Carbonyl Cyanide m-Chlorophenylhydrazine (CCCP) Reverses Resistance to Colistin, but Not to Carbapenems and Tigecycline in Multidrug-Resistant Enterobacteriaceae

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Background: Carbapenems (CAR), colistin (CST), and tigecycline (TGC) alone or in combination therapy has become the last-resort antibiotics for treating infections caused by multidrug resistant (MDR) bacteria. However, resistance to these reserve antibiotics are increasingly being reported worldwide. Hence, the quest to find other agents that will synergistically restore the efficacy of these antibiotics have increased.

Methods: Sixty-three clinical Enterobacteriaceae isolates comprising of Klebsiella pneumoniae (n = 24), Enterobacter spp. (n = 15), Serratia marcescens (n = 12), Citrobacter freundii (n = 8), Escherichia coli (n = 2), and K. oxytoca/michiganensis (n = 2) with known carbapenem resistance mechanisms and undescribed CST and TGC resistance mechanisms were subjected to broth microdilution and meropenem (MEM) disc synergy test in the presence and absence of carbonyl cyanide m-chlorophenylhydrazine (CCCP), a H⁺ conductor (protonophore).

Results and conclusions: Susceptibility to MEM, imipenem (IMP), CST, and TGC was found in only 2, 0, 17, and 9 isolates respectively. Addition of CCCP reversed resistance to CST, TGC, IMP, and MEM in 44, 3, 0, and 0 isolates respectively; CST had the highest mean minimum inhibitory concentration (MIC) fold change (193.12; p < 0.0001) post CCCP compared to that of MEM (1.70), IMP (1.49) and TGC (1.16). Eight isolates tested positive for the MEM-CCCP disc synergy test. We concluded that CCCP reverse CST resistance in CST-resistant Enterobacteriaceae. Although CCCP is an experimental agent with no therapeutic value clinically, further studies are necessary to decipher the mechanisms underlying the CST-CCCP synergy to inform the development of adjuvants that could be therapeutically effective in CST-resistant infections.

Keywords: carbapenem, efflux pumps, colistin, CCCP, protonophore, tigecycline
INTRODUCTION

Bacterial resistance to last-resort antibiotics viz., carbapenems (CAR), colistin (CST), and tigecycline (TGC), continues to pose a major clinical challenge to infection treatment and management throughout the world (Osei Sekyere, 2016; Osei Sekyere et al., 2016b). Among Gram-negative bacteria, Enterobacteriaceae are commonly implicated in resistance to carbapenems through mechanisms such as carbapenemases, porin downregulation, and/or efflux upregulation (Patel and Bonomo, 2013; Sekyere et al., 2015; Osei Sekyere et al., 2016a). On the other hand, resistance to CST is mediated by lipid A modifications through chromosomal mutations (in mcrB, pmrAB, phoPQ, and/or pmrHFIJKL) or the plasmid-borne mcr-1/2 gene (Olaitan et al., 2014; Osei Sekyere, 2016; Pragasam et al., 2017). TGC is a glycosylcycline that is known to affect protein synthesis by binding to the ribosomal RNA; however, the major mechanism of resistance to TGC is hyperexpression of RND-type efflux pumps (Osei Sekyere et al., 2016b). Mutations in regulatory genes (sexS, ramA, marA, and rraA) leads to up-regulation of AcrAB-ToIC intrinsic RND-type multidrug efflux pumps and TGC resistance (Osei Sekyere et al., 2016b). In addition, acquisition and hyper expression of oqxAB exogenous RND-type multidrug efflux genes confers resistance to TGC (Osei Sekyere et al., 2016b; Pournaras et al., 2016). Due to the importance of the cell envelope in mediating resistance to antibiotics through its physicochemical properties, porin channels, and efflux pumps, attention is being drawn to protonophores such as carbonyl cyanide m-chlorophenylhydrazine (CCCP), which is used as an experimental agent with no therapeutic value clinically (Li et al., 2015), as models to study the interactions between antibiotics and the cell envelope's components (plasma membrane, cell wall and capsule; Spindler et al., 2011; Mohamed et al., 2016; Ni et al., 2016). Protonophores (e.g., CCCP) reduce ATP production and increase membrane permeability in bacteria (Spindler et al., 2011; Park and Ko, 2015; Mohamed et al., 2016; Ni et al., 2016) by interfering with the transmembrane electrochemical gradient and proton motive force (Spindler et al., 2011; Yu et al., 2015). By depolarizing the plasma membrane and reducing ATP production, protonophores such as CCCP can indirectly affect the activity of proton pumps and cellular metabolism to cause cell death (Spindler et al., 2011; Yu et al., 2015). CAR as β-lactam antibiotics, act on the cell wall by inhibiting peptidoglycan synthesis to cause cell lysis (Sekyere et al., 2015; Mohamed et al., 2016). CST is a polymyxin that acts on lipopolysaccharides of the cell membrane to displace Ca\(^{2+}\) ions and lyse the cell membrane (Yu et al., 2015; Mohamed et al., 2016; Osei Sekyere et al., 2016b). Due to the association of the bacterial cell envelope (cell wall, cell membrane, and capsule) with the resistance of these important reserve antibiotics, and the ability of protonophores to influence the permeability and energy of the cell envelope, we sought to investigate the effect of CCCP on the resistance of these antibiotics. CCCP has been reported to reduce efflux activity in CAR-resistant Gram-negative bacteria such that resistance to CAR was reduced or reversed (Huang et al., 2008). It has also been shown to effectively reduce or reverse CST resistance in some Gram-negative bacteria (Park and Ko, 2015; Ni et al., 2016), and its effect on TGC resistance has been shown to be relatively lower or insignificant (He et al., 2015). For instance, the study by Ni et al. (2016) involved only two Klebsiella pneumoniae species out of all the Gram-negative samples/isolates used in that study. Hence, these two K. pneumoniae species, of which only one was colistin-resistant, were the only Enterobacteriaceae species used. The rest were all Gram-negative non-fermenters such as Acinetobacter baumannii, Pseudomonas aeruginosa, and Stenotrophomonas maltophilia. The study by Park and Ko (2015) also involved only A. baumannii and no Enterobacteriaceae. Therefore, to our knowledge, this is surely the only study involving a true representation and substantial population of Enterobacteriaceae.

