Molecular mechanism of antibiotic resistance induced by mono- and twin-chained quaternary ammonium compounds

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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
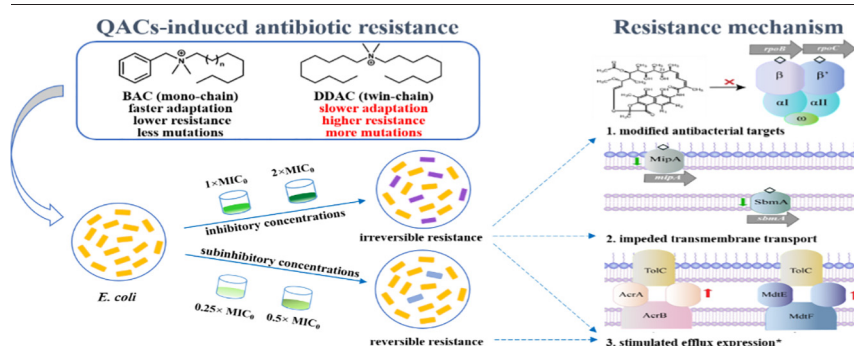
ABSTRACT

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.

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

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}, +331%), 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; Kreuzinger et al., 2007; Li and Brownawell, 2010; Martinez-Carballo 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 antibiotic resistance under changing environmental conditions (Andersson 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 *sdeAB* 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 didecyldimethylammonium 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 60-day subculturing process. Mutations were identified by whole-genome sequencing to infer 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, $C_6H_5CH_2N(CH_3)_2RCl$ (R = C_8H_{17} to $C_{18}H_{37}$)) and didecyldimethylammonium chloride (DDAC, $C_{22}H_{48}ClN$) 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 MillQ 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 OD_{600} 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 OD_{600} 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 OD_{600} of each well using a microplate reader (Tecan, Austria). The MICs were determined as the concentration that inhibited growth by 90% (MIC90) based on OD_{600}. For simplicity, we used MIC instead of MIC90 in the rest. The MIC value of the ancestor strain was defined as the initial MIC (MIC_0). Detailed detection methods of reactive oxygen species (ROS) production, cell membrane permeability and transmission electron microscope (TEM) after exposure to QACs below or above MIC_0 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 × MIC_0. 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 × MIC_0 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 × MIC_0 of BAC or DDAC exposure were diluted 1:10^6 and spread on LB agar plates containing individual antibiotics at 1 × MIC_0 (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 × MIC_0 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, 0.5DDAC-Chl-R, 0.5DDAC-Tet-R, 0.5DDAC-Amp-R, 2DDAC-Chl-R, 2DDAC-Tet-R, 2DDAC-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 Chl-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 $0.8 MIC_0$ of respective antibiotics (Chl for the Chl-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 $2^{-\triangle\triangle Ct}$ method and the results were showed as $\log_2$ 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 MIC_0 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-MIC_0 levels were significant, whereas those under subinhibitory concentrations ($0.25 \times$ and $0.5 \times MIC_0$) were insignificant (Fig. S1a and S1c). As QACs are positively charged and act on cell membrane, cell membrane damage was observed at exposure concentration $> 0.5 \times MIC_0$ (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, $2 \times MIC_0$) and 43d (80 mg/L, $4 \times MIC_0$), and a similar pattern was observed during DDAC exposure on 21d (9 mg/L, $2 \times MIC_0$) and 43d (18 mg/L, $4 \times MIC_0$). The ultimate MIC values of BAC and DDAC were 80 mg/L and 18 mg/L (approximately $4 \times MIC_0$), 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 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 MIC_0 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 $0.5 \times MIC_0$-mutants exhibited high resistance to Rif (MIC values were 10.67 ± 4.62 to 26.67 ± 9.24 times of the WT strains, $p < 0.0001$),
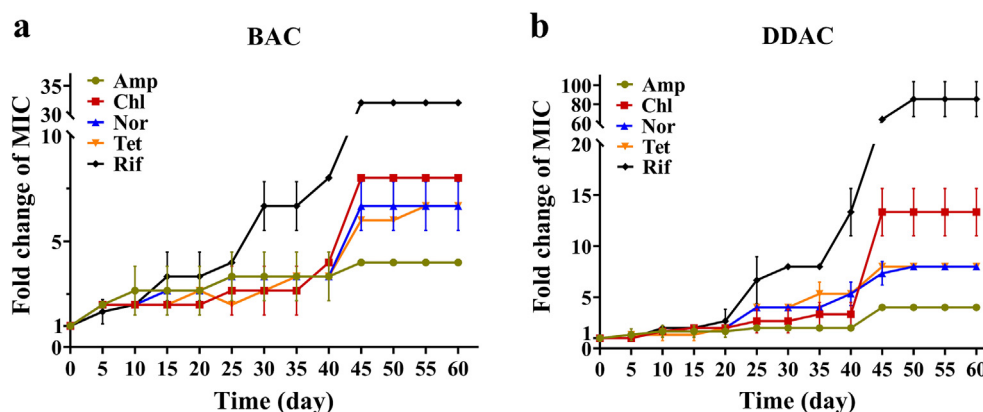

