Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Molecular mechanism of antibiotic resistance induced by mono- and twin-chained quaternary ammonium compounds

Yin Jia a, b, Huijie Lu a, Lizhong Zhu a, b, * a College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China b Zhejiang Provincial Key Laboratory of Organic Pollution Process and Control, Hangzhou 310058, China

HIGHLIGHTS
• Increasing concentrations of QACs induced irreversible antibiotic resistance
• Twin-chained DDAC induced higher resistance and more mutations than mono-chained BAC
• 17 mutated genes were identified with direct correlations with antibiotic resistance.
• Mutations causing upregulated efflux pump was a main antibiotic resistance mechanism.

GRAPHICAL ABSTRACT

ABSTRACT
The usage of quaternary ammonium compounds (QACs) as disinfectants has increased dramatically since the outbreak of COVID-19 pandemic, leading to potentially accelerated emergence of antibiotic resistance. Long-term exposure to subinhibitory level QACs can lead to multidrug resistance, but the contribution of mutagenesis to resistance evolution is obscure. In this study, we subcultured E. coli K-12 under subinhibitory (0.25 × and 0.5 × Minimum Inhibitory Concentration, MIC) or inhibitory (1 × and 2 × MIC) concentrations of benzalkonium chloride (BAC, mono-chained) or didecyldimethylammonium chloride (DDAC, twin-chained) for 60 days. The sensitivity of QAC-adapted cells to five typical antibiotics decreased significantly, and in particular, the MIC of rifampicin increased by 85 times. E. coli adapted faster to BAC but developed 20–167% higher antibiotic resistance with 56% more mutations under DDAC exposure. The broader mutations induced by QACs, including negative regulators (acrR, marR, soxR, and crp), outer membrane proteins and transporters (mipA and sbmA), and RNA polymerase (rpoB and rpoC), potentially contributed to the high multi-drug resistance. After QACs stresses were removed, the phenotypic resistance induced by subinhibitory concentrations of QACs was reversible, whereas that induced by inhibitory concentrations of QACs was irreversible. The different patterns and molecular mechanism of antibiotic resistance induced by BAC and DDAC is informative to estimating the risks of broader QACs present at varied concentrations in the environment.

ARTICLE INFO
Editor: Fang Wang

Keywords
Benzalkonium chloride
Didecyldimethylammonium chloride
Antibiotic resistance
Whole-genome sequencing
Mutation

1. Introduction
Antimicrobial resistance (AMR) poses serious threats to public health globally. The abuse of non-antibiotic chemicals greatly promotes the emergence and spread of AMR (Baker-Austin et al., 2006; Li et al., 2016; Wang et al., 2020). Since the coronavirus pandemic in 2020, the use of disinfectants (e.g., surface cleaners, sanitizers) has increased dramatically across the world, which is expected to increase AMR in pathogenic microbes in the coming years (Mahoney et al., 2021). Among the 597 products listed in the U.S. Environmental Protection Agency’s (EPA) List N: Disinfectants

http://dx.doi.org/10.1016/j.scitotenv.2022.155090
Received 15 February 2022; Received in revised form 17 March 2022; Accepted 3 April 2022
Available online 6 April 2022

0048-9697/© 2022 Elsevier B.V. All rights reserved.
for Use Against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), 277 contain quaternary ammonium compounds (QACs) as active ingredients (United States Environmental Protection Agency, 2022). QACs are a group of cationic surfactants widely used as broad-spectrum antimicrobial agents in medical, industrial and household applications, which interact with negatively charged cell membranes to cause destruction and leakage of cell contents (Jennings et al., 2015; Tandukar et al., 2013). QACs environmental concentrations typically range from less than 1 μg/L to 2.8 mg/L, while the surge of their usage during the COVID-19 pandemic has resulted in significant increases in untreated wastewater (from 478 g day−1 to 2061 g day−1, + 351%) and residential dust (from 36.3 μg/g to 58.9 μg/g, + 62%) and other environments (Clara et al., 2007; Ferrer and Furlong, 2001; Hora et al., 2020; Kreuziger et al., 2007; Li and Brownawell, 2010; Martinez-Carballe et al., 2007; Alygizakis et al., 2021; Sun et al., 2003; Zhang et al., 2015; Zheng et al., 2020).

Bacterial phenotypic resistance to antibiotics generally increases in response to QACs, as measured by minimal inhibitory concentration (MIC) (Langsrud et al., 2004; McBain et al., 2004; Mechin et al., 1999). De novo mutation is an important way for bacteria to acquire resistance to varying environmental conditions (Anderson and Hughes, 2014; Gullberg et al., 2011; Long et al., 2016). However, previous studies mostly focused on phenotypic resistance indicated by changes in MIC upon exposure of QACs at constant, subinhibitory concentrations. In a previous study, MICs to antibiotics increased by 3.5- to 7.5-fold after QACs exposure (Bore et al., 2007; Capita et al., 2019; Forbes et al., 2019; Maertens et al., 2020; Soumet et al., 2012). Only a few studies have attempted to attribute the phenotypic resistance to specific gene mutations. For example, knocking out the putative efflux pump gene adeAB in QACs-resistant strains resulted in restored susceptibility to most QACs and antibiotics (Maseda et al., 2009). Examining de novo mutations in a high-throughput manner would allow better elucidation of QACs-induced resistance mechanism. This study aimed to explore the relationship between increased resistance and de novo mutations caused by QACs, and investigate whether different mutations would be developed under constant subinhibitory concentration versus increasing inhibitory concentration of QACs. To achieve this objective, we characterized the phenotypic and genotypic resistance of E. coli in response to two most frequently used QACs, benzalkonium chloride (BAC, mono-chained) and didecylmethylammonium chloride (DDAC, twin-chained). It was hypothesized that resistance induced by QACs were different according to the alkyl chain length of QACs that interact with cell membrane. Two sets of exposure experiments were conducted: continuous exposure to subinhibitory concentrations of QACs, and exposure to increasing inhibitory concentrations of QACs. The MICs of antibiotics were detected through the phenotypic resistance to specific antibiotic strains. Considering the same mutations would allow better elucidation of QACs-induced resistance mechanism. The stability of antibiotic resistance upon alleviating QACs stresses were evaluated. Outcomes from this study are expected to greatly improve current understandings of QACs-induced antibiotic resistance as a result of intensive disinfection, especially during the COVID-19 pandemic.

