significant reduction in the MIC values of meropenem and chloramphenicol after chlorhexidine adaptation (see Table S2 in the supplemental material). There was also no significant change (>2-fold) in MIC values of other antiseptics after chlorhexidine adaptation for any of the strains tested.

Due to increased disinfectant/antiseptic resistance often being associated with increased efflux, the MICs for chlorhexidine were retested in the presence of the known efflux pump inhibitors (EPIs) carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and phenylalanine-arginine-β-naphthylamide (PaβN). In all of the WT strains, the addition of CCCP gave a significant reduction in the MIC, with values reduced from between 8 and 32 mg/liter to between 0.5 and 4 mg/liter (8- to 16-fold reductions). The addition of CCCP also reduced the MIC of chlorhexidine in all of the chlorhexidine-adapted strains, with an MIC reduction of between 32-fold (M109 CA) and 256- to 512-fold (MGH 78578 CA and NCTC 13443 CA). In each case, the MICs of the WT and chlorhexidine-adapted strains, in the presence of CCCP, were either identical or within a 2-fold dilution (Table 1). The addition of CCCP also increased susceptibility to colistin in both chlorhexidine-adapted and WT strains. For PaβN, no reduction was seen in the MIC for the WT or chlorhexidine-adapted strain (data not shown).

The fitness of strains following chlorhexidine adaptation was assessed. Following chlorhexidine adaptation, virulence in the wax moth (*Galleria mellonella*) was reduced in several strains (NCTC 13443 CA, MGH 78578 CA, M109 CA, and NCTC 13368 CA), but not in M3 CA and NCTC 13439 CA (see Fig. S1 in the supplemental material). To investigate whether the observed loss of virulence was due to a growth defect, growth curves were assessed. A reduction in the growth rate was observed for some isolates, but it did not always correlate with loss of virulence (data not shown).

Whole-genome sequencing analysis of chlorhexidine-adapted strains. To understand what mechanisms are responsible for increased tolerance to chlorhexidine, all the chlorhexidine-adapted strains and their respective parental counterparts were whole-genome sequenced. Several genetic changes (both indels and nonsynonymous single nucleotide polymorphisms [SNPs]) were identified in the chlorhexidine-adapted strains (Table 2; see Table S3 in the supplemental material). Four out of the six strains had nonsynonymous SNPs in the *phoPQ* two-component regulator system, and four out of the six strains showed mutations in a putative Tet repressor gene (here called *smvR* [regulator of *smvA*]) adjacent to and divergently transcribed from a putative homologue of the methyl viologen resistance gene *smvA*. In three of these four strains, a truncated Tet repressor protein was observed, either through a missense mutation (M3 CA), deletion of the C terminus (M109 CA), or a nonsynonymous SNP leading to the formation of a premature stop codon (NCTC 13443 CA). In the fourth strain (NCTC 13439 CA), the entire *smvR* gene was deleted, along with downstream sequences, which included the nitrate extrusion protein gene (*narU*) and genes encoding proteins involved in nitrate reductase (*narZ* and *narY*). In two strains (M3 CA and NCTC 13443 CA), mutations in both *smvR* and *phoPQ* were identified.

In all adapted strains, genetic changes were identified in genes in addition to *phoPQ* and/or *smvR*. Mutations were observed in genes associated with the outer membrane or lipopolysaccharide (LPS), e.g., *lptD* in NCTC 13443 CA. Strain MGH 78578 CA contained a mutation in the DNA mismatch repair gene *mutS*, which explains the increased frequency of mutations after chlorhexidine adaptation in the strain. Two strains also contained alterations in the outer membrane protein MipA (MltA-interacting protein).

