Journal of Hazardous Materials 470 (2024) 134102

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Research Paper

NaClO Co-selects antibiotic and disinfectant resistance in *Klebsiella pneumonia*: Implications for the potential risk of extensive disinfectant use during COVID-19 pandemic

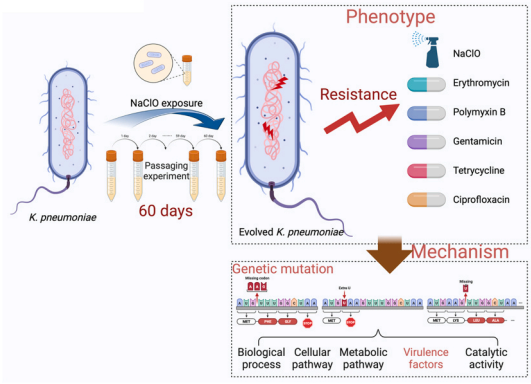
Zeyou Chen [a], Yulin Zhang [a], Daqing Mao [b], Xiaolong Wang [a,*], Yi Luo [a,c,**]

[a] College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Nankai University, Tianjin 300071, China
[b] School of Medicine, Nankai University, Tianjin, China
[c] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, China

HIGHLIGHTS

- NaClO enhanced the resistance of *K. pneumonia* to both NaClO and antibiotics.
- Evolved resistant strains showed fitness costs by decreasing their growth rates.
- NaClO exposure affect pathogenicity of *K. pneumonia* by genetic mutations.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
NaClO
K. pneumoniae
Antibiotic resistance
Disinfectant resistance
Gene mutations

ABSTRACT

The inappropriate use of antibiotics is widely recognized as the primary driver of bacterial antibiotic resistance. However, less attention has been given to the potential induction of multidrug-resistant bacteria through exposure to disinfectants. In this study, *Klebsiella pneumonia*, an opportunistic pathogen commonly associated with hospital and community-acquired infection, was experimentally exposed to NaClO at both minimum inhibitory concentration (MIC) and sub-MIC levels over a period of 60 days. The result demonstrated that NaClO exposure led to enhanced resistance of *K. pneumonia* to both NaClO itself and five antibiotics (erythromycin, polymyxin B, gentamicin, tetracycline, and ciprofloxacin). Concurrently, the evolved resistant strains exhibited fitness costs, as evidenced by decreased growth rates. Whole population sequencing revealed that both concentrations of NaClO exposure caused genetic mutations in the genome of *K. pneumonia*. Some of these mutations were known to be associated with antibiotic resistance, while others had not previously been identified as such.

* Corresponding author.
** Corresponding author at: College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Nankai University, Tianjin 300071, China.
E-mail addresses: loong91@126.com (X. Wang), luoy@nankai.edu.cn (Y. Luo).

https://doi.org/10.1016/j.jhazmat.2024.134102
Received 15 October 2023; Received in revised form 1 February 2024; Accepted 19 March 2024
Available online 27 March 2024

In addition, 11 identified mutations were located in the virulence factors, demonstrating that NaClO exposure may also impact the pathogenicity of *K. pneumoniae*. Overall, this study highlights the potential for the widespread use of NaClO-containing disinfectants during the COVID-19 pandemic to contribute to the emergence of antibiotic-resistant bacteria.

Environmental Implication: Considering the potential hazardous effects of disinfectant residues on environment, organisms and biodiversity, the sharp rise in use of disinfectants during COVID-19 pandemic has been considered highly likely to cause worldwide secondary disasters in ecosystems and human health. This study demonstrated that NaClO exposure enhanced the resistance of *K. pneumonia* to both NaClO and five antibiotics (erythromycin, polymyxin B, gentamicin, tetracycline, and ciprofloxacin), highlighting the widespread use of NaClO-containing disinfectants during the COVID-19 pandemic may increase the emergence of antibiotic-resistant bacteria in the environment.