Moreover, previous studies did not consider the effect of CCCP on CAR, CST, and TGC simultaneously or in tandem (Park and Ko, 2015; Mohamed et al., 2016; Ni et al., 2016). This study attempts to analyse the interaction between CCCP and CAR, CST, and TGC in reversing IMP, MEM, CST, and TGC resistance from a CCCP-antibiotic synergy perspective with the hope that future studies will investigate potential ionophores that can serve as adjuvants to restore the potency of these reserve antibiotics.

MATERIALS AND METHODS

Ethical Approval

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal Ethical approved this study under the reference number BE040/14.

Bacterial Strains

A collection of 63 clinical isolates of multiple lineages comprising of K. pneumoniae (n = 24), Enterobacter species (n = 15), Serratia marcescens (n = 12), Citrobacter freundii (n = 8), Klebsiella oxytoca (n = 2), and Escherichia coli (n = 2) with well-described carbapenem resistance mechanisms (mainly bla\(^{NDM-1}\) and bla\(^{GES-5}\) and unknown CST and TGC resistance mechanisms were used (Table 1); known and well-described CST (mcr-1/2, mutations and insertional inactivation of mcrB, pmrAB, phoPQ, and pmrHFIJKL) and TGC (mutations in acrAB, ramA, ramAR, marABR, and soxS) resistance mechanisms were not found in 48 isolates when their genomes were compared to that of wild type strains (TGC-susceptible strains of each species): K. pneumoniae MGH78578 (accession number CP000647); E. cloacae NCTC 9394 (accession number FP929040.1); E. coli ATCC 25922 (accession number CP009072); S. marcescens ATCC 13880 (accession number KN050642.1). The isolates were obtained from a private pathology laboratory in Durban, South Africa. The isolates were sourced from 10 private hospitals in Durban by the private pathology laboratory.

Forty-eight of the isolates had been previously characterized using whole genome sequencing (WGS; bioproject number PRJNA287968) to characterize their resistome, and 12 had been characterized by PCR (Osei Sekyere et al., 2016a) to identify their carbapenem resistance mechanisms; three isolates had been
### TABLE 1 | Effect of CCCP on the MICs of MEM, IMP, CST, and TGC as well as on the zone diameter of MEM on clinical Enterobacteriaceae isolates.