TABLE 2 Chromosomal genetic changes after exposure to chlorhexidine

Strain and gene name	Type of change	Change[b]	MGH 78578 equivalent based on NCBI reference sequence NC_009648.1	Function
M109				
wcaJ	SNP	Q399STOP		CPS biosynthesis glycosyltransferase
yfiN	SNP	A173V	KPN_RS15690	Diguanylate cyclase
smvR	Deletion	400-bp Del	KPN_RS10110	TetR family transcriptional regulator
NCTC 13439[a]				
mipA	SNP	Q98STOP	KPN_RS06390	MltA-interacting protein
rarA	SNP	W37R	KPN_RS15910	AraC family transcriptional regulator
smvR	Deletion	Complete Del	KPN_RS10110	TetR family transcriptional regulator
narU	Deletion	Complete Del	KPN_RS10115	Nitrite extrusion protein 2
narZ	Deletion	Complete Del	KPN_RS10120	Nitrate reductase A subunit alpha
narH	Deletion	Complete Del	KPN_RS10125	Nitrate reductase A subunit beta
narJ	Deletion	First 130 aa Del	KPN_RS10130	Nitrate reductase molybdenum cofactor assembly chaperone
M3				
phoP	SNP	E82K	KPN_RS06075	PhoP family transcriptional regulator
ackA	SNP	S274F	KPN_RS14420	Acetate kinase
smvR	Deletion of 5 bp after nucleotide 22	Truncation of 72 aa (normally 191 aa)	KPN_RS10110	TetR family transcriptional regulator
—[c]	Deletion (G) after nucleotide 445	Truncation of 174 aa (normally 184 aa)	KPN_RS17785	Isopentenyl-diphosphate delta-isomerase
NCTC 13443[a]				
sufD	SNP (synonymous)		KPN_RS11525	Fe-S cluster assembly protein
phoQ	SNP	A20P	KPN_RS06070	Two-component sensor protein
lptD	SNP	Y625N	KPN_RS00270	LPS assembly outer membrane complex protein
smvR	SNP	W125STOP	KPN_RS10110	TetR family transcriptional regulator
—	Insertion (T) after nucleotide 375	Truncation of 125 aa (normally 235 aa)	KPN_RS14015	Membrane protein; putative permease
NCTC 13368				
acoK	SNP	E253A		LuxR family transcriptional regulator

(Continued on next page)

TABLE 2 (Continued)

Strain and gene name	Type of change	Change[b]	MGH 78578 equivalent based on NCBI reference sequence NC_009648.1	Function
rpsA	SNP	V122F	KPN_RS05050	30S ribosome protein S1
yfiO (*bamD*)	SNP	V27E	KPN_RS15655	Part of outer membrane protein complex
phoP	SNP	Y98C	KPN_RS06075	PhoP family transcriptional regulator
MGH 78578[a]				
–	SNP	G121S	KPN_RS01550	Phosphonate ABC transporter substrate-binding protein
–	SNP	A276V	KPN_RS11605	5-Methyltetrahydropteroyltriglutamate homocysteine methyltransferase
mutS	SNP	T115P	KPN_RS16580	DNA mismatch repair protein
	SNP	R46C	KPN_RS24340	LuxR family transcriptional regulator
surA	SNP	L74P	KPN_RS00265	Peptidyl-prolyl *cis-trans* isomerase
phoQ	SNP	L348Q	KPN_RS06070	Two-component sensor protein
–	SNP	N30S	KPN_RS04685	Aldose dehydrogenase
–	SNP	N32S	KPN_RS20410	Transcription accessory protein
–	Insertion (A)	In repeat region AAGCTAA		
comA	Insertion (C) after nucleotide 122	Truncation of 136 aa (normally 170 aa)	KPN_RS08585	Competence protein
–	Insertion (G) after nucleotide 131	Truncation of 172 aa (normally 329 aa)	KPN_RS10945	Nitrate ABC transporter substrate-binding protein
–	Insertion (T) after nucleotide 4423	Elongation of 5 aa (normally 1,490 aa)	KPN_RS15800	Hypothetical protein
–	Insertion (GG) after nucleotide 1920	Truncation of 649 aa (normally 1,332 aa)	KPN_RS25360	Helicase
nuoC-nuoD	Insertion (GGG) after nucleotide 1382	Extra glycine	KPN_RS14365	NADH-quinone oxidoreductase subunit C/D
mipA	Insertion (G) after nucleotide 195	Truncation of 72 aa (normally 275 aa)	KPN_RS06390	MltA-interacting protein MipA
nikE	Insertion (C) after nucleotide 87	Truncation of 81 aa (normally 263 aa)	KPN_RS20815	ATP-binding protein of nickel transport system

[a]There was evidence of plasmid loss in the strain following chlorhexidine adaptation.
[b]aa, amino acids; Del, deletion; STOP, stop codon.
[c]–, name not known.