2. Materials and methods

2.1. QACs, antibiotics, and test strain

Two QACs, benzalkonium chloride (BAC, C6H5CH2N(CH3)2RCl (R = C6H13) and didecylmethylammonium chloride (DDAC, C12H25N(CH3)2Cl) were purchased from Sigma-Aldrich (USA). Ampicillin (Amp), chloramphenicol (Chl), norfloxacin (Nor) were obtained from Aladin (China), and tetracycline (Tet) and rifampin (Rif) were from Macklin (China). The five antibiotics with different antibacterial mechanisms have been widely applied worldwide, and were representational in previous studies on antibiotic resistance (Table S1). The solvent used for dissolving BAC, DDAC, Amp, Nor, Tet, Rif was MilliQ water, while the solvent for Chl was 95% ethanol.

The model bacterium used in this study was Escherichia coli K-12 MG1655 (E. coli). Luria-Bertani (LB) medium was used for culture growth.

E. coli stock from −80 °C freezer was inoculated on LB agar plate using the streak plate method and incubated at 37 °C for 24 h. Then a single colony was isolated from the plate and subsequently grown in liquid LB for 12 h at 37 °C, and 150 rpm to reach an OD600 of 1.0. The cell suspension was used for subsequent experiments. All the experiments were performed with biological triplicates.

2.2. Determination of minimum inhibitory concentrations (MICs)

To identify the initial exposure concentrations of QACs, the MICs of DDAC and BAC were tested. Briefly, an overnight bacteria culture was diluted with sterile 0.9% NaCl solution to an OD600 of 0.1 using a spectrophotometer colorimetric (Shimadzu, Japan). Next, 96-well plates were prepared and 10 μL bacteria suspension was added to each well containing 90 μL of serially, 2-fold diluted BAC or DDAC in Mueller-Hinton Broth (MHB) medium. For the growth control group, 10 μL bacteria suspension was added to 90 μL MHB medium without QACs. For the negative control group, 10 μL sterile 0.9% NaCl solution was used instead of bacteria suspension. For the blank control group, only 100 μL of MHB medium was added. The plates were incubated at 37 °C for 24 h to measure the OD600 of each well using a microplate reader (Tecan, Austria). The MICs were determined as the concentration that inhibited growth by 90% (MIC90) based on OD600. For simplicity, we used MIC instead of MIC90 in the rest. The MIC value of the ancestor strain was defined as the initial MIC (MIC0). Detailed detection methods of reactive oxygen species (ROS) production, cell membrane permeability and transmission electron microscope (TEM) after exposure to QACs below or above MIC0 are described in Test S1-S2.

2.3. QACs exposure and bacteria subculturing

To determine any possible adaptation to QACs, the strains were exposed to increasing subinhibitory concentrations of BAC or DDAC, respectively. The initial exposure concentrations of QACs were based on 0, 0.25, 0.5, 1 × MIC0. The 48-well plates were used for bacterial culture. Specifically, 10 μL overnight culture of wild-type (WT) E. coli was firstly added to each well containing 490 μL of serially, 2-fold diluted QACs in LB medium. After growing at 37 °C and 150 rpm for 24 h, 10 μL of the suspension were taken from each well to the next well of a new plate containing the same concentration of QACs. When growth was observed at the highest inhibitory concentration the suspension was additionally transferred into a well with twice the previous concentration. The cultures were subcultured for 60 days and they eventually adapted to 2 × MIC0 of BAC or DDAC, respectively. During the 60-day exposure, the MIC fold changes of five antibiotics for E. coli that adapted to the highest concentrations of QACs at that time were measured every 5 days.

2.4. Isolation of resistant mutants and hereditary stability test

For the resistance mutant isolation, the last cell cultures under 0, 0.5, 2 × MIC0 of BAC or DDAC exposure were diluted 1:106 and spread on LB agar plates containing individual antibiotics at 1 × MIC0 (i.e., 16 mg/L Chl, 10 mg/L Tet, 8 mg/L Amp, 8 mg/L Rif, 0.5 mg/L Nor). The plates were incubated at 37 °C for 24 h. Colonies grown on the selective plates were considered as potential antibiotic resistance mutants. Three mutants were randomly picked up from each selective plate. The MICs of five antibiotics for these mutants were determined. Considering the same mutations of mutants selected from Amp and Nor plates, as well as the Chl and Rif plates, mutants selected from Chl, Tet and Amp plates were presented in the following sections.