Fig. 1. Fold changes in the MIC values of *E. coli* subcultures against five antibiotics under BAC (a) and DDAC (b) exposure. Amp: ampicillin, Chl: chloramphenicol, Nor: norfloxacin, Tet: tetracycline, Rif: rifampin. The subcultures with the highest resistance to BAC and DDAC were tested for antibiotic resistance at each sampling point with biological triplicates.

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 $2 \times MIC_0$-mutants to multiple antibiotics remained in their P5 subcultures (Table S4). The resistance of $2 \times MIC_0$-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 $0.5 \times MIC_0$-mutants and $2 \times MIC_0$-mutants with the highest

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

$2 \times MIC_0$-mutants harbored more mutated genes than the $0.5 \times MIC_0$-mutants (16 v.s. 2, Fig. 3b and Table S5). *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
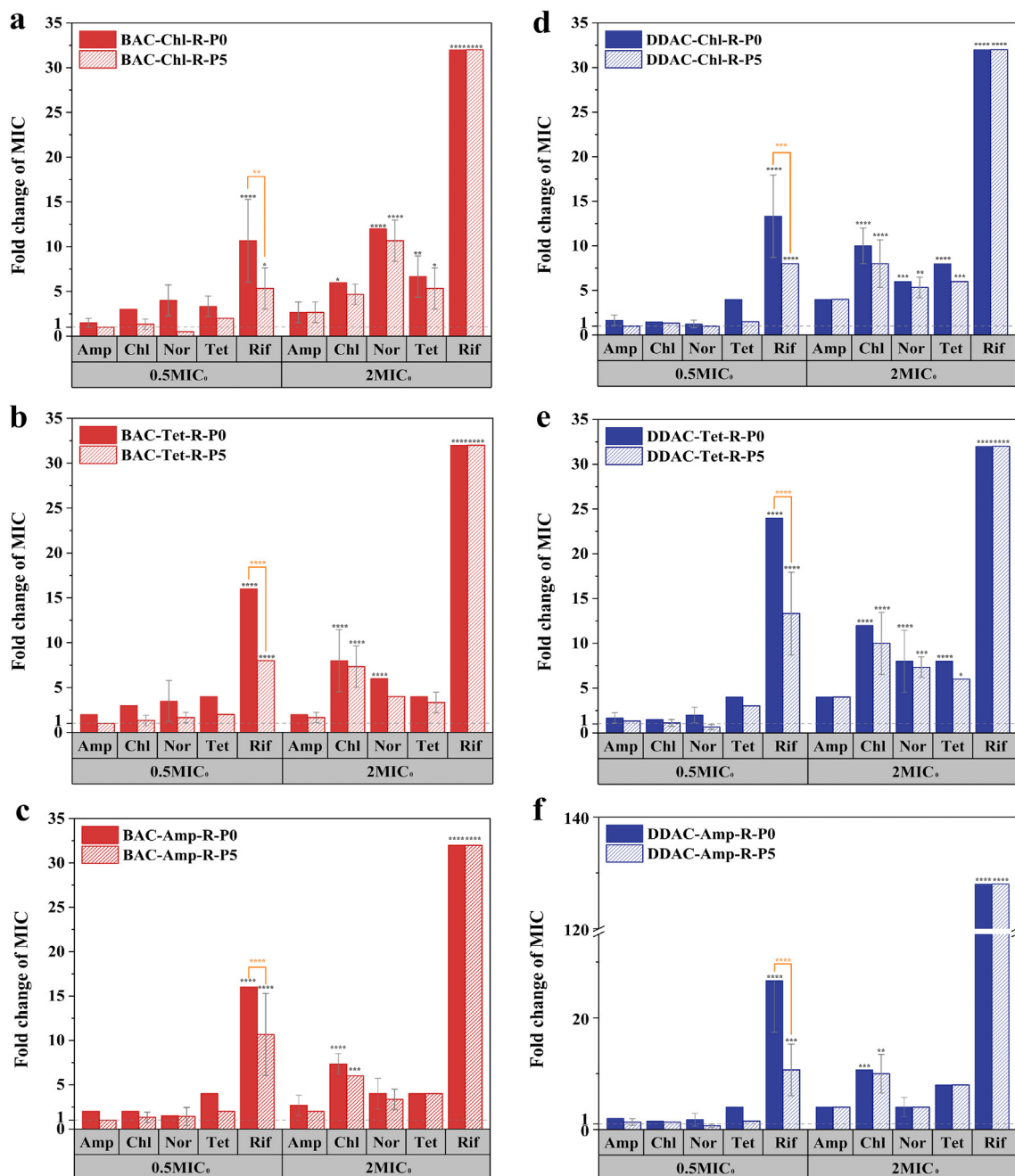