TABLE 3 MIC and MBC values of CHD and CST after plasmid complementation[a]

Strain	Plasmid	Description	CHD		CST	
			MIC (mg/liter)	MBC (mg/liter)	MIC (mg/liter)	MBC (mg/liter)
M109 CA	pACYC-184 alone	Empty vector	32	32	2–4	2–4
	pACYC M3 *smvR* WT	*smvR* from strain M3	8	8	2	2
	pACYC M3 *smvR* CHD	*smvR* from strain M3 CA	32	32	2–4	2–4
	pACYC 13443 *smvR* WT	*smvR* from strain NCTC 13443	8	8	2	2
	pACYC 13443 *smvR* CHD	*smvR* from strain NCTC 13443 CA	32	32	2–4	2–4
	pACYC *phoPQ* WT	*phoPQ* from strain NCTC 13443	32–64	32–64	1–2	2
	pACYC *phoQ* A20P	*phoPQ* from strain NCTC 13443 CA	32–64	64	64	64–>64
M109 WT	pACYC-184 alone	Empty vector	8–16	8–16	0.5–1	2–4
	pACYC *phoPQ* WT	*phoPQ* from strain NCTC 13443	8–16	8–32	0.5–1	2–4
	pACYC *phoQ* A20P	*phoPQ* from strain NCTC 13443 CA	8–16	16–32	32–64	64
25	pACYC-184 alone	Empty vector	8–16	8–16	0.5	2–4
	pACYC *phoPQ* WT	*phoPQ* from strain NCTC 13443	8–16	16–32	0.5–1	1–4
	pACYC *phoQ* A20P	*phoPQ* from strain NCTC 13443 CA	8–16	16–64	32–64	32–>64

[a]Levels of resistance to CHD and CST were measured after electroporation of the plasmids into the strains listed. All the MICs are shown as ranges of the results of at least three independent experiments.

All other mutations were present in only one strain out of six following chlorhexidine adaptation. There was also evidence for plasmid loss following chlorhexidine adaptation, which explains the loss of antibiotic resistance in certain strains, e.g., meropenem in NCTC 13443 CA.

Chlorhexidine adaptation experiments were repeated independently in the same strains, and again, mutations in *smvR* and *phoPQ* were observed (data not shown). Therefore, since all chlorhexidine-adapted strains had mutations in *phoPQ* and/or *smvR*, further analysis concentrated on these genes.

Role of SmvR and PhoPQ in resistance to chlorhexidine. To define the role of *smvR* in increasing resistance to chlorhexidine and colistin, pACYC-184 plasmids containing either the WT or the CA *smvR* version from NCTC 13443 and M3 (see Table 2 for individual mutations) were introduced into strain M109 CA (which has a deletion in the C terminus of *smvR*). Introduction of the wild-type version of *smvR* from either NCTC 13443 or M3 resulted in a decrease in the MIC values of CHD compared to the wild-type value (from 32 to 8 mg/liter), whereas the introduction of the CA *smvR* from either NCTC 13443 or M3 had no effect on the MIC value of CHD (Table 3), indicating that in M109 CA, the deletion of *smvR* increased resistance to chlorhexidine. No change in the MIC value of colistin was observed following introduction of either the CA or WT *smvR* version from NCTC 13443 or M3 into M109 CA. To confirm the roles of *smvA* and *smvR* in elevated resistance to chlorhexidine, we examined 10 preantibiotic era *K. pneumoniae* Murray strains that do not possess a homologue to the genes (10). These strains were highly susceptible to chlorhexidine, with MIC values ranging from 1 to <0.5 mg/liter compared to 8 to 32 mg/liter for the 26 Murray isolates that do contain *smvA* and *smvR*.