| Isolate | BMD MIC (mg/L) | WGS\textsuperscript{†}/PCR/CNP\textsuperscript{‡}-confirmed carbapenemases | CCCP-MEM disc synergy test\textsuperscript{§} |
|---------|----------------|-------------------------------------------------|---------------------------------------------|
| **Escherichia coli ATCC 25922** | | | |
| | MEM | MEM+CCCP (\(\mu\text{g}\text{/mL}\)) | IMP\textsuperscript{**} | IMP+CCCP (\(\mu\text{g}\text{/mL}\)) | TGC\textsuperscript{††} | TGC+CCCP (\(\mu\text{g}\text{/mL}\)) | CST\textsuperscript{†††} | CST+CCCP (\(\mu\text{g}\text{/mL}\)) | | |
| ≤0.25 | ≤0.25 (1) | 0.25 | ≤0.25 (1) | ≤0.25 | ≤0.25 (1) | 0.5 | 0.5 (1) | Not determined | – |
| **K. pneumoniae ATCC BAA 1706** | | | | | | | | | | |
| 0.5 | ≤0.25 (≥2) | 0.5 | ≤0.25 (≥2) | ≤0.25 | ≤0.25 (1) | 0.25 | 0.25 (1) | Not determined | – |
| **K. pneumoniae** | | | | | | | | | | |
| C (UNN_S3) | 256 | 128 (2) | 128 | 64 (2) | 4 | 4 (1) | 128 | 1 (128) | bl\textsubscript{NDM}−1 | – |
| D (UNN_S4) | 512 | 128 (4) | 128 | 64 (2) | 8 | 4 (2) | 256 | 0.5 (512) | bl\textsubscript{NDM}−1 | – |
| J (UNN_S9) | 512 | 512 (1) | 256 | 256 (1) | 2 | 2 (1) | 256 | 1 (256) | bl\textsubscript{NDM}−1 | – |
| J (UNN_S10) | 128 | 128 (1) | 256 | 256 (1) | 4 | 4 (1) | 0.5 | 0.5 (1) | bl\textsubscript{NDM}−1 | – |
| 3_S2 | 128 | 128 (1) | 128 | 128 (1) | 2 | 2 (1) | 64 | 0.25 (256) | bl\textsubscript{OXA−232} | – |
| 12_S5 | 64 | 64 (1) | 128 | 128 (1) | 4 | 4 (1) | 64 | 0.25 (256) | bl\textsubscript{NDM}−1 | – |
| 13_S6 | 128 | 128 (1) | 64 | 64 (1) | 1 | 1 (1) | ≤0.5 | ≤0.25 (≥2) | bl\textsubscript{NDM}−1 | – |
| 15_S8 | 16 | 16 (1) | 128 | 128 (1) | 2 | 2 (1) | 128 | 1 (128) | bl\textsubscript{OXA−5} | – |
| 18_S10 | 512 | 256 (2) | 512 | 256 (2) | 4 | 4 (1) | 256 | 1 (256) | bl\textsubscript{OXA−5} | + \\textsuperscript{+++} |
| 20_S11 | 256 | 128 (2) | 128 | 64 (2) | 8 | 8 (1) | 64 | 0.25 (256) | bl\textsubscript{NDM}−1 | – |
| 21_S12 | 512 | 256 (2) | 128 | 128 (1) | 4 | 4 (1) | 64 | 0.5 (128) | bl\textsubscript{NDM}−1 | – |
| 29_S13 | 512 | 256 (2) | 128 | 128 (1) | 8 | 8 (1) | 256 | 0.5 (512) | bl\textsubscript{NDM}−1 | – |
| 30_S14 | 256 | 256 (1) | 128 | 128 (1) | 2 | 2 (1) | 128 | 0.5 (256) | bl\textsubscript{OXA−5} | – |
| 32_S15 | 128 | 128 (1) | 32 | 32 (1) | 4 | 4 (1) | 0.5 | 0.25 (2) | bl\textsubscript{NDM}−1 | – |
| 34_S16 | 512 | 512 (1) | 512 | 512 (1) | 2 | 1 (2) | 256 | 1 (256) | bl\textsubscript{OXA−5} | – |
| 35_S17 | 512 | 256 (2) | 512 | 512 (1) | 8 | 8 (1) | 256 | 1 (256) | bl\textsubscript{OXA−5} | – |
| 36_S18 | 128 | 128 (1) | 128 | 128 (1) | 2 | 2 (1) | 64 | 0.25 (256) | bl\textsubscript{NDM}−1 | – |
| 46 | 16 | 16 (1) | 32 | 32 (1) | 4 | 2 (1) | 32 | 0.125 (256) | bl\textsubscript{OXA−5} | – |
| 52_S26 | 512 | 512 (1) | 256 | 256 (1) | 1 | 1 (1) | 16 | 0.125 (128) | bl\textsubscript{NDM}−1 | – |
| 53_S27 | 256 | 128 (2) | 128 | 64 (2) | 4 | 4 (1) | 256 | 1 (256) | bl\textsubscript{OXA−5} | – |
| 60 | 512 | 256 (2) | 128 | 128 (1) | 1 | 1 (1) | 64 | 1 (64) | CNP\textsuperscript{+++} | + |
| 66 | 512 | 512 (1) | 256 | 256 (1) | 2 | 2 (1) | 1 | 0.125 (8) | bl\textsubscript{NDM}−1 | – |
| 70 | 512 | 256 (2) | 256 | 128 (2) | 4 | 2 (2) | 256 | 1 (256) | CNP\textsuperscript{+++} | – |
| **S. marcescens** | | | | | | | | | | |
| B (UNN38_S2) | >512 | 64 (>8) | 512 | 256 (2) | 4 | 4 (1) | 256 | 1 (256) | bl\textsubscript{NDM}−1 | – |
| E (UNN41_S5) | 128 | 64 (2) | 128 | 128 (1) | 2 | 2 (1) | 256 | 0.25 (1024) | bl\textsubscript{NDM}−1 | + |
| G (UNN43_S7) | 16 | 16 (1) | 512 | 256 (2) | 4 | 2 (2) | 256 | 4 (64) | bl\textsubscript{NDM}−1 | – |
| K (UNN47_S11) | 128 | 64 (2) | 256 | 256 (1) | 4 | 4 (1) | 128 | 2 (64) | bl\textsubscript{NDM}−1 | – |
| L (UNN_S12) | 128 | 64 (2) | 128 | 128 (1) | 4 | 4 (1) | 64 | 2 (32) | bl\textsubscript{NDM}−1 | + |