Fig. 2. Antibiotic MICs of putative mutants BAC-Chl-R (a), BAC-Tet-R (b), BAC-Amp-R (c), DDAC-Chl-R (d), DDAC-Tet-R (e), DDAC-Amp-R (f) at the end of subculturing process (P0) and 5 days after cultivation in the absence of BAC or DDAC (P5) with biological triplicates. The mutants were isolated from antibiotic 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: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
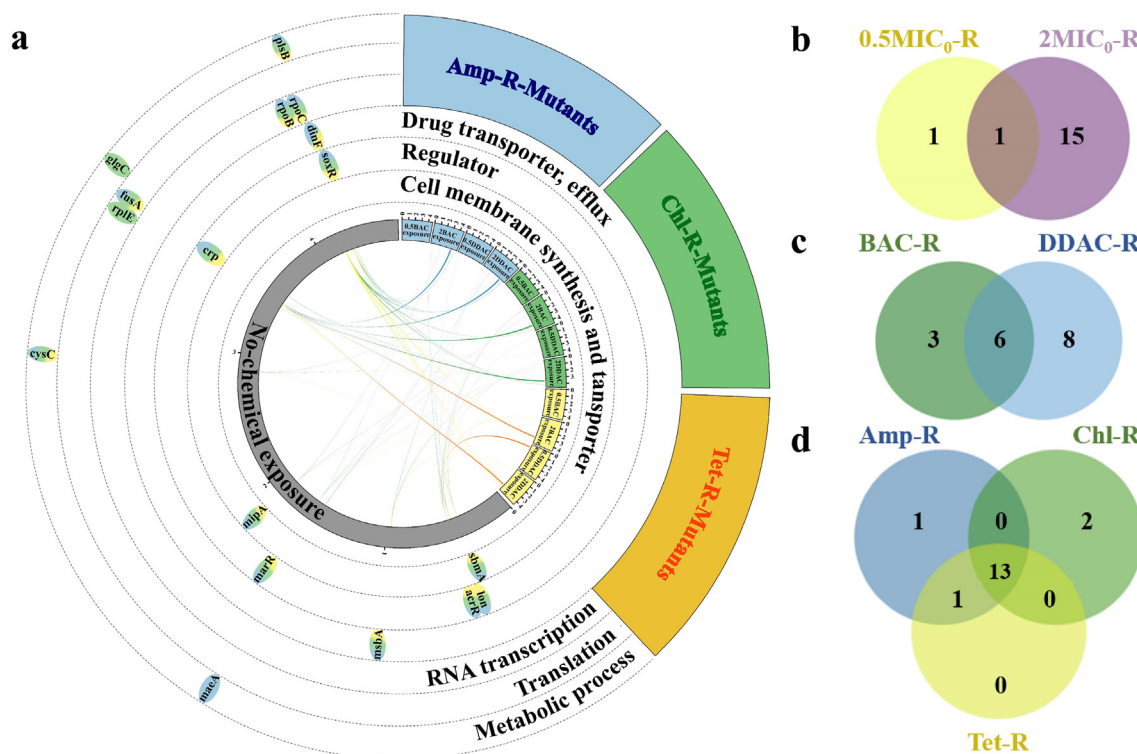
Fig. 3. BAC and DDAC induced genetic mutations. (a) Genetic mutations identified in the sequenced resistance mutants. Mutated genes were grouped based on function and marked on the circular genome of *E. coli*. Yellow, blue, and green colors represent mutants isolated from antibiotic plates containing Tet, Amp, Chl, respectively; (b) Venn diagram showing the number of shared (brown) and unique mutated genes in $0.5MIC_0$-R (yellow) and $2MIC_0$-R (violet) mutants; (c) Venn diagram showing the number of gene mutations shared (cyan) and unique mutated genes in BAC-R (green) and DDAC-R (blue) mutants; (d) Venn diagram showing the number of gene mutations shared (light green) and unique mutated genes in Amp-R (blue), Chl-R (green) and Tet-R (yellow). Detailed information of the mutated genes can be found in Table S4.