According to MICs values, mutants with the highest resistance under 0.5, 2 × MIC0 of BAC or DDAC exposure were selected from Chl, Tet and Amp plates (namely 0.5BAC-Chl-R, 0.5BAC-Tet-R, 0.5BAC-Amp-R, 2BAC-Chl-R, 2BAC-Tet-R, 2BAC-Amp-R, 0.5DADC-Chl-R, 0.5DADC-Tet-R, 0.5DADC-Amp-R, 2DADC-Chl-R, 2DADC-Tet-R, 2DADC-Amp-R). Then they were diluted 1:1000 and subcultured in LB media without QACs at 37 °C and 150 rpm, the media were changed every 24 h. After 5 days, the
MICs of five antibiotics for these resubcultured mutants were tested again to see whether the resistance were still well kept.

2.5. DNA extraction, whole-genome sequencing (WGS), and variant detection

Mutants with the highest resistance and untreated WT E. coli were cultured in 5 mL liquid LB at 37 °C and 150 rpm. Genomic DNA was extracted using TIANamp Bacteria DNA Kit (Tiangen, China). The harvested DNA was detected by the agarose gel electrophoresis and quantified by NanoDrop™ One (Thermo Scientific, USA).

Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations. The whole genome of WT E. coli was sequenced using Illumina HiSeq PE 150 platform at the Beijing Allwegene Technology Co., Ltd. All good quality paired reads were assembled using the SPAdes (v3.13.0) (Bankevich et al., 2012) into a number of scaffolds. Finally, scaffolds with larger than 500 bp were selected for subsequent analysis. The trimmed reads of WT E. coli were aligned against the E. coli K-12 MG1655 genome from Genbank (NC_000913.3) to assemble the WT E. coli genome.

All isolated mutants DNA was sequenced on an Illumina HiSeq 4000 instrument using 2 × 150 bp paired-end reads. After sequencing, efficient sequencing was compared with WT E. coli genome using BWA software and then marked the repeated reads using SAMtools (Li et al., 2009) software. The SAMtools was also used to detect single nucleotide polymorphism (SNP) and filter SNPs with PE reads less than 4. Annotation of SNP, Insertion/Deletion (InDel) used ANNOVAR (Wang et al., 2010) software. In addition, the statistical analysis was calculated and plotted using R and Perl.

2.6. Quantitative reverse transcription PCR (RT-qPCR)

Based on the SNP/InDel detection, the Chi-R, Tet-R and Amp-R mutants as well as WT E. coli were tested relatively genes expression by RT-qPCR. The strains were cultured in liquid LB with 0.8MIC0 of respective antibiotics (Chi for the Chi-R mutant, Tet for the Tet-R mutant, Amp for the Amp-R mutant) at 37 °C and 150 rpm for 6 h. Bacteria in logarithmic growth were harvested by centrifugation. The total RNA was extracted using a RNAprep Pure Cell/Bacteria Kit (Tiangen, China). RNA samples were converted to cDNA with a FastKing RT Kit (Tiangen, China). RT-qPCR was performed using SuperReal PreMix (SYBR Green) (Tiangen, China) with transcribed cDNA templates on StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) following the instruction. The primers were listed in the Table S2. The relative expression was calculated by the 2−ΔΔCt method and the results were showed as log2 fold change (LFC) (Livak and Schmittgen, 2001). All experiments in this study were carried out with independent biological replicates and repeated at least 3 times.

3. Results and discussion

3.1. Different levels of QACs exposure led to variations in multidrug resistance and stability

The MIC0 values of BAC and DDAC were 20 mg/L and 4.5 mg/L, respectively. ROS production and cell morphology changes in response to QACs at above-MIC0 levels were significant, whereas those under subinhibitory concentrations (0.25 × and 0.5 × MIC0) were insignificant (Fig. S1a and S1c). As QACs are positively charged and act on cell membrane, cell membrane damage was observed at exposure concentration > 0.5 × MIC0 (Fig. S1b). Notably, all exposure concentrations were much lower than the critical micelle concentrations (BAC: 1.41 g/L, DDAC: 0.67 g/L), and thus QACs acted as single molecules or aggregation of several molecules in the solution (Test S3 and Fig. S2).