(Continued)
| Isolate | BMD\(^{-}\) MIC (mg/L) | WGS\(^{T}/\)PCR/CNP\(^{T}\)- confirmed carbapenemases | CCCP-MEM disc synergy test\(^{i}\) |
|---------|------------------------|---------------------------------|-----------------|
| MEM | MEM+CCCP\(^{\alpha}\) | IMP\(^{**}\) | IMP+CCCP\(^{\alpha}\) | TGC\(^{j}\) | TGC+CCCP\(^{\alpha}\) | CST\(^{i}\) | CST+CCCP\(^{\alpha}\) |
| 7_S3 | 64 | 64 (1) | 32 | 32 (1) | 2 | 2 (1) | 128 | 1 (128) | bla\(_{NDM-1}\) | – |
| 45_S21 | 32 | 32 (1) | 32 | 8 (4) | 4 | 4 (1) | 128 | 2 (64) | – | – |
| 56_S29 | 512 | 512 (1) | 256 | 128 (2) | 2 | 2 (1) | 256 | 1 (256) | bla\(_{NDM-1}\) | – |
| 59_S30 | 512 | 256 (2) | 256 | 128 (2) | 2 | 2 (1) | 128 | 2 (64) | bla\(_{NDM-1}\) | – |
| 67_S33 | 256 | 128 (2) | 512 | 256 (2) | 4 | 4 (1) | 64 | 2 (32) | bla\(_{NDM-1}\) | – |
| 68_S34 | 64 | 64 (1) | 32 | 32 (1) | 2 | 2 (1) | 128 | 1 (128) | bla\(_{NDM-1}\) | – |
| 71_S36 | >512 | 512 (> 1) | 128 | 64 (2) | 4 | 4 (1) | 128 | 2 (64) | bla\(_{NDM-1}\) | – |
| **E. cloacae (UNLESS OTHERWISE STATED IN THE FOOTNOTE)** | | | | | | | | | |
| A\(^{***}\) (UNN37_S1) | 64 | 64 (1) | 32 | 16 (2) | 2 | 1 (2) | 64 | 1 (4) | bla\(_{NDM-1}\) | – |
| F\(^{††††}\) (UNN44_S6) | 512 | 512 (1) | 128 | 128 (1) | 4 | 4 (1) | 128 | 0.25 (512) | bla\(_{NDM-1}\) | – |
| H\(^{††††}\) (UNN44_S8) | >512 | 512 (> 1) | 128 | 64 (2) | 2 | 1 (2) | 64 | 1 (64) | bla\(_{NDM-1}\) | + |
| 1_S1 | 2 | 2 (1) | 64 | 64 (1) | 2 | 2 (1) | 128 | 1 (128) | – | – |
| 16_S9\(^{†††††}\) | 256 | 256 (1) | 128 | 128 (1) | 2 | 2 (1) | 16 | 0.25 (64) | bla\(_{NDM-1}\) | – |
| 28 | 128 | 64 (2) | 64 | 32 (2) | 1 | 1 (1) | 256 | 0.5 (512) | bla\(_{NDM-1}\) | – |
| 41 | 512 | 512 (1) | 256 | 256 (1) | 1 | 1 (1) | 256 | 0.5 (512) | bla\(_{NDM-1}\) | – |
| 43_S20\(^{†††††}\) | >512 | 512 (> 1) | 64 | 64 (1) | 4 | 4 (1) | 128 | 1 (128) | bla\(_{NDM-1}\) | – |
| 49_S24\(^{†††††}\) | 512 | 256 (2) | 128 | 128 (1) | 2 | 2 (1) | 256 | 2 (128) | bla\(_{NDM-1}\) | + |
| 51_S25 | >512 | 512 (> 1) | 128 | 64 (2) | 0.25 | 0.25 (1) | 128 | 1 (128) | bla\(_{NDM-1}\) | – |
| 54 | 512 | 256 (2) | 128 | 64 (2) | 0.5 | 0.5 (1) | 32 | 0.125 (256) | bla\(_{NDM-1}\) | – |
| 55_S28\(^{†††††}\) | 512 | 256 (2) | 256 | 128 (2) | 2 | 2 (1) | 128 | 0.5 (256) | bla\(_{NDM-1}\) | – |
| 63_S31\(^{†††††}\) | 512 | 256 (2) | 64 | 64 (1) | 4 | 4 (1) | 64 | 0.5 (128) | – | – |
| 66_S32 | 512 | 512 (1) | 256 | 128 (2) | 4 | 4 (1) | 128 | 0.5 (128) | – | – |
| 74 | 512 | 128 (4) | 256 | 128 (2) | 1 | 0.5 (2) | 128 | 2 (64) | bla\(_{NDM-1}\) | – |
| **E. coli** | | | | | | | | | |
| 10_S4 | >512 | 512 (> 1) | 512 | 512 (1) | 4 | 2 (2) | 64 | 0.125 (512) | bla\(_{NDM-5}\) | – |
| 22 | 512 | 256 (2) | 256 | 256 (1) | 1 | 0.5 (2) | 128 | 2 (64) | bla\(_{NDM-1}\) | – |
| **C. freundii** | | | | | | | | | |
| 4 | >512 | 512 (> 1) | 512 | 512 (1) | 0.5 | 0.5 (1) | 1 | 0.125 (8) | bla\(_{NDM-1}\) | – |
| 9 | 256 | 256 (1) | 128 | 128 (1) | 2 | 1 (2) | 16 | 0.5 (32) | bla\(_{NDM-1}\) | – |
| 14_S7 | 64 | 64 (1) | 32 | 32 (1) | 2 | 2 (1) | 1 | 0.125 (8) | – | – |
| 17 | 256 | 128 (2) | 256 | 128 (2) | 1 | 1 (1) | 128 | 0.5 (256) | CNP+ | – |
Table 1 (Continued)