1. Introduction

Antimicrobial resistance has become a serious public health problem threatening human health, which now causes at least 700,000 people to die globally every year [1]. The use of disinfectants has exploded globally since the outbreak of COVID-19 due to their high efficacies in controlling the spread of the coronavirus. Statistics indicate that the global sales of surface disinfectants soared to 4.5 billion US dollars in 2020 alone, marking a more than 30% increase compared to pre-pandemic levels [2]. In 2020, the U.S. EPA alone approved 545 disinfectant products for COVID-19 disinfection. [3] Large-scale use of disinfectants can bring many negative effects on the ecological environment [4]. Among them, the extensive use of disinfectants is known to induce bacteria to develop disinfectant resistance, which is potentially connected with antibiotic resistance [5–7]. Thus, the large-scale use of disinfectants during the COVID-19 pandemic has the potential to exacerbate the already grave issue of bacterial antibiotic resistance, posing serious threat to human health.

Although there are significant differences in the bactericidal mechanism between disinfectants and antibiotics, increasing evidence demonstrates that the use of disinfectants can contribute to bacterial resistance against both disinfectants and antibiotics [8–10]. For example, Mc Cay et al. found that continuous exposure to sublethal concentrations of benzalkonium chloride significantly increased the resistance of *Pseudomonas aeruginosa* to antibiotic ciprofloxacin [11]. Hou *et al.* found that the resistance of *P. aeruginosa* to ceftazme, chloromycin, and ampicillin increased by 1.4–5.6 times upon exposure to NaClO [12]. However, some opposite phenomena in which bacteria often tend to be sensitive to antibiotics when developing disinfectant resistance were also observed in previous studies [13–17]. Therefore, it still needs more studies to explore bacterial antibiotic resistance following exposure to disinfectants. The documented mechanisms for concurrent bacterial resistance to antibiotics and disinfectants can be grouped into two categories: cross-resistance and co-resistance. Regarding cross-resistance, certain bacterial efflux pumps (which expel toxic substances from cells) may have multiple substrates, encompassing both antibiotics and disinfectants. Consequently, the overexpression of these efflux pumps can make bacteria resistant to both antibiotics and disinfectants simultaneously. For example, the efflux pump, *MexCD-OprJ*, endows cross-resistance of *P. aeruginosa* to the antimicrobial triclosan and multiple antibiotics [18]. Another type of mechanism is co-resistance, in which antibiotic resistance genes and disinfectant resistance genes are located in the same mobile genetic elements, such as plasmid, integron, and gene cassette [5]. After acquiring these mobile genetic elements, bacteria would have co-resistance to disinfectants and antibiotics.

Klebsiella pneumonia, a member of the genus *Klebsiella* of *Enterobacteriaceae*, is a rod-shaped, gram-negative, lactose-fermenting bacterium. It is ubiquitous in the environment and has been associated with a variety of human infections [19–23]. Now, *K. pneumoniae* is considered the most common cause of hospital-acquired pneumonia in the United States, accounting for 3− 8% of all nosocomial bacterial infections [24]. More seriously, the worldwide spread of multidrug-resistant *K. pneumoniae* has become a public health threat, which caused an estimated 2700 deaths in 2017 in the United States alone [25]. Since the outbreak of SARS-CoV-2, disinfectants have been widely used worldwide, among which chlorine-containing disinfectants represented by NaClO are particularly common. Nevertheless, improper disinfectant usage may indiscriminately select multidrug-resistant *K. pneumoniae*, resulting in serious risks to human health. Presently, there is still a lack of reports on the resistance of *K. pneumoniae* to multiple antibiotics after exposure to disinfectants, as well as the associated mechanisms.

Through the NaClO exposure experiment, this study investigated the influence of NaClO disinfection on the development of *K. pneumoniae* resistance to multiple antibiotics and explored the underlying genetic mutations using whole-genome sequencing. The results demonstrated that NaClO, at both minimum inhibitory concentration (MIC) and sub-MIC levels, coselected for resistance to NaClO and antibiotics. The whole-genome analysis identified chromosomal genetic mutations known to be involved in antibiotic resistance, along with genes not previously recognized for their role in antibiotic resistance. This study expanded our understanding of the mechanisms in NaClO-induced antibiotic resistance and provided important hints for accelerating antibiotic resistant bacteria from overuse of disinfectants during COVID-19 pandemic.

2. Experimental section

2.1. Strain

To get closer to a strain that could infect humans, a *K. pneumoniae* bacterium isolated from the feces of a healthy human without any apparent or self-declared disease and no recent medication experience for nearly six months before the fecal collection was used in this study. The genome sequence of this strain has been submitted to the NCBI database, and its accession number is NZ_VKKD00000000. This strain was cultured in lysogeny broth (LB) media on a horizontally moving shaker at 180 rpm and 37 ℃, which was regarded as the ancestor (Kpn. o), and used as the inoculum for the evolutionary experiments. This strain has a minimum inhibitory concentration (MIC) to NaClO of 275 mg/L, which was measured using a Mueller-Hinton microbroth dilution method [26].