During the subculturing process, the rate of MIC increase was not constant (Fig. 1). BAC exposure led to increased MIC on 3d (40 mg/L, 2 × MIC0) and 43d (80 mg/L, 4 × MIC0), and a similar pattern was observed during DDAC exposure on 21d (9 mg/L, 2 × MIC0) and 43d (18 mg/L, 4 × MIC0). The ultimate MIC values of BAC and DDAC were 80 mg/L and 18 mg/L (approximately 4 × MIC0), respectively. The initial MIC values of Amp, Chl, Nor, Tet and Rif against E. coli were 8, 16, 0.25, 10 and 8 mg/L, respectively. The MIC values of antibiotics increased rapidly from before to after these adaptations occurred (0–5d and 40–45d for BAC, 20–25d and 40–45d for DDAC). In the last 15 days (40–60d), the MIC values remained unchanged (Table S3). The final MIC values of Amp, Chl, Nor, Tet and Rif were 32, 128, 1.7 ± 0.3, 66.7 ± 11.5, 256 mg/L after BAC exposure, and 32, 213.3 ± 37.0, 2, 80, 682.7 ± 147.8 mg/L after DDAC exposure. The adaptation to BAC occurred faster than that of DDAC, and as expected, MIC values of the five tested antibiotics also increased earlier under BAC exposure than that of DDAC (0–20d). Compared to mono-chained BAC, twin-chained DDAC replaces the benzyl group of BAC with an additional long carbon side chain, exhibiting better antimicrobial activity, and thus the strains under BAC exposure adapt faster to QACs (Wang et al., 2018). However, in terms of resistance to antibiotics, the MIC values of Chl, Tet, and Rif under DDAC exposure increased to higher degrees than that of BAC exposure. Among the five antibiotics, Rif had the greatest variations in MIC values (increased to 32.00 and 85.33 ± 18.48 times of MIC0 under BAC exposure, and 32, 213.3 ± 37.0, 2, 80, 682.7 ± 147.8 after DDAC exposure. The adaptation to BAC occurred faster than that of DDAC, and as expected, MIC values of the five tested antibiotics also increased earlier under BAC exposure than that of DDAC (0–20d). Compared to mono-chained BAC, twin-chained DDAC replaces the benzyl group of BAC with an additional long carbon side chain, exhibiting better antimicrobial activity, and thus the strains under BAC exposure adapt faster to QACs (Wang et al., 2018). However, in terms of resistance to antibiotics, the MIC values of Chl, Tet, and Rif under DDAC exposure increased to higher degrees than that of BAC exposure. Among the five antibiotics, Rif had the greatest variations in MIC values (increased to 32.00 and 85.33 ± 18.48 times of MIC0 under BAC and DDAC exposure, respectively). This indicated potentially stable acquisition of genetic resistance to Rif in addition to an expression of intrinsic resistance, which generally leads to a small increase in resistance.

To investigate whether E. coli would lose the antibiotic resistance developed under different levels of QACs exposure, the mutants were cultivated for another 5 days without QACs (endpoint subculture: P5, Fig. 2). The 0.5 × MIC0-mutants exhibited high resistance to Rif (MIC values were 10.67 ± 4.62 to 26.67 ± 9.24 times of the WT strains, p < 0.0001),
which decreased significantly in the P5 subcultures (MIC values were 5.33 ± 2.31 to 13.33 ± 4.62 times of the WT strains, \( p < 0.05 \)), indicating a rapid loss of resistance. On the other hand, the high resistance of \( 2 \times \text{MIC}_0 \)-mutants to multiple antibiotics remained in their P5 subcultures (Table S4). The resistance of \( 2 \times \text{MIC}_0 \)-mutants was inheritable, and the mutagenic effects occurred during adaptation rather than exposure to QACs at subinhibitory concentrations. As bacteria proliferate, antibiotic resistance continuously increased, posing higher risks of gene transfer in the environment.

### 3.2. Genetic mutations under QACs exposure

To understand the molecular mechanism of QACs-induced antibiotic resistance, the \( 0.5 \times \text{MIC}_0 \)-mutants and \( 2 \times \text{MIC}_0 \)-mutants with the highest MIC values against five antibiotics were selected for WGS. A total of 19 SNPs and 1 Indel (\( \text{acrR} \) indel mutation with 1-bp insertion resulted in a frameshift) in 17 genes were detected (Table S5). Except for the synonymous mutation of \( \text{fusA} \), the remaining SNPs were nonsynonymous, which can be grouped into six categories according to their functions: (i) RNA transcription (\( \text{rpoB}, \text{rpoC} \)), (ii) regulator (\( \text{acrR}, \text{marR}, \text{soxR}, \text{crp}, \text{lon} \)), (iii) drug transporter, efflux pumps (\( \text{msbA}, \text{dinF} \)), (iv) cell membrane synthesis and transporter (\( \text{mipA}, \text{sbmA} \)), (v) translation (\( \text{fusA}, \text{rplE} \)) and (vi) metabolic processes (\( \text{plsB}, \text{glgC}, \text{maeA}, \text{cysC} \)) (Fig. 3a).

\( 2 \times \text{MIC}_0 \)-mutants harbored more mutated genes than the \( 0.5 \times \text{MIC}_0 \)-mutants (16 v.s. 2, Fig. 3b and Table S5). \( \text{E. coli} \) inclined to acquire resistance through gene mutations during the adaptation to QACs. Mutation is one of the basic processes by which genetic variation emerges in bacterial

---

**Fig. 2.** Antibiotic MICs of putative mutants BAC-Chl-R (a), BAC-Tet-R (b), BAC-Amp-R (c), DDAC-Chl-R (d), DDAC-Tet-R (e), DDAC-Amp-R (f) at the end of subculturing process (P0) and 5 days after cultivation in the absence of BAC or DDAC (P5) with biological triplicates. The mutants were isolated from antibiotic plates inoculated with subcultures exposed to BAC or DDAC at 0.5MIC and 2MIC for 60 days. Values on the y-axis represent fold changes in MIC compared to those of WT strains. Statistically significant differences were denoted by asterisks: *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \).
populations (Didelot and Maiden, 2010). $2 \times \text{MIC}_0$-mutants could pass on their resistance to offsprings without continued QACs exposure (Fig. 2). In contrast, the resistance of $0.5 \times \text{MIC}_0$-mutants may originate from upregulated expression of intrinsic resistance under QACs, and the cells returned to the sensitive state upon withdrawal of QACs stresses.