| Isolate | MEM | MEM+CCCP (A"") | IMP*** | IMP+CCCP (Δ) | TGC# | TGC+CCCP (Δ) | CST‡§ | CST+CCCP (Δ) | WGS†/PCR/CNP‡-confirmed carbapenemases | CCCP-MEM disc synergy test†§ |
|---------|-----|----------------|--------|--------------|------|--------------|-------|--------------|------------------------------------------|-----------------------------|
| 26      | 512 | 218 (2)        | 128    | 32 (4)       | 0.5  | 0.5 (1)      | 256   | 0.5 (512)    | blNDM−1                                  | +                           |
| 27      | >512| 512 (>−1)      | 128    | 64 (2)       | 0.5  | 0.5 (1)      | 32    | 0.125 (256)  | blNDM−1                                  | −                           |
| 48_S23  | 512 | 256 (2)        | 128    | 64 (2)       | 2    | 2 (1)        | 0.25  | 0.25 (1)     | blNDM−1                                  | −                           |
| 72      | 512 | 256 (2)        | 512    | 128 (2)      | 1    | 1 (1)        | 128   | 2 (64)       | blNDM−1                                  | −                           |

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| 2**     | 2   | 2 (1)         | 16     | 8 (2)        | 1    | 1 (1)        | 32    | 0.125 (256)  | CNP+                                     | −                           |
| 69_S35†††††† | 512 | 512 (1) | 128 | 128 (1) | 2 | 2 (1) | 64 | 1 (64) | blNDM−1 | − |