2.2. Evolution experiment

The schematic of the evolution experiment upon NaClO exposure is shown in Fig. 1. The entire exposure experiment was performed in 15 mL-plastic centrifuge tubes. Three experimental groups of sub-MIC exposure group, MIC exposure group, and non-exposed control group were set up. To be specific, *K. pneumoniae* grown in 5 mL LB media containing 140 and 280 mg/L NaClO was named as sub-MIC and MIC groups (denoted as M and H), respectively. Correspondingly, bacteria grown in LB without NaClO were considered the unexposed control group (denoted as C). Each treatment comprised 5 parallel populations. Every 24 h of cultivation on a horizontally moving shaker at 180 rpm and 37 ℃, 50 μL bacterial suspension was transferred to fresh LB

containing the same concentrations of NaClO or without NaClO. In this way, the whole passaging experiment was repeated for 60 days. During the experiment, the bacterial suspension was diluted into 30% glycerol solution at volume ratio of 1:1 every five days and stored at − 80 ℃ for subsequent experiments and analyses. To avoid the strains being contaminated, the entire evolution experiment was carried out on a super clean bench. In addition, the bacterial suspensions collected every five days were also appropriately diluted and spread on the LB solid medium, and the monoclonal colonies were selected for PCR amplification to identify their purity, as detailed in the Supporting Information (SI).

2.3. Determination of MIC for NaClO and antibiotics

Mueller-Hinton microbroth dilution method was used to detect MIC of the ancestral and evolved populations against NaClO and five representative antibiotics (erythromycin, polymyxin B, gentamicin, tetracycline, and ciprofloxacin). Briefly, 100 μL bacterial suspension was spiked into MHB media containing either different concentrations of NaClO or antibiotics in 96-well plate. The used concentration range for MIC determination was NaClO (200–425 mg/L), erythromycin (60–600 mg/L), polymyxin B (0.1–51.2 mg/L), gentamicin (0.1–51.2 mg/L), tetracycline (0.1–51.2 mg/L), and ciprofloxacin (0.1–51.2 mg/L). The experiment was repeated three times. After adding samples, the 96-well plate was incubated at 37 ℃ for 24 h, and the absorbance of each well was measured at 620 nm using a multimode microplate reader. The minimum concentration that completely inhibited bacterial growth was determined as the MIC.

2.4. Measurement of bacterial growth curve

For measurement of growth curves of ancestral and evolved *K. pneumoniae*, bacterial suspensions at each sampling time point were also centrifuged at 8000 *g* at 4 ℃ for 10 min to collect cell pellets, and further washing 3 times with phosphate-buffered saline (PBS) to remove residual NaClO and metabolites and then re-suspended in PBS at 10^7 – 10^8 CFU/mL. Subsequently, 10 μL cell suspension was inoculated into 200 μL sterile LB medium in 96-well plate and incubated at 37 ℃ for more than 12 h. During the incubation, the OD_{600} of bacterial suspension was detected every 30 min using multimode microplate reader. The growth dynamics of the strains were fitted in Origin 2017 software, and the corresponding maximum growth rate and lag time were calculated.

2.5. Detection of bacterial biofilm formation capacity

Biofilm formation capacity of the ancestral and evolved populations was measured using crystal violet staining [27]. Briefly, the overnight culture of the strains was diluted to OD_{600} = 1 with sterile PBS solution, and 2 μL was taken into a 96-well plate containing 198 μL fresh LB medium, which was incubated at 37 ℃ for 48 h. The 96-well plates were then washed with sterilized distilled water and repeated twice to remove the planktonic bacteria. After draining the wells, 125 μL crystal violet solution (0.1%) was added to each well and allowed to stand for 10 min. After dyeing, the 96-well plates were cleaned twice with sterilized distilled water to remove crystal violet dye and non-tightly bound bacteria. After air drying for 30 min, 200 μL 30% acetic acid solution was added to each well to dissolve crystal violet. Finally, the absorbance at 570 nm was recorded, and all treatments were repeated five times.