populations (Didelot and Maiden, 2010). $2 \times MIC_0$-mutants could pass on their resistance to offsprings without continued QACs exposure (Fig. 2). In contrast, the resistance of $0.5 \times MIC_0$-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 *ropC* encoding RNA polymerase (RNAP), and *acrR*, *marR*, *soxR*, *crp* encoding the regulators of AcrAB-TolC 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*, *fusA*, *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 (Haunreiter et al., 2019). Mutations in *rpoB* burdened a fitness cost in the absence of antibiotics, while compensatory mutations in *rpoC* could restore the fitness of rifampin-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-TolC (*acrR*, *soxR*, *marR*) and MdtEF (*crp*). AcrAB-TolC, a tripartite system consisting of a periplasmic protein (AcrA), a transporter protein (AcrB) and an accessory protein channel (TolC), 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 → Glu) 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; Woebking 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
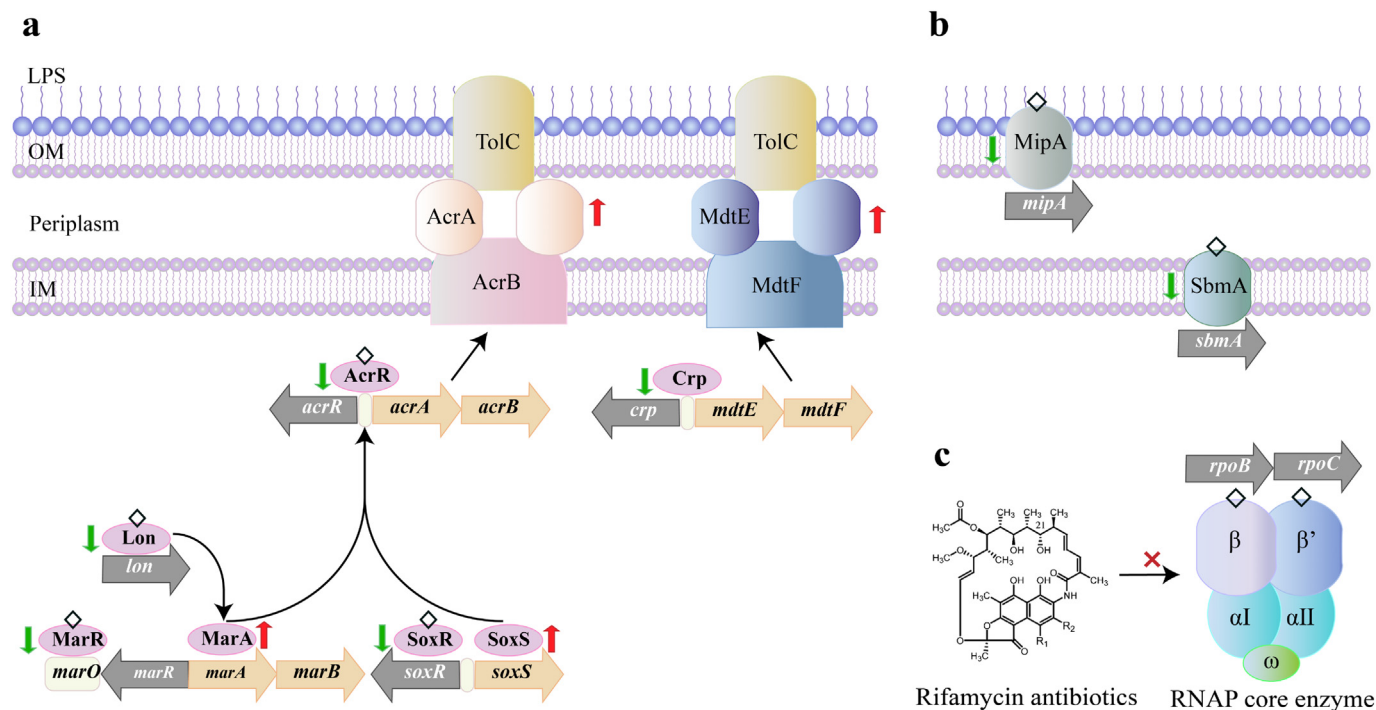
Fig. 4. Proposed antibiotic resistance mechanism of QACs-induced *E. coli* mutants: (a) stimulated efflux expression; (b) impeded transmembrane transport; (c) modified antibacterial targets. Mutated genes were marked in gray. Proteins (in ellipses) with changes in amino acid sequences were denoted by diamonds. Green/red arrows represent down/up-regulations in gene expressions. LPS: lipopolysaccharide, OM: outer membrane, IM: inner membrane.