For *phoPQ*, introduction of pACYC *phoPQ* WT or pACYC *phoQ* A20P plasmids into M109 CA showed no increase in MIC values of chlorhexidine, but the presence of pACYC *phoQ* A20P increased the MIC of colistin from 2 to 64 mg/liter (Table 3). As M109 CA already had an elevated MIC for chlorhexidine, perhaps obscuring any effect of additional PhoPQ mutations, both plasmids were electroporated into wild-type M109 and another clinical *K. pneumoniae* strain, 25. Again, there was no increase in the MIC values of chlorhexidine, but there was an increase in resistance to colistin following the introduction of pACYC *phoQ* A20P (from 0.5 to 1 mg/liter to 32 to 64 mg/liter) (Table 3).

Analysis of changes in gene expression following chlorhexidine adaptation. To further understand the mechanism(s) of chlorhexidine adaptation in *K. pneumoniae* and whether chlorhexidine adaptation is related to mutations in *smvR* and *phoPQ*, the expression levels of specific genes were observed in both WT and CA strains. Besides

TABLE 4 Expression levels of select genes following CA or CST adaptation

Strain	Fold upregulation[a]							Mutation	Fold increase in MIC over WT	
	phoP	*phoQ*	*smvA*	*smvR*	*pmrK*	*pmrD*	*pagP*		CHD	CST
M109 CA			10					Δ*smvR* C terminus (CT)	4	None
NCTC 13439 CA			19		12	3		Δ*smvR*	16	>32
M3 CA	15	9	18	7	64	19	7	PhoP E82K, Δ*smvR*	8	>32
NCTC 13443 CA	5	5	27	6	26	6	2	PhoQ A20P, Δ*smvR* CT	16	>32
NCTC 13368 CA	8	7			18	6		PhoP Y98C	16	>32
MGH 78578 CA	6	8			24	6		PhoQ L348Q	16	>32
M109 WT pACYC *phoQ* A20P[b]		3			7	2	4	*phoQ* A20P on plasmid	None	64
KP16 CST	5	4			20	5	7	PhoQ T244N	None	>64
M3 CST	8	5			38	7	4	PhoQ L348Q	None	>64
51851 vs 46704	10	9			60	11	6	MgrB Q30STOP	None	>64
NCTC 13438 CST					55			PmrB D150Y	None	>64

[a]With respect to wild-type levels, unless otherwise indicated.
[b]Significant fold upregulation compared to WT or pACYC *phoPQ* WT.

smvR and *phoPQ*, other genes analyzed included genes known to be (in)directly regulated by PhoPQ (*pmrD*, *pmrK*, and *pagP*) and *smvA* (Table 4).

Strains with deletions or truncations of *smvR* (M109 CA, NCTC 13439 CA, NCTC 13443 CA, and M3 CA) all showed upregulation in the expression of *smvA* (10- to 27-fold). Strain M109 CA (which was the only strain that did not show increased colistin resistance following chlorhexidine adaptation) showed no change in the expression levels of *phoPQ*. For strains with mutations in *phoPQ* (NCTC 13368 CA, MGH 78578 CA, M3 CA, and NCTC 13443 CA), significant upregulation of *phoP* (5- to 15-fold), *phoQ* (5- to 9-fold), *pmrK* (18- to 64-fold), and *pmrD* (6- to 19-fold) was observed. Interestingly, one strain (NCTC 13439 CA) did show upregulation of *pmrD* (3-fold) and *pmrK* (12-fold) despite there being no upregulation of *phoPQ* expression. Strains with mutations in *PhoPQ*, but not *smvR* (NCTC 13368 CA and MGH 78578 CA), had unaltered expression levels of *smvR* and *smvA* following chlorhexidine adaptation. Strains with mutations in both *phoPQ* and *smvR* had upregulated *pagP* expression (2- to 7-fold); no upregulation of *pagP* was seen in strains with mutations in only *phoPQ* or *smvR*.

The expression levels of genes (*phoP*, *phoQ*, *pmrD*, *pmrK*, *pagP*, *smvA*, and *smvR*) were assessed in strain M109 with pACYC *phoQ* A20P or pACYC *phoPQ* WT. There was slight upregulation in *phoQ* (3-fold) and elevated levels of *pmrK* (7-fold), *pmrD* (2-fold), and *pagP* (4-fold) when pACYC *phoQ* A20P was present versus pACYC *phoPQ* WT (Table 4).