BAC and DDAC exposure commonly induced 6 mutations in rpoB and rpoC encoding RNA polymerase (RNAP), and acrR, marR, soxR, crp encoding the regulators of AcrAB-ToIC and MdtEF efflux pumps (Fig. 3c). All of these genes have documented associations with antibiotic resistance (discussed in Section 3.3). In addition, the mutated genes induced by DDAC included msbA, dinF, fuaA, rplE, plsB, glgC, maeA and cySC, which were mainly related to drug transport, bacterial protein synthesis and metabolism. BAC induced mutations in mipA, sbmA and lon, which were mainly related to cell membrane structure, transporter, and efflux related proteins. The different antibiotics used for screening mutants had limited effects on gene mutations (Fig. 3d), indicating that mutations did occur during the 960-generation, 60-day subculturing process.

### 3.3. Association of mutated genes with antibiotic resistance

Among the mutated genes identified, rpoB encodes the beta subunit of RNAP, which is the target of rifampicin (Jindani et al., 2003). It was reported that rpoB could evolve quickly upon rifampicin exposure (Goldstein, 2014). Mutations in specific positions of the coding sequences are known to affect the binding of drugs to RNAP, leading to drug resistance (Hausreiter et al., 2019). Mutations in rpoB burdened a fitness cost in the absence of antibiotics, while compensatory mutations in rpoC could reduce the fitness of rifampicin-resistant bacteria (Brandis et al., 2012; Comas et al., 2012; de Vos et al., 2013; Tawgozy and Qarasnji, 2020). The specific SNPs detected in rpoB (Asp342 → Gly) and rpoC (Ala787 → Val) have not been described so far. However, the same site was mutated (Ala787 → Thr) in a plasmid-borne rpoC constructed for E. coli (Ito and Nakamura, 1996). These mutations occurred in 2BAC-R and 2DDAC-R strains (Table S5), and they were deemed to play important roles in acquiring heritable rifampicin resistance (Fig. 4c).

Another major class of mutated genes were regulators of multidrug efflux pumps, e.g., AcrAB-ToIC (acrR, soxR, marR) and MdtEF (crp). AcrAB-ToIC, a tripartite system consisting of a periplasmic protein (AcrA), a transport protein (AcrB) and an accessory protein channel (ToIC), is the main multidrug efflux machinery in E. coli, endowing bacteria with resistance to broad-spectrum antibiotics (Du et al., 2015; Okusu et al., 1996; Ramos et al., 2014). The expression of acrAB was regulated by local repressor genes, including the adjacent acrR gene, as well as by marR and soxR genes. Mutations in these genes might result in acrAB overexpression and multidrug resistance (Bialek-Davenet et al., 2011). In this study, two mutations in acrR occurred in 0.5DDAC-Tet-R (Trp4 → Arg) and BAC-induced mutants (except 0.5BAC-R and 2BAC-Amp-R, Table S5). The specific SNPs in soxR (Leu95 → Ser) and marR (Val45 → Gly) were detected in 2BAC-R and 2DDAC-R mutants (Table S5). Furthermore, the mutation in lon (Lys35 → Glu) detected in 2BAC-R mutants was also considered to influence the expression of acrAB, because MarA, a positive regulator governing acrAB expression, was identified as a downstream target of Lon protease (Bhaskarla et al., 2016). Similarly, the mutations in crp, a repressor of MdtEF, might increase the expression of mdtEF genes, leading to antibiotics extrusion (Hirakawa et al., 2006). The variations in crp occurred in 2DDAC-R (Asp54 → Gly), 2BAC-R (Gln171 → Lys), 2BAC-R and 2DDAC-R (Phe63 → Ser) (Table S5). These mutations have not been reported before.

Mutations also occurred in drug transporter genes. The 2DDAC-R mutants had a mutation in the msbA (Ala247 → Val) gene (Table S5). It encodes the inner membrane ATP-binding cassette (ABC) transporter MsbA, which can extrude antibiotics and toxic ions from the cell (Alexander et al., 2018; Reuter et al., 2003; Woebbing et al., 2008). It has been reported that mutations in the msbA gene of Pseudomonas aeruginosa were related to antibiotic resistance (Diez-Aguilar et al., 2021). Therefore, we suspected
the increased antibiotic resistance could be partially attributed to the mbdA mutation in E. coli. Another mutated gene related to drug transport was dinF, encoding the DNA damage-inducible protein F, a member of the multi-antimicrobial extrusion superfamily associated with SOS response (Brown et al., 2007). The mutations of the dinF gene exhibited increasing susceptibility to quinolones antibiotics and low ability to grow in the presence of various toxic compounds (e.g., antibiotics, phytoalexins, and detergents) (Brown et al., 2007; Tocci et al., 2013). Conversely, in our study, the mutants of 2DDAC-Amp-R and 2DDAC-Tet-R with mutation in dinF (Leu339 → Ile) had increased resistance to ciprofloxacin and other antibiotics (Table S4). A recent study showed that in the absence of dinF, E. coli were tolerant to periplanetasin-4 (a novel antimicrobial peptide), which damaged DNA and initiated SOS response by accumulating nitric oxide (a free radical with high toxicity) (Lee et al., 2021). In this context, the dinF gene mutation may have similar consequences by influencing the intracellular damage pathway which deserves experimental validation.

The mutations in mipA (Gly31 → Asp) and sbmA (Arg37 → Pro) in 2BAC-R mutants were associated with cell membrane structure and transporters (Table S5). mipA encodes an outer membrane protein called MltA-interacting protein (MipA), a novel antibiotic resistance-related protein. The deletion of mipA in E. coli resulted in the increase in MICs of kanamycin, chloramphenicol and aureomycin (Dan-feng et al., 2015). The sbmA gene encodes an inner membrane protein (SbmA) that contains eight transmembrane regions, which can transport peptide antibiotics into cytoplasm, and thereby its correlation with increased antibiotic resistance was weak. rsbA encoding the 50S ribosomal subunit protein L5 is important to cell viability, whereas its relationship with antibiotic resistance has not been reported.