the increased antibiotic resistance could be partially attributed to the *msbA* 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 thus its mutation may increase the resistance to these antibiotics (Corbalan et al., 2013; Cristobal et al., 2008; Mattiuzzo et al., 2007).

fusA and *rplE* genes are associated with translation. Mutations in *fusA*, which encodes the drug target of antibiotic fusidic acid (elongation factor G), could be related to antibiotic resistance (O'Neill et al., 2007). Nevertheless, the *fusA* mutation in 2DDAC-R mutant was synonymous (Table S5), and thereby its correlation with increased antibiotic resistance was weak. *rplE* encoding the 50S ribosomal subunit protein L5 is important to cell viability, whereas its relationship with antibiotic resistance has not been reported.

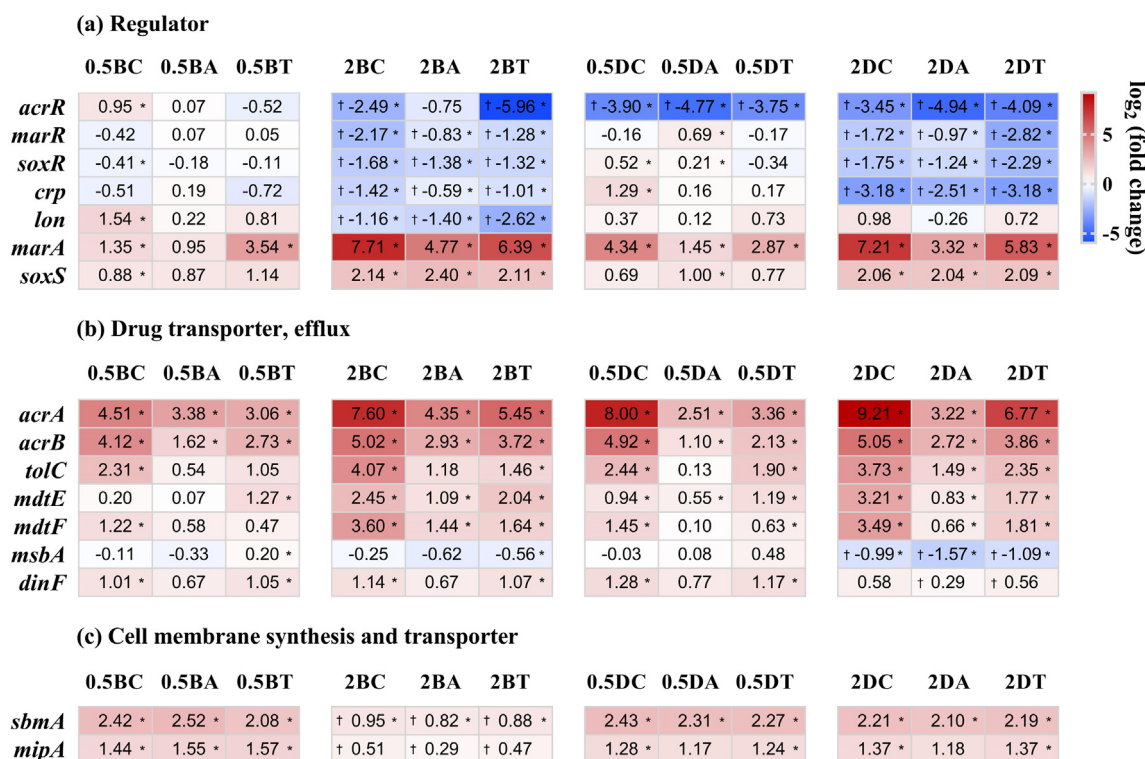QACs induced various non-canonical mutations of metabolic genes. *plsB* gene is involved in glycerol-3-phosphate metabolism, which plays an important role in phospholipid synthesis and persister formation (a