Adaptation to colistin does not lead to an increase in resistance to chlorhexidine. Since adaptation to chlorhexidine has been shown to lead to development of colistin resistance, we wanted to understand if the reverse was true. When *K. pneumoniae* strains were adapted to colistin (here called CST strains), the MIC values for colistin increased (from 1 to 2 mg/liter to 64 to >64 mg/liter), but the susceptibility to chlorhexidine remained the same as that of preexposure strains (Table 4). When whole-genome analysis was carried out on these isolates, strain M3 CST was shown to have a mutation (PhoQ L348Q) identical to that found in strain MGH 78578 CA and no other mutations.

To understand whether colistin-resistant *K. pneumoniae* strains had *phoPQ* expression profiles similar to those of chlorhexidine-adapted strains, we analyzed four strains with different colistin resistance mutations: strains 16 CST (PhoQ T244N), M3 CST (PhoQ L348Q), 51851 (Δ*mgrB*), and NCTC 13438 CST (PmrB D150Y). All of the strains had CST MIC values of 64 mg/liter and CHD MIC values of 16 to 32 mg/liter. Transcriptional analysis showed that these strains had elevated levels of *pmrK* (20- to 60-fold) and, with the exception of 13438 CST, elevated levels of *phoP* (5- to 10-fold), *phoQ* (4- to 9-fold), *pmrD* (5- to 11-fold), and *pagP* (4- to 7-fold). Strains that had mutations in *phoQ* had very similar elevated expression levels compared to chlorhexidine-adapted strains containing mutations in *phoP* or *phoQ* (Table 4).

DISCUSSION

This study has shown that adaptation of clinical *K. pneumoniae* isolates to chlorhexidine exposure can lead to not only stable resistance to chlorhexidine, but also cross-resistance to colistin. This has important clinical implications for the treatment of infections by and outbreaks of MDR (particularly carbapenem-resistant) *K. pneumoniae* isolates, given their increasing prevalence in hospitals (11). Many carbapenem-resistant *K. pneumoniae* isolates are susceptible to very few antibiotics, notably, colistin; treatment often involves combination therapy including colistin (12). Therefore, any potential loss of colistin efficacy has implications for treatment of these infections. While chlorhexidine has been successfully used as part of a multifaceted intervention to reduce the prevalence of carbapenem-resistant *K. pneumoniae* in hospitals (13), the observation that exposure to chlorhexidine leads to colistin resistance means that eradication of potentially colistin- and carbapenem-resistant isolates is very problematic. Since the isolates have also acquired increased resistance to chlorhexidine, this also makes prevention of colonization with the isolates more difficult, which has the potential to either prolong existing outbreaks or lead to new outbreaks.

Genome analysis of chlorhexidine-adapted strains identified mutations in *phoPQ* and/or *smvR*. SmvR is a putative Tet repressor family protein encoded by a gene that is adjacent to and transcribed divergently from the *smvA* gene. Adaptation to chlorhexidine resulted in disruption of *smvR* by either complete deletion or production of a truncated version. SmvA is an efflux pump of the major facilitator superfamily (MFS) and has been implicated in methyl viologen resistance and the efflux of acriflavine and other quaternary ammonium compounds (QACs) in *Salmomella enterica* serovar Typhimurium (14, 15). Upregulation of *smvA* was confirmed in all the strains with nonfunctional SmvR, suggesting that SmvR acts as a repressor of *smvA* and that increased expression of *smvA* leads to increased chlorhexidine resistance. Divergent expression from the same promoter is commonly observed in genes that are negatively regulated by Tet repressors (16). Examples of TetR homologues involved in resistance to biocides are QacR in *Staphylococcus aureus* and EnvR and NemR in *Escherichia coli* (17–19).

The observation that *K. pneumoniae* (Murray) isolates, which do not possess homologues to *smvA* and *smvR*, are highly susceptible to chlorhexidine again suggests that *smvA* is an important efflux pump in chlorhexidine resistance. *E. coli* does not possess a homologue to *smvA* and is significantly more susceptible than *Klebsiella* to chlorhexidine (20). However, potential homologues of *smvA* and *smvR* are found in most species of *Enterobacteriaceae*, as well as other Gram-negative MDR pathogens, e.g., *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. This potentially means that SmvA homologues mediate reduced susceptibility to polycationic antiseptics in other bacteria and that a similar mechanism of adaptation, with deletion of *smvR*, may be observed in other clinically important pathogens. In *A. baumannii*, deletion of another Tet repressor (*adeN*) that is adjacent to an efflux pump operon (*adeIJK*) resulted in acquisition of increased resistance to triclosan (21).