*Microbroth dilution: EUCAST breakpoints (2016) were used for the interpretation.
†Whole genome sequencing results. Known resistance mechanisms of tigecycline and colistin could not be found in the isolates.
‡Carba NP Test: isolates for which PCR or WGS had not been used to confirm their carbapenemase production were confirmed for carbapenemase production using the Carba NP test.
§This was measured with meropenem (MRP) discs and CCCP, an efflux inhibitor. A difference of more than 5mm between the MRP discs and IMP-CCCP discs zone diameters were interpreted as positive for efflux-mediated resistance.
**MIC fold change between antibiotic alone and antibiotic+CCCP i.e. ratio of MIC of antibiotic alone to that of antibiotic+CCCP.
††Impenem. EUCAST resistance breakpoint for imipenem and meropenem (MRP) is >8mg/L; susceptibility breakpoint for both antibiotics is ≤2mg/L.
***Tigecycline. EUCAST resistance breakpoint for tigecycline is >2 mg/L; susceptibility breakpoint is ≤1mg/L.
‡‡Colistin. EUCAST resistance breakpoint for colistin is >2mg/L; susceptibility breakpoint is ≤2mg/L.
** Negative result: the difference in zone diameter between IMP and IMP-CCCP discs is less than 5mm.
†††Positive result: the difference between the IMP and IMP-CCCP discs’ zone diameters is ≥5 mm.
‡‡‡Carba NP test negative for carbapenemase production.
§§§Carba NP test positive for carbapenemase production.
****Enterobacter asburiae.
††††Enterobacter asburiae.
†††††Enterobacter cloacae complex “Hoffmann cluster III”.
††††††Enterobacter kobei.
‡‡‡‡Enterobacter cloacae complex “Hoffmann cluster IV”.
‡‡‡‡‡Enterobacter asburiae.
§§§§Enterobacter asburiae.
§§§§§Klebsiella oxytoca.
†††††††Klebsiella michiganensis.
subjected to the Carba NP test (Table 1; Osei Sekyere et al., 2016a,c) to determine if they were carbapenemase producers. CST and TGC resistance mechanisms could not be investigated in 15 isolates that had not been subjected to whole genome sequencing (WGS; Table 1). Five out of the 63 isolates (60, 45_S21, 1_S1, 65_S32, and 14_S7) were non-carbapenemase-producing whilst K. pneumoniae ATCC BAA 1706 (KP 1076) and E. coli ATCC 25922, which were susceptible to all the antibiotics, were used as controls (Pragasam et al., 2016; Table 1).

Antibiotics, CCCP, VRP, and RSP
Antibiotics, namely MEM, IMP, CST, TGC, and CCCP, which is used herein as an experimental protonophore; as well as efflux pump inhibitors (EPIs) viz., verapamil (VRP) and reserpine (RSP) in powder form were purchased from Sigma-Aldrich (St Louis, USA). VRP is a calcium (Ca²⁺) channel blocker and RSP is a plant-derived efflux pump inhibitor that block MATE, SMR, and ABC-type efflux pumps, particularly in Gram-positive bacteria and in mycobacteria (by VRP); their use in Gram-negative bacteria is so far minimal although efflux inhibition has been reported (Li et al., 2015; Radchenko et al., 2015; Pule et al., 2016). Solutions of VRP were prepared in deionized water whilst that of CCCP was kept constant. EUCAST breakpoints (Supplementary Table S1) to determine if they were carbapenemase producers. The MICs of CCCP, RSP, and VRP were calculated as the ratio of the MICs of the antibiotic alone to that of the antibiotic plus CCCP. A fold change of ≥8 was adopted as significant. The mean MIC fold change per antibiotic per specie was calculated with the following equation:

\[
\text{1/total sample size (n)} \times \text{sum (MIC fold change } \times \text{frequency of fold change)}.
\]

Where the “frequency of fold change” is the number of times a particular MIC fold change was recorded for that antibiotic and specie. Fold changes of >1, ≥2, >8 were used as 1, 2, and 8, respectively, in the mean fold change analysis. The mean fold change per species was translated into a graph using Microsoft Excel™ 2016 (Figure 1).