QACs induced various non-canonical mutations of metabolic genes, plsB gene is involved in glycerol-3-phosphate metabolism, which plays an important role in phospholipid synthesis and persister formation (a specialized cell state that exhibit multidrug tolerance, including the ability to survive by antibiotics killing) (Lewis, 2008; Spoering et al., 2006). plsB gene mutation could diminish the rate of phospholipid synthesis, resulting in dormancy and increase in the level of persisters (Spoering et al., 2006). Based on this, we suspected the specific SNP in plsB (Gly130 → Ser) occurring in 2DDAC-R mutants may contribute to the increase of antibiotic resistance (Table S5). The exact mechanism deserves further investigation. Mutations in glgC, maeA and cysC, which encode ADP-glucose pyrophosphorylase, malate dehydrogenase and adenylyl-sulfate kinase, respectively, may significantly affect metabolism. For example, the mutations in glgC could empower evolved bacteria with abilities to inhibit or kill their ancestors (Lemmonnier et al., 2008).

Fig. 4. Proposed antibiotic resistance mechanism of QACs-induced E. coli mutants: (a) stimulated efflux expression; (b) impeded transmembrane transport; (c) modified antibacterial targets. Mutated genes were marked in gray. Proteins (in ellipses) with changes in amino acid sequences were denoted by diamonds. Green/red arrows represent down/up-regulations in gene expressions. LPS: lipopolysaccharide, OM: outer membrane, IM: inner membrane.

3.4. Expression of efflux related genes

Based on the association of mutated genes with antibiotic resistance, we hypothesized that certain genetic mutations could influence gene expressions contributing to antibiotic resistance, especially those related to efflux pumps and cell membrane structure (Fig. 4a-b). To verify this hypothesis, we measured the expression levels of mutated genes involving (i) regulator, (ii) drug transporter and efflux, and (iii) cell membrane structure and transporter.

Antibiotics extrusion is an intrinsic resistance mechanism of E. coli, i.e., strains without mutations of efflux pump related genes could also have up-regulated expressions of these genes (Fig. 5b). On the other hand, mutations of negative regulators can enhance the expression of this intrinsic resistance. This occurred during the subculturing or adaptation of E. coli to QACs. Specifically, mutated regulator genes (acrR, marR, soxR, crp, lon) showed decreases in expression, but the expression of positive regulatory genes (marA, soxS) acting on the downstream of these regulators increased (Fig. 5a). As a result, the expression of genes coding for multidrug efflux pumps (acrA, acrB, tolC, mdtE, mdtF) increased (Fig. 5b). For example, gene acrR, a negative regulator of AcrAB-TolC efflux, showed a decreased LFC of −3.90, −4.77, −3.75, −2.49, −0.75, −5.96, −3.45,
−4.94, −4.09 in the 0.5DDAC-R, 2BAC-R, 2DDAC-R mutants, respectively, while its expression in the 0.5BAC-R mutant without acrR mutation remained unchanged (Fig. 5a). The expressions of other negative regulatory genes were downregulated (marR, soxR in 2BAC-R and 2DDAC-R; lon in 2BAC-R) (Fig. 4a). In contrast, the positive regulatory genes (marA, soxS) were up-regulated. Genes coding for AcrAB-TolC efflux pumps (acrA, acrB, tolC) were upregulated. Similarly, the genes related to MdtEF efflux pumps (mdtE, mdtF) had higher expression in 2BAC-R and 2DDAC-R with mutations in the negative regulatory gene crp, which itself showed decreased expression (Fig. 5a-b).

The LFC reduced to −0.99, −1.57, −1.09 for msbA and 0.58, 0.26, 0.56 for dinF (Fig. 5b). This appeared to contradict our findings of increasing antibiotic resistance in 2DDAC-R mutants, as MsbA and DinF were responsible for extruding antibiotics (Alexander et al., 2018; Brown et al., 2007; Reuter et al., 2003; Tocci et al., 2013; Woebking et al., 2008). Additionally, the LFC values of these genes were below 2. Therefore, we suspected that AcrAB-TolC and MdtEF efflux pumps, rather than the two drug transporters, played a major role in extruding antibiotics. LFC of 2BAC-R with sbmA and mipA mutations decreased to 0.95, 1.08, 1.08 (sbmA) and 0.51, 0.29, 0.47 (mipA), although the expression still increased compared to the WT strain (Fig. 5c). The above results indicated that mutations in sbmA and mipA led to decreases in their expressions, which may in turn reduce the transport of antibiotics via these two membrane transporters.

4. Conclusions

Compared with mono-chained BAC, twin-chained DDAC led to slower development but higher level of antibiotic resistance in E. coli. Mutated genes conferring QAC-induced antibiotic resistance were associated with negative regulators (acrR, marR, soxR, crp), cell membrane synthesis and transporters (mipa, sbmA), and RNAp (rpoB, rpoC). At sub-MIC concentrations, QACs-induced antibiotic resistance arose through upregulated gene expressions was largely reversible when QACs stresses were removed. Exposure at 2 × MIC resulted in mutations that facilitated the development of stronger and irreversible resistance. DDAC with long alkyl chain had higher hydrophobicity, stronger antibacterial activity, more gene mutations and higher resistance levels, which may have a greater impact on the enhancement of environmental resistance. The evolution and potential propagation of antibiotic resistance in response to increasing environmental concentrations of QACs during the pandemic, in particular DDAC, deserves critical attention.