specialized cell state that exhibit multidrug tolerance, including the ability to survive by 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 (Lemonnier et al., 2008). Similarly, a clear link between mutations of these genes and antibiotic resistance is lacking.

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,

(a) Regulator

	0.5BC	0.5BA	0.5BT	2BC	2BA	2BT	0.5DC	0.5DA	0.5DT	2DC	2DA	2DT
acrR	0.95 *	0.07	-0.52	† -2.49 *	-0.75	† -5.96 *	† -3.90 *	† -4.77 *	† -3.75 *	† -3.45 *	† -4.94 *	† -4.09 *
marR	-0.42	0.07	0.05	† -2.17 *	† -0.83 *	† -1.28 *	-0.16	0.69 *	-0.17	† -1.72 *	† -0.97 *	† -2.82 *
soxR	-0.41 *	-0.18	-0.11	† -1.68 *	† -1.38 *	† -1.32 *	0.52 *	0.21 *	-0.34	† -1.75 *	† -1.24 *	† -2.29 *
crp	-0.51	0.19	-0.72	† -1.42 *	† -0.59 *	† -1.01 *	1.29 *	0.16	0.17	† -3.18 *	† -2.51 *	† -3.18 *
lon	1.54 *	0.22	0.81	† -1.16 *	† -1.40 *	† -2.62 *	0.37	0.12	0.73	0.98	-0.26	0.72
marA	1.35 *	0.95	3.54 *	7.71 *	4.77 *	6.39 *	4.34 *	1.45 *	2.87 *	7.21 *	3.32 *	5.83 *
soxS	0.88 *	0.87	1.14	2.14 *	2.40 *	2.11 *	0.69	1.00 *	0.77	2.06 *	2.04 *	2.09 *