The addition of the EPI CCCP increased susceptibility to chlorhexidine in both the wild-type and chlorhexidine-adapted strains, suggesting that efflux pumps known to be affected by the uncoupling agent, including MFS-type pumps, such as *smvA*, might be involved. In colistin-resistant chlorhexidine-adapted strains, CCCP also increased susceptibility to colistin. This is consistent with other studies suggesting a role for efflux in colistin resistance in a range of Gram-negative pathogens (22). CCCP, due to its function as a general uncoupler of proton motive force, may affect metabolic activity, which has been linked to increased susceptibility to colistin in *A. baumannii* (23) and chlorhexidine in *P. aeruginosa* (24). Indeed, growth of both the WT and CA strains was diminished following the addition of CCCP.

The cross-resistance to colistin following chlorhexidine adaptation is likely due to upregulation of the operon containing *pmrK*. This operon functions to modify LPS by cationic replacement of the phosphate groups with 4-amino-4-deoxy-L-arabinose (L-Ara4N). This reduces the lipid A net negative charge and causes a reduction in the

binding affinity of colistin (25). PhoPQ has been linked to modification of LPS and the outer membrane content (26–28) and regulates genes involved in colistin resistance, including *pmrD* and *pmrAB* (29, 30). Mutations in PhoPQ in *K. pneumoniae* have already been shown to be associated with colistin and cationic peptide resistance and with lipid A modification (29, 31–33). Quantitative PCR (qPCR) analysis of strains with *phoPQ* mutations showed significant upregulation in the expression levels of *phoPQ* (at least 5-fold). For these strains, genes regulated directly or indirectly by *phoPQ* (*pmrD* and *pmrK*) were also upregulated. This upregulation was consistent for *K. pneumoniae* strains that are resistant to both colistin and chlorhexidine or colistin alone. However, there are examples of mutations in PhoQ leading to elevated *phoPQ* expression, but not development of colistin resistance (34). In this study, one chlorhexidine-adapted strain (NCTC 13439 CA) had elevated colistin resistance but no detected mutations in *phoPQ* and did not show upregulation of their expression. However, the strain did have upregulated *pmrD* and *pmrK* expression levels, which suggested that the genes were activated independently of *phoPQ*. The expression levels of *pmrK* are regulated by another two-component regulator implicated in colistin resistance, *pmrAB*. While expression levels of *pmrAB* may be regulated by PhoPQ (through PmrD), *pmrAB* is also transcribed, independently of PhoPQ, from a constitutive promoter within the upstream *pagB* gene (26). Expression of *pmrD* may be subject to regulation by a second, unknown system based on the observation that in *E. coli* expression of *pmrD* was not abolished following inactivation of *phoPQ* (35). In *K. pneumoniae*, upregulation of *pmrD* following adaptation to colistin was not always linked to upregulation of *phoQ* (36). The presence of a mutation in the RarA regulator in NCTC 13439 CA is consistent with upregulation of *rarA* in *K. pneumoniae* enhancing growth on polymyxin B (37). The mutated amino acid (W37R) is predicted to directly interact with DNA, according to the crystal structure of the related regulator MarA (38).