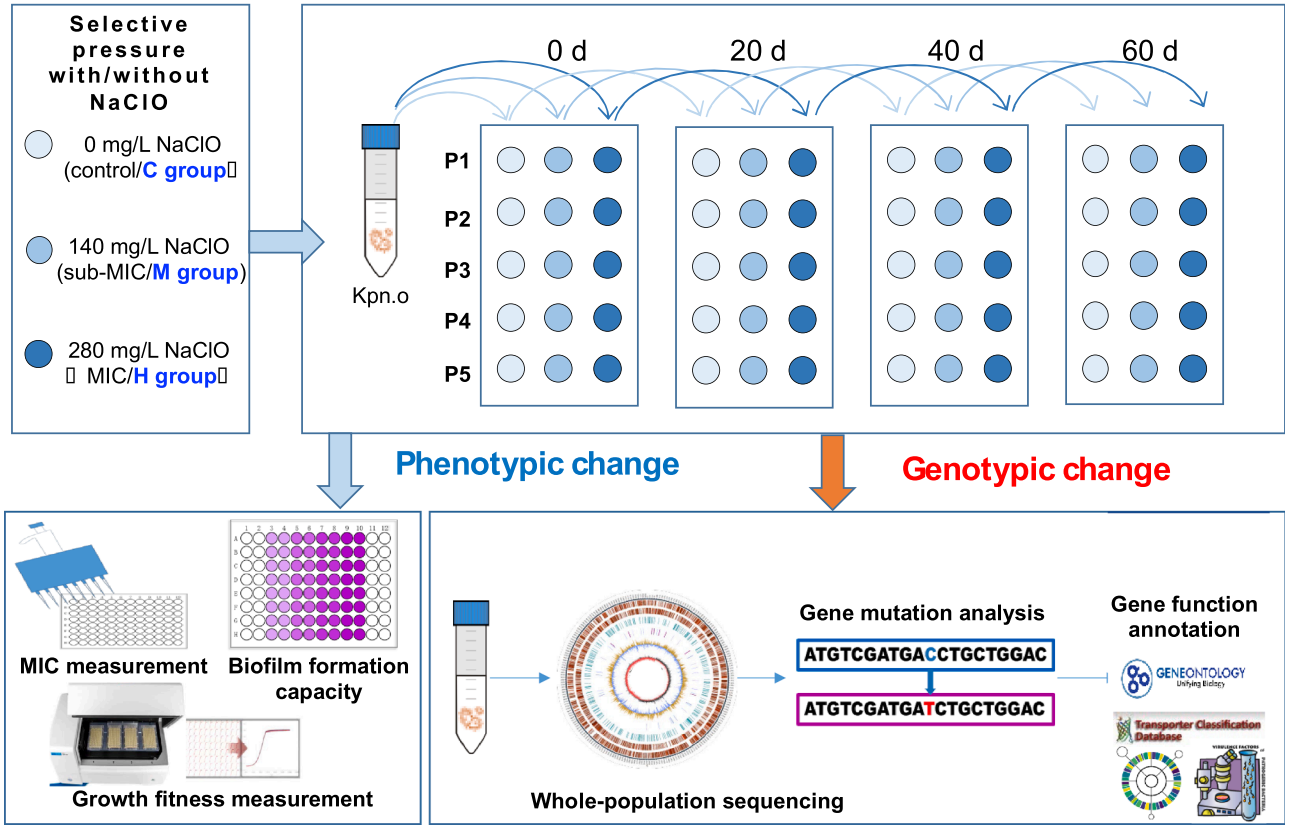


Fig. 1. Schematic diagram of NaClO exposure experiment. A total of five parallel populations (P1-P5) of *K. pneumoniae* (Kpn.o) under each exposure condition were serially passaged every 24 h into fresh LB medium containing NaClO at the same exposure levels for up to 60 days. The exposure conditions include 0 mg/L, 140 mg/L, and 280 mg/L of NaClO, which are denoted as C, M, and H groups, respectively. Populations during the exposure periods were subject to phenotypic change analysis and whole-population sequencing.