CRediT authorship contribution statement

Yin Jia: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. Huijie Lu: Investigation, Visualization, Writing – original draft, Writing – review & editing. Lizhong Zhu: Conceptualization, Investigation, Funding acquisition, Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (22193061 and 21836003) and National Key Research and Development Program of China (2020YFC1806903).
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.155090.

References

Alexander, M.K., Miu, A., Beck, M., Stensdal, B., Drancourt, M., Fournier, P., 2009. Conservation of plasmid-borne ampicillin resistance in Escherichia coli adapted to benzalkonium chloride or exposed to stress-inducers. J. Appl. Microbiol. 106, 208–214. https://doi.org/10.1111/j.1365-2672.2008.02140.x.

Lee, M.K., Hwang, J.S., Lee, D.G., 2007. DNA elcF elcin nitric oxide signaling induced by Periplasm873a from American Cockroach in Escherichia coli.Curr. Microbiol. 76, 3550–3561. https://doi.org/10.1007/s00284-007-02615-5.

Lemonnier, M., Levin, B.R., Rome, G., Garber, K., Baquero, M.-R., Mercante, J., Lemichere, E., Baquero, F., Blazquez, J., 2008. The evolution of contact-dependent inhibition in non-pathogenic Pseudomonas aeruginosa isolates. J. Antimicrob. Chemother. 65, 987–992. https://doi.org/10.1093/jac/dkn006.

Liu, X., Brownwell, B.J., 2010. Quaternary ammonium compounds in urban estuarine sediments – a class of contaminants in need of increased attention? Environ. Sci. Technol. 44, 7561–7568. https://doi.org/10.1021/es1011669.

Li, H., Handshock, B., Wysock, A., Fenello, T., Ruan, J., Homer, N., Muth, G., Abecasis, G., Durbin, R., Genome Project Data, P., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2087. https://doi.org/10.1093/bioinformatics/btp352.

Li, D., Zhang, Y.Z., He, M., Gu, A., 2012. Water disinfection byproducts induce antibiotic resistance role of environmental pollutants in resistance phenomena. Environment. Sci. Technol. 50, 3193–3201. https://doi.org/10.1021/es505113s.

Lival, K.J., Smitttig, C.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25, 402–408. https://doi.org/10.1016/S1046-623X(01)00368-9.

Long, H., Miller, S.F., Strauss, A., Zhao, C., Cheng, L., Ye, Z., Griffin, K., Te, R., Lee, H., Chen, C.-C., Lynch, M., 2016. Antibiotic treatment enhances the genome-wide mutation rate of target cells. Proc. Natl. Acad. Sci. U. S. A. 113, 2498–2505. https://doi.org/10.1073/pnas.1602180113.

Maertens, H., Demeyere, K., De Reu, K., Dewulf, J., Vanhautegheim, D., Van Coillie, E., Meyer, H., Muyldermans, S., Demedts, P., 2018. Novel dsRNA-resistant protein activator of the Rho GTPase Rac1 and GTPase-activating protein of Rho proteins. PNAS. 115, 2503–2508. https://doi.org/10.1073/pnas.1717724115.

McBain, A.J., Ledder, R.G., Moore, L.E., Catrenich, C.E., Gilbert, P., 2004. Effects of the chemical content of untreated wastewater of Athens, Greece under the SARS-CoV-2 pandemic and beyond: Consideration of environmental implications. Environ. Sci. Technol. 48, 2588–2594. https://doi.org/10.1021/es001742v.

Mechling, J., Criado-Brusque, L., Helander, C., Fiedler, R.G., Fiedler, C.E., Gilbert, P., 2004. Effects of quaternary-ammonium based formulations on bacterial community dynamics and antimicrobial susceptibility. Appl. Environ. Microbiol. 70, 3449–3456. https://doi.org/10.1128/aem.70.7.3449-3456.2004.

Mechling, J., Criado-Brusque, L., Helander, C., Fiedler, R.G., Fiedler, C.E., Gilbert, P., 2003. Consideration of quaternary-ammonium based formulations on bacterial community dynamics and antimicrobial susceptibility. Appl. Environ. Microbiol. 70, 3449–3456. https://doi.org/10.1128/aem.70.7.3449-3456.2004.

Meurer, P., Fournier, P., 2007. Estimation of plasmid-borne ampicillin resistance in Escherichia coli K-12. J. Bacteriol. 189, 6597–6608. https://doi.org/10.1128/JB.00967-07.

Molina, J., Delgado-Rojo, M., 2007. Benzo[a]pyrene diol epoxide-induced DNA adducts in human bronchial epithelial cells: Clinical relevance of genotoxic damage. Toxicol. Appl. Pharmacol. 222, 277–287. https://doi.org/10.1016/j.taap.2007.07.003.

Mougin, M., Ballivet, N., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7. https://doi.org/10.1371/journal.ppat.1002043.

Nishino, K., Yamaguchi, A., 1996. Selection and characterization of blueprint plasmid–resistant Escherichia coli strains. J. Gen. Microbiol. 142, 197–208. https://doi.org/10.1099/0022166x-142-1-197.