The results demonstrate that PhoPQ mutations arise through chlorhexidine adaptation and can be clearly linked to colistin resistance. However, the data suggest that additional factors are important in mediating resistance to chlorhexidine, and they may operate independently of PhoPQ. qPCR analysis using colistin-adapted strains with specific mutations in PhoQ (T244N and L348Q) showed levels of *phoPQ* expression very similar to those of chlorhexidine-adapted strains containing PhoPQ mutations (NCTC 13368 CA, NCTC 13443 CA, M3 CA, and MGH 78578 CA), suggesting that increased levels of *phoPQ*, *pmrD*, and *pmrK* expression, while important for colistin resistance, are not sufficient for increased resistance to chlorhexidine. This observation was reinforced by colistin resistance, but not chlorhexidine resistance, being transferred on pACYC *phoQ* A20P, which again caused upregulation of *phoQ*, *pmrD*, and *pmrK* relative to pACYC *phoPQ* WT. Identical PhoQ mutations (L348Q) were present in both strains M3 CST and MGH 78578 CA, suggesting that this mutation alone is not sufficient to lead to increased chlorhexidine resistance but is enough to generate colistin resistance. Interestingly, upregulated *pagP* expression was observed in colistin-adapted strains where upregulated *phoPQ* expression was also observed (KP16 CST, M3 CST, and 51851). For chlorhexidine-adapted strains, those with mutations in *phoPQ* but not *smvR* showed no *pagP* upregulation. *pagP* expression does not appear to be dependent upon specific allelic changes, since both MGH 78578 CA and M3 CST have mutations in *phoQ* (L348Q), but only M3 CST showed upregulated *pagP* expression. These observations could imply that upregulated *pagP* expression is antagonistic to chlorhexidine resistance. M3 CA and NCTC 13443 CA have elevated *pagP* levels but also have mutations in *smvR*, in addition to mutations in *phoPQ*. We have demonstrated that *smvR* has a role in chlorhexidine resistance, and it is plausible that mutations in *smvR* "mask" any negative effects on chlorhexidine resistance caused by elevated *pagP* expression. PagP causes palmitoylation of lipid A, which has been found not to contribute to colistin resistance in other pathogens (39), but its role in chlorhexidine resistance has not been investigated. It is possible that increased palmitoylation of lipid A reduces the frequency of other lipid A modifications that are important in chlorhexidine resistance. Future work

will focus on understanding the role of lipid A modification in chlorhexidine resistance, including the roles of PhoPQ, PagP, and MarA.

It is also plausible that mutations in *phoPQ* are secondary-site or compensatory mutations that enable the resistant strain to recover its fitness and virulence following the initial development of biocide resistance (40). PhoPQ is a global regulator and regulates a number of genes important in fitness and virulence (41–43). Again, further work is needed to investigate this.

Overall, this study has identified a novel mechanism of resistance to chlorhexidine (*smvA-smvR*) that may potentially operate in a number of different species. Clearly, increased *smvA* expression is important for chlorhexidine adaptation in *K. pneumoniae*, but it is not the only mechanism and may operate in conjunction with other regulatory processes. Chlorhexidine adaptation is also associated with the generation of mutations in PhoPQ that affect a number of known regulatory targets (notably, *pmrD* and *pmrK*). Upregulation of these genes also correlates with the presence of colistin resistance. The fact that increased colistin and chlorhexidine resistance may occur in clinical isolates without significant loss of fitness/virulence highlights the potential challenges associated with critical infection control procedures and the use of chlorhexidine as an antiseptic to control health care-associated infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *K. pneumoniae* isolates used in this study are clinical strains with a variety of antibiotic resistance markers, e.g., bla_{NDM-1} and bla_{SHV-18}, and have been described previously (9, 10, 44). *K. pneumoniae* was adapted to chlorhexidine as described in a previous study (9). Additional experiments in adaptation to chlorhexidine or colistin were also performed. Briefly, strains were cultured at quarter MICs of chlorhexidine or colistin and passaged every 2 days into fresh medium containing double the previous concentration of chlorhexidine/colistin. This continued for six passages. Stability was measured by passaging the strains 10 times in the absence of selective pressure (chlorhexidine/colistin). All the strains were grown in tryptic soy broth (TSB) with aeration on tryptic soy agar (TSA) plates at 37°C unless otherwise stated. Chlorhexidine digluconate (Sigma) was used throughout and was diluted in sterile water unless otherwise stated.