2.6. Whole-population sequencing

In this study, to identify the genetic mutation upon NaClO exposure, the ancestral population (Kpn.o), one population (C-3) from the unexposed control group, and four populations from the exposed treatments (M-3 and M-5 from sub-MIC exposure group; H-2 and H-5 from MIC exposure group) on the 60th day were used to analyze the genome. In this study, we employed whole-population sequencing instead of single-clone sequencing to mitigate potential biases arising from the random selection of clones from the population. To be specific, 1 mL of the overnight bacterial culture was centrifuged at 16,000 *g* for 2 min to obtain the bacterial pellets. Bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit according to the manufacturer's instructions. The extracted DNA was then sent to Beijing Baimaike Biotechnology Co., Ltd. for whole-population sequencing using Nanopore sequencing platform. All data generated during this study are available in publicly accessible databases. Bacterial genome sequencing data can be accessed through the Sequence Read Archive of NCBI (BioProject accession number PRJNA1069381).

Sequence data analysis proceeded as follows. Firstly, quality control was conducted on the raw reads, and the subreads with low quality and excessively short length (< 2000 bp) were filtered out. Subsequently, Canu v1.5 software was used to assemble the filtered clean reads [28]. The assembled results were further refined and corrected using Racon v3.4.3 software. Circlator v1.5.5 software was used to produce correct representations of circular DNA structures [29]. Following this, Pilon v1.22 software was used for additional error correction, resulting in the genome with higher accuracy for subsequent analysis [30]. After that, open reading frames (ORFs) in contigs were predicted and assembled using Prodigal v2.6.3 software [31]. Antibiotic resistance genes were annotated using RGI [32] based on the Comprehensive Antibiotic Resistance Database (CARD), and BlastP [31] was used to compare the main general databases, such as Nr, PHI, etc. Based on the comparison results of Nr database blast, Blast2GO v2.5 was used to annotate the gene function according to the GO database [33]. The genome sequences of six sequenced *K. pneumoniae* strains (Kpn.o, C-3, M-3, M-5, H-2, H-5) were used to construct an evolutionary tree using phyML software to study the evolutionary relationship between strains [34]. The C-3 strain in the control group was taken as the reference strain. The collinearity relationship between the reference strain and the other five strains was determined by MCScanX software [35]. MUMmer software was used to compare the sequence of the reference strain and the other five strains, and the single-nucleotide polymorphism (SNP) and the insertions and deletions (InDel) mutations in the compared results were extracted and summarized.

3. Results and discussion

3.1. NaClO exposure increased NaClO resistance

The effect of NaClO exposure on the development of resistance of *K. pneumoniae* to NaClO was investigated first, the minimum inhibition concentrations (MIC) of NaClO for the populations collected from different experimental groups (unexposed control, exposed treatments at sub-MIC and MIC levels) on the 5th, 15th, 30th, 45th, and 60th days of exposure were measured. As shown in Fig. 2a, with increasing exposure time, the resistance of five parallel *K. pneumoniae* populations at three experimental groups to NaClO all gradually increased, indicating the selection of NaClO-resistance regardless of NaClO exposure or not. It was quite interesting to observe that bacterial population resistance to NaClO also persistently increased in the unexposed control group (Fig. 2b), although an insignificant decrease was exhibited from day 45 to day 60 (ANOVA, $p > 0.05$). One possible hypothesis was that LB media itself could exert certain selective pressure on the *K. pneumoniae*, leading to adaptive evolution. Given that the used *K. pneumoniae* was isolated from human feces, the nutrient-rich LB media used in the evolution experiment could create substantially different growth conditions compared to the gastrointestinal tract, thereby inducing bacterial adaptive evolution. This phenomenon has been reported in *E. coli* grown in nutrient-rich media, where significant changes were observed in both physiological and mutational responses [36]. However, the evolved resistance to NaClO observed in the unexposed control was generally lower than that observed in the NaClO-exposed treatments. Additionally, the evolved populations selected by MIC level exposure showed the highest resistance to NaClO (Fig. 2b and c). These results indicated that long-term exposure to NaClO can lead bacteria to develop NaClO resistance, and this was not surprise as many previous studies have demonstrated the selection of disinfectant-resistant microbes following extensive use of disinfectants [37–39].