Okusu, H., Ma, D., Nikaido, H., 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multi-antibiotic resistance (Mar) mutants. J. Bacteriol. 178, 306–308. https://doi.org/10.1128/JB.178.8.306-308.1996.
O’Neill, A.J., McLaws, F., Kahninger, G., Henniksen, A.S., Chopra, L. 2007. Genetic basis of resistance to fusidic acid in staphylococci. Antimicrob. Agents Chemother. 51, 1737–1740. https://doi.org/10.1128/AAC.01542-06.

Ramos, P.I., Picão, R.C., Almeida, L.G., Lima, N.C., Girardello, R., Vivan, A.C., Xavier, D.E., Barcellos, F.G., Pelisson, M., Vespero, E.C., Mêdigue, C., Vasconcelos, A.T., Gales, A.C., Nicolas, M.F., 2014. Comparative analysis of the complete genome of KPC-2-producing Klebsiella pneumoniae Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms. BMC Genom. 15, 54. https://doi.org/10.1186/1471-2164-15-54.

Reuter, G., Janvilisri, T., Venter, H., Shahi, S., Balakrishnan, L., van Veen, H.W., 2003. The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities. J. Biol. Chem. 278, 35193–35198. https://doi.org/10.1074/jbc.M306226200.

Soumet, C., Fourreau, E., Legrandois, P., Maris, P., 2012. Resistance to phenicol compounds following adaptation to quaternary ammonium compounds in Escherichia coli. Vet. Microbiol. 158, 147–152. https://doi.org/10.1016/j.vetmic.2012.01.030.

Spoering, A.L., Vulic, M., Lewis, K., 2006. GlpD and PlsB participate in persister cell formation in Escherichia coli. J. Bacteriol. 188, 5136–5144. https://doi.org/10.1128/jb.00369-06.

Soumet, C., Fourreau, E., Legrandois, P., Maris, P., 2012. Resistance to phenicol compounds following adaptation to quaternary ammonium compounds in Escherichia coli. Vet. Microbiol. 158, 147–152. https://doi.org/10.1016/j.vetmic.2012.01.030.

Spoering, A.L., Vulic, M., Lewis, K., 2006. GlpD and PlsB participate in persister cell formation in Escherichia coli. J. Bacteriol. 188, 5136–5144. https://doi.org/10.1128/jb.00369-06.

Sun, H.F., Takata, A., Hata, N., Kasahara, I., Taguchi, S., 2003. Transportation and fate of cationic surfactant in river water. J. Environ. Monit. 5, 891–895. https://doi.org/10.1039/b308988f.

Tandukar, M., Oh, S., Tezel, U., Konstantinidis, K.T., Pavlostathis, S.G., 2013. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. Environ. Sci. Technol. 47, 9730–9738. https://doi.org/10.1021/es400570k.

Tawgozy, F.H.A., Qurami, B.K., 2020. Molecular analysis of rifampicin resistance conferring mutations in mycobacterium tuberculosis. Baghdad Sci. J. 17, 444–451. https://doi.org/10.21123/bsj.2020.17.2.0444.

Tocci, N., Iannelli, F., Boidoni, A., Ciuna, M.L., Decorosi, F., Viti, C., Pozzi, G., Ricci, S., Oggoni, M.R., 2013. Functional analysis of pneumococcal drug efflux pumps associates the MATE DinF transporter with quinolone susceptibility. Antimicrob. Agents Chemother. 57, 248–253. https://doi.org/10.1128/aac.01298-12.

United States Environmental Protection Agency, 2022. List N: Disinfectants for Use Against SARS-CoV-2. https://www.epa.gov/pesticide-registration/list-n-disinfectants-use-against-sars-cov-2. (Accessed 15 February 2022).

de Vos, M., Mueller, B., Borrell, S., Black, P.A., van Helden, P.D., Warren, R.M., Gagneux, S., Victor, T.C., 2013. Putative compensatory mutations in the rpoC gene of rifampin-resistant mycobacterium tuberculosis are associated with ongoing transmission. Antimicrob. Agents Chemother. 57, 827–832. https://doi.org/10.1128/aac.01541-12.

Wang, K., Li, M.Y., Hakonarson, H., 2010. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 38, 7. https://doi.org/10.1093/nar/gkq603.

Wang, S.P., Wang, H.H., Ren, B.A., Li, X.D., Wang, L., Zhou, H., Weir, M.D., Zhou, X.D., Masri, R.M., Oastes, T.W., Cheng, L., Xu, H.H.K., 2018. Drug resistance of oral bacteria to new antibacterial dental monomer dimethyaminohexadecyl methacrylate. Sci. Rep. 8, 11. https://doi.org/10.1038/s41598-018-23831-3.

Wang, Y., Lu, J., Engelstadter, J., Zhang, S., Ding, P.B., Mao, L.K., Yuan, Z.G., Bond, P.L., Guo, J.H., 2020. Non-antibiotic pharmaceuticals enhance the transmission of exogenous antibiotic resistance genes through bacterial transformation. ISME J. 14, 2179–2196. https://doi.org/10.1038/s41396-020-0679-2.

Woebbing, B., Velamakanni, S., Federici, L., Seeger, M.A., Murakami, S., van Veen, H.W., 2008. Functional role of transmembrane helix 6 in drug binding and transport by the ABC transporter MsbA. Biochemistry 47, 10904–10914. https://doi.org/10.1021/bi800778d.

Zheng, G., Filippelli, G.M., Salamova, A., 2020. Increased indoor exposure to commonly used disinfectants during the COVID-19 pandemic. Environ. Sci. Technol. Lett. 7, 760–765. https://doi.org/10.1021/acs.estlett.0c00587.