Determination of MIC/MBC. The MICs of various antibiotics and disinfectants/antiseptics for *K. pneumoniae* isolates were determined using a broth microdilution method with a starting inoculum of 1×10^5 CFU/ml. The optical density at 600 nm (OD_{600}) was measured after 20 h of static incubation at 37°C, and the MIC was defined as the lowest concentration of antibiotic/disinfectant at which no bacterial growth was observed. A change in the MIC was considered significant if at least a 4-fold increase/decrease was observed in three replicate experiments. The EPIs PaβN and CCCP were added at concentrations of 25 and 10 mg/liter, respectively, where required. For MBC testing, 10 μl of suspension was removed from each well of the MIC microtiter plate where no bacterial growth was observed, along with the two wells immediately below the MIC where growth was observed. These were spotted on TSA plates and incubated at 37°C for 24 h. The MBC was defined as the lowest concentration of antibiotic/disinfectant at which no bacterial growth was observed in three replicate experiments.

***G. mellonella* killing assays.** 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 essentially as described by Wand et al. (45). The data were analyzed by the Mantel-Cox method using Prism software version 6 (GraphPad, San Diego, CA, USA).

Whole-genome sequencing of *K. pneumoniae* strains. Genomic DNA was purified using a Wizard genomic DNA purification kit (Promega). DNA was tagged and multiplexed with the Nextera XT DNA kit (Illumina). Whole-genome sequencing of *K. pneumoniae* isolates was performed by PHE-GSDU (Public Health England Genomic Services and Development Unit) on an Illumina (HiSeq 2500) with paired-end read lengths of 150 bp. A minimum 150 Mb of Q30 quality data were obtained for each isolate. FastQ files were quality trimmed using Trimmomatic (46). SPAdes 3.1.1 was used to produce draft chromosomal assemblies, and contigs of less than 1 kb were filtered out (47). FastQ reads from chlorhexidine-exposed isolates were subsequently mapped to their respective wild-type preexposure chromosomal sequence using BWA 0.7.5 (48). Bam format files were generated using Samtools (49), and VCF files were constructed using GATK2 Unified Genotyper (version 0.0.7) (50). They were further filtered using the following filtering criteria to identify high-confidence SNPs: mapping quality, >30; genotype quality, >40; variant ratio, >0.9; read depth, >10. All the above-described sequencing analyses were performed using PHE Galaxy (51). BAM files were visualized in Integrative Genomics Viewer (IGV) version 2.3.55 (Broad Institute). Changes in *phoPQ* and *smvR* regions were verified using Sanger sequencing (Beckmann Genomics, Takeley, United Kingdom) and analyzed using DNAStar Lasergene 10.

Plasmid complementation in *K. pneumoniae*. *phoPQ* and *smvR* genes from both wild-type and chlorhexidine-adapted strains were amplified using primers listed in Table S1 in the supplemental material. They were digested with ClaI and XbaI and ligated into the pACYC-184 cloning vector (NEB).

Plasmids were checked for correct sequence using Sanger sequencing. The resultant plasmids were electroporated into *K. pneumoniae* strains using a MicroPulser (Bio-Rad) and the following settings: 2.5 kV, 25 μF, 200 Ω, and a 0.2-mm-gap cuvette. Subsequent plasmid insertion was tested using the primers pACYC seqF (CGTTTTCAGAGCAAGAGATTAC) and pACYC seqR (GCATTGTTAGATTTCATACACG).

Quantitative PCR. qPCR was used to measure the expression of *phoP*, *phoQ*, *pmrD*, *pmrK*, *pagP*, *smvR*, and *smvA* in the chlorhexidine-adapted and respective WT strains using primers listed in Table S1 in the supplemental material. Triplicate overnight cultures grown in TSB were back diluted to an OD_{600} of 0.1, harvested using RNA protect bacteria reagent (Qiagen) at mid-log phase (OD_{600} of 0.5), and RNA extracted using the RNeasy minikit (Qiagen), including on-column DNase treatment according to the manufacturer's instructions. In addition, 5 μg RNA was treated with a DNA-free kit (Ambion), of which 0.2 μg RNA was reverse transcribed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. qPCR was carried out in at least triplicate on each sample using a StepOnePlus real-time PCR system and Fast SYBR green master mix (Life Technologies). Data were analysed using Expression Suite Software version 1.0.3 (Life Technologies) using *gapA*, *rpoB*, and *infB* as endogenous controls and taking primer efficiency into account.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01162-16.

TEXT S1, PDF file, 0.3 MB.

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