$\log_2$ (fold change) 5 0 -5

(b) Drug transporter, efflux

	0.5BC	0.5BA	0.5BT	2BC	2BA	2BT	0.5DC	0.5DA	0.5DT	2DC	2DA	2DT
acrA	4.51 *	3.38 *	3.06 *	7.60 *	4.35 *	5.45 *	8.00 *	2.51 *	3.36 *	9.21 *	3.22 *	6.77 *
acrB	4.12 *	1.62 *	2.73 *	5.02 *	2.93 *	3.72 *	4.92 *	1.10 *	2.13 *	5.05 *	2.72 *	3.86 *
tolC	2.31 *	0.54	1.05	4.07 *	1.18	1.46 *	2.44 *	0.13	1.90 *	3.73 *	1.49 *	2.35 *
mdtE	0.20	0.07	1.27 *	2.45 *	1.09 *	2.04 *	0.94 *	0.55 *	1.19 *	3.21 *	0.83 *	1.77 *
mdtF	1.22 *	0.58	0.47	3.60 *	1.44 *	1.64 *	1.45 *	0.10	0.63 *	3.49 *	0.66 *	1.81 *
msbA	-0.11	-0.33	0.20 *	-0.25	-0.62	-0.56 *	-0.03	0.08	0.48	† -0.99 *	† -1.57 *	† -1.09 *
dinF	1.01 *	0.67	1.05 *	1.14 *	0.67	1.07 *	1.28 *	0.77	1.17 *	0.58	† 0.29	† 0.56

(c) Cell membrane synthesis and transporter

	0.5BC	0.5BA	0.5BT	2BC	2BA	2BT	0.5DC	0.5DA	0.5DT	2DC	2DA	2DT
sbmA	2.42 *	2.52 *	2.08 *	† 0.95 *	† 0.82 *	† 0.88 *	2.43 *	2.31 *	2.27 *	2.21 *	2.10 *	2.19 *
mipA	1.44 *	1.55 *	1.57 *	† 0.51	† 0.29	† 0.47	1.28 *	1.17	1.24 *	1.37 *	1.18	1.37 *

Fig. 5. The expressions of selected mutated genes related to regulator (a), drug transporter and efflux (b), and cell membrane synthesis and transporter (c) in mutants with biological triplicates. Red/blue indicate up/down-regulated genes. $\log_2$ fold change |LFC| ≥ 2 and *$p < 0.05$ are the criteria for differentially expressed genes. The dagger (†) represents strains harboring specific mutation. The abbreviations of mutants provided the information regarding QACs, exposure concentration, and antibiotics. Specifically, 0.5 BC: 0.5BAC-Chl-R, 0.5BA: 0.5BAC-Amp-R, 0.5BT: 0.5BAC-Tet-R, 2 BC: 2BAC-Chl-R, 2BA: 2BAC-Amp-R, 2BT: 2BAC-Tet-R, 0.5 DC: 0.5DDAC-Chl-R, 0.5DA: 0.5DDAC-Amp-R, 0.5DT: 0.5DDAC-Tet-R, 2 DC: 2DDAC-Chl-R, 2DA: 2DDAC-Amp-R, 2DT: 2DDAC-Tet-R.

−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, 0.82, 0.88 (*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.

Appendix A. Supplementary data

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

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