RESULTS
RSP and VRP Have No Effect on IMP, MEM, CST, and TGC Resistance
The MICs of CCCP, RSP, and VRP in the various species ranged from 8 to 64, 512 to >512, and 512 to >512 mg/L, respectively (Supplementary Table S1). The concentration of CCCP used (10 mg/L) was well below its MIC except for one isolate that had an MIC of 8 mg/L for which 5 mg/L CCCP was used; hence, CCCP toxicity on the bacterial cells used in this study is minimal or null. Addition of sub-MICs of RSP and VRP to the antibiotics resulted in no change in the antibiotics’ MICs (Supplementary Table S1; fold change = 1). Moreover, the influence of MFS, ABC, and SMR efflux pumps that are not inhibited by RSP and VRP in Gram-negative bacteria on these isolates’ MICs cannot be excluded: MATE-type and RND types of efflux pumps are believed to be affected by RSP and VRP respectively (Ribera et al., 2002; Shinabarger et al., 2011; Surendranath et al., 2014; Li et al., 2015; Radchenko et al., 2015).

Per the MICs of CCCP, RSP, and VRP alone as well as that of RSP and VRP plus IMP, MEM, CST, and TGC shown in Supplementary Table S1, there were no changes in the MIC of the antibiotics upon addition of the inhibitors. The fold change was one for all species and antibiotics. Due to the mechanism of resistance RSP and VRP as actual EPIs, the role of efflux pumps...
in conferring resistance to the antibiotics and the probability of CCCP affecting the antibiotics’ MICS indirectly through efflux pumps was ascertained.

**CCCP Interacts with CST, but Not with CAR and TGC**

CCCP reversed resistance to CST in 44 isolates and reduced CST MIC by several (from 2 to 1024) folds in almost all the isolates, with a mean fold change of 193.12; 49 isolates had a CST MIC fold change between 64 and 512 and three had no change at all. CST p-values ranged from <0.0099 to <0.0001 in the various species; see Supplementary Table S3. CCCP reversed resistance to TGC in only three isolates, and the overall mean fold change in TGC MICs was 1.16. CCCP could not reverse resistance to MEM and IMP, and their average MIC fold changes were 1.70 and 1.49, respectively. The mean MIC fold change per species is shown in Figure 1 and the p-values for CST mean fold change per species are shown in Supplementary Table S3; that of IMP, MEM and TGC are not shown as they were far below the ≥eight-fold change cut off and also had insignificant p-values. Susceptibility to TGC, CST, MEM, and IMP in the absence of CCCP was respectively observed in 17, nine, two and zero isolates.

Table 1 summarizes the MICs of MEM, IMP, CST, and TGC alone as well as with CCCP, the carbapenem resistance mechanism of the isolates and the results of the MEM-CCCP disc synergy test. The high level of resistance to MEM (with MICs between 2 and 512 mg/L), IMP (with MICs between 16 and 512 mg/L) and CST (with MICs between 0.5 and 512 mg/L) for all isolates is easily seen from Table 1.

### Four Out of Five MEM-CCCP Positive Isolates Had a Two-Fold MIC Reduction in MEM

Only eight isolates (E, H, L, 18_S10, 26, 36_S18, 49_S24, and 60) tested positive for the CCCP-MEM disc synergy test (Table 1) and all but two (36_S18 and H) of these had a two-fold reduction in MEM MIC upon addition of CCCP. Isolate 60 was the only CAR-resistant isolate that both produced no carbapenemase and tested positive for the MEM-CCCP test.

### CCCP Had No Effect on the MEM and IMP MICs of Non-carbapenemase-producing Isolates

Four of the five CAR-resistant but non-carbapenemase positive isolates (1_S1, 14_S7, 45_S21, 60, and 65_S32) had no change in IMP and MEM MICs; only isolate 60 had an MIC fold change of 2 upon CCCP addition.

### DISCUSSION

Increasing resistance to antibiotics of last-resort is making the research for novel antibiotics as well as for adjuvants that will potentiate the effect of existing ones a present necessity (Osei Sekyere, 2016). Important antibiotics such as β-lactams (e.g., CAR) and CST are known to respectively act on the bacterial cell wall and cell membrane to cause cell lysis and death (Sekyere et al., 2015; Mohamed et al., 2016; Osei Sekyere et al., 2016b). Moreover, RND-type multidrug efflux pump AcrAB-TolC, which is a major contributor to intrinsic multidrug resistance in Enterobacteriaceae, is driven by the proton motive force and is
found in the cell membrane (Ricci et al., 2014; Li et al., 2015; He et al., 2015). These suggest the important role of the cell wall and cell membrane (with embedded efflux pumps) as potential drug targets and as a bacterial defense mechanism against antibiotics. Due to the activity of CCCP as a protonophore that reversibly binds protons (H+) and transports them across the cell membrane, leading to membrane depolarization, eradication of the electrochemical concentration gradient (ECG) and reduced ATP production by ATP synthase (Spindler et al., 2011; Yu et al., 2015; Ni et al., 2016), it was used as an experimental model to investigate how protonophores' effect on the cell membrane could affect the potency of the most important reserve antibiotics: CAR, CST, and TGC.