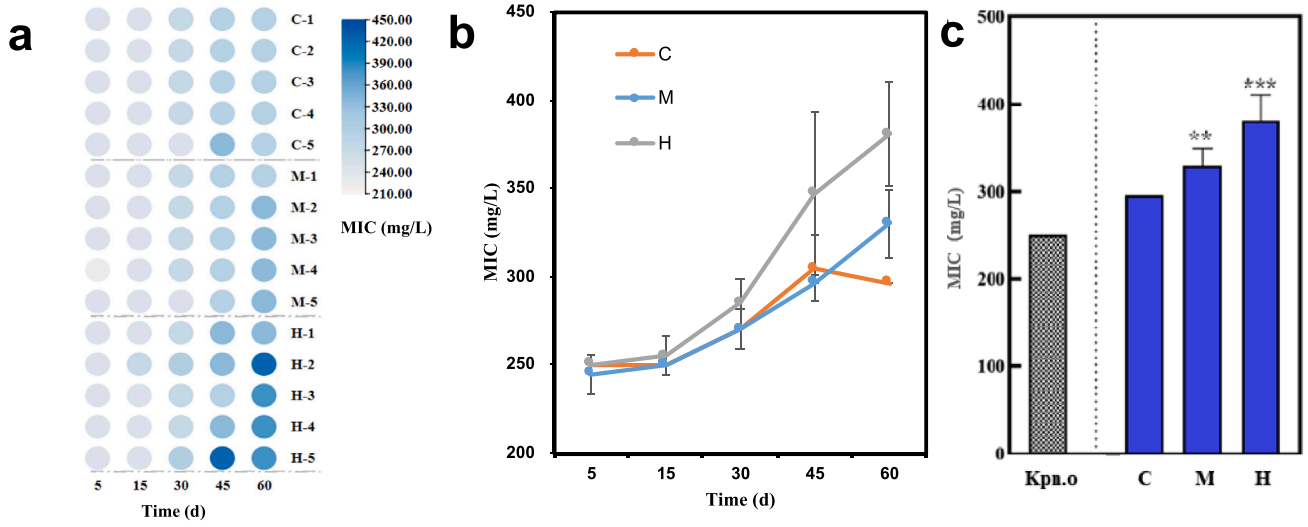


Fig. 2. NaClO exposure increases the resistance of *K. pneumoniae* population to NaClO. (a) Evolution of NaClO resistance over time for all five populations at different treatments, C-1 to C-5: NaClO-unexposed control; M-1 to M-5: NaClO-exposed treatment at sub-MIC level; H-1 to H-5: NaClO-exposed treatments at MIC level; (b) Change of average MIC of NaClO for the populations grown in three treatments over time; (c) Comparison of NaClO resistance for the ancestral population (Kpn.o) and the evolved populations from three experimental groups after 60-days exposure. ANOVA test: * * $p < 0.01$, * ** $p < 0.001$.

3.2. NaClO exposure co-selected bacterial resistance to multiple antibiotics

To explore the effect of NaClO exposure on the development of antibiotic resistance of *K. pneumoniae*, the resistance of evolved populations collected from different treatments at different sampling times to tetracycline, ciprofloxacin, gentamicin, polymyxin B, and erythromycin was measured. These five antibiotics were purposely selected because they belong to different classes of antibiotics and have inconsistent antibacterial mechanisms (Fig. 3). The results showed that the resistance of the populations from unexposed control to the five antibiotics generally decreased with increasing time (Fig. 3a and b), which contrasts with the trend of bacterial NaClO resistance over time in the control group (Fig. 2b). This indicated that the evolved NaClO resistance selected by the LB medium could decrease bacterial antibiotic resistance. Collateral sensitivity refers to an evolutionary trade-off between antibiotic resistance mechanisms in bacteria, where resistance to one antibiotic increases susceptibility to another antibiotic [40,41]. Thus, the observed phenomena in the unexposed control may indicate collateral sensitivity between NaClO resistance and antibiotic resistance. However, this type of collateral sensitivity was not observed in the NaClO-exposed populations. On the contrary, we found that NaClO exposure coselected the resistance of *K. pneumoniae* populations to studied antibiotics. This was indicated by the fact that populations from NaClO-exposed groups generally exhibited higher resistance to five antibiotics compared to the evolved populations in the unexposed control (Fig. 3b). Meanwhile, the effect of NaClO on increasing bacterial antibiotic resistance showed inconsistent dose-response relationship for the different antibiotics (Fig. 3b and c). For the tetracycline- and erythromycin-resistance, the MIC level of NaClO had a stronger stimulant effect than the sub-MIC level, whereas ciprofloxacin, gentamicin, and polymyxin B, the sub-MIC concentration exposure had larger promotional effect. This inconsistency might be related to the discrepant antibacterial mechanism of different antibiotics. Both tetracycline and