This study has shown a potential synergy between CCCP and CST in reversing CST resistance among multidrug resistant (MDR) Enterobacteriaceae, which can be explored further to find adjuvants that can restore the efficacy of CST in treating CST-resistant infections. Mohamed et al. (2016) recently showed that CST disrupted both the cell membrane and cell wall to cause cell lysis and death, and that the effect of CST on the cell wall is similar to that of β-lactams. Ni et al. (2016) argued that CCCP's depolarization of the cell membrane might restore the negative charges, which are neutralized or reduced in CST-resistant isolates, thus making the resistant cells susceptible to CST again. On the other hand, Park and Ko (2015) proposed that the reduction in ATP production, and subsequently a reduced metabolic activity after treating cells with CCCP, could be responsible for the enhanced activity of CST as no change was observed in the expression levels of adeABC and adeIJK efflux genes in both resistant and susceptible isolates.

Using the natural producer of CST, Paenibacillus polymyxa, Yu et al. (2015) showed that the addition of Ca2+, and to a lesser extend Mg2+, reduced the bactericidal effect of CST. They thus showed that Ca2+ and/or Mg2+ depletion was important in enhancing CST activity. Putting these findings together with our own, we hypothesize that CCCP depolarization of the membrane potential and reduction of ATP levels leads to a depletion of or imbalance in Ca2+ and/or Mg2+ levels in CST-resistant isolates, which facilitates the easier binding of CST to the lipid A to cause cell lysis and death. However, this hypothesis would need to be verified experimentally. The limited role of efflux pumps in conferring CST resistance has also been observed by other researchers (Park and Ko, 2015; Ni et al., 2016). This is not surprising given that CST mainly acts on the lipid A of the outer lipopolysaccharide membrane (Sekyere et al., 2015).

RSP and VRP could not reverse or reduce IMP, MEM, and TGC MICs (Table 1), providing evidence that they are most likely not involved in conferring resistance to IMP, MEM, CST, and TGC (Osei Sekyere et al., 2016b). Notably, the inactivity of CCCP as well as phenylalanine-arginine β-naphthylamide (PaBN), RSP, and VRP to reverse TGC have been reported, corroborating our findings (Park and Ko, 2015). Thus, the two-fold reduction in TGC MIC by CCCP in 10 isolates as well as the reversal of TGC resistance in three isolates (Table 1) would need further studies to ascertain the mechanism behind this observation.

The possibility that carbapenemases might overshadow the effect of increased efflux pumps activity in the isolates is most likely minimal as RSP and VRP had no effect on the isolates' CAR resistance. Moreover, among the non-carbapenemase producing but CAR-resistant isolates, there was no change in CAR MIC after adding CCCP except in only isolate 60. We hypothesize that the depolarization of the plasma membrane, the subsequent cytoplasmic ion imbalance and reduction of ATP production by CCCP might affect the optimal activity of carbapenemases, which require energy and zinc (in the case of NDM-1) to function (Osei Sekyere et al., 2015) further studies will be necessary to substantiate this.

CCCP enlarged the inhibition zones around the MEM disc on the CCCP-MEM disc synergy test to various sizes (data not shown), albeit a cut-off of ≥5 mm was adopted as positive according to literature (Huang et al., 2008). A pattern of two-fold MIC reduction in MEM after adding CCCP was observed in all but two isolates that tested positive for the MEM-CCCP. Thus, experimental CCCP does have some effect on carbapenem (MEM) resistance that could be investigated further.

Although the peptidomimetic efflux-pump inhibitor, PaBN, is commonly used in Gram-negative bacteria, its effect on CST in Enterobacteriaceae has been found to be insignificant (Opperman and Nguyen, 2015; Park and Ko, 2015).

We thus concluded that CCCP reverses CST resistance in CST-resistant Enterobacteriaceae. Although CCCP is an experimental agent with no therapeutic value clinically, further studies are necessary to decipher the mechanisms underlying the CST-CCCP synergy to inform the development of adjuvants that could be therapeutically effective in CST-resistant infections.

**AUTHOR CONTRIBUTIONS**

JO designed the study, undertook the laboratory work, results analysis and manuscript preparation and formatting. DA took part in the study design and laboratory work, tabulated the BMD results and took part in the revision of the manuscript for scientific merit.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00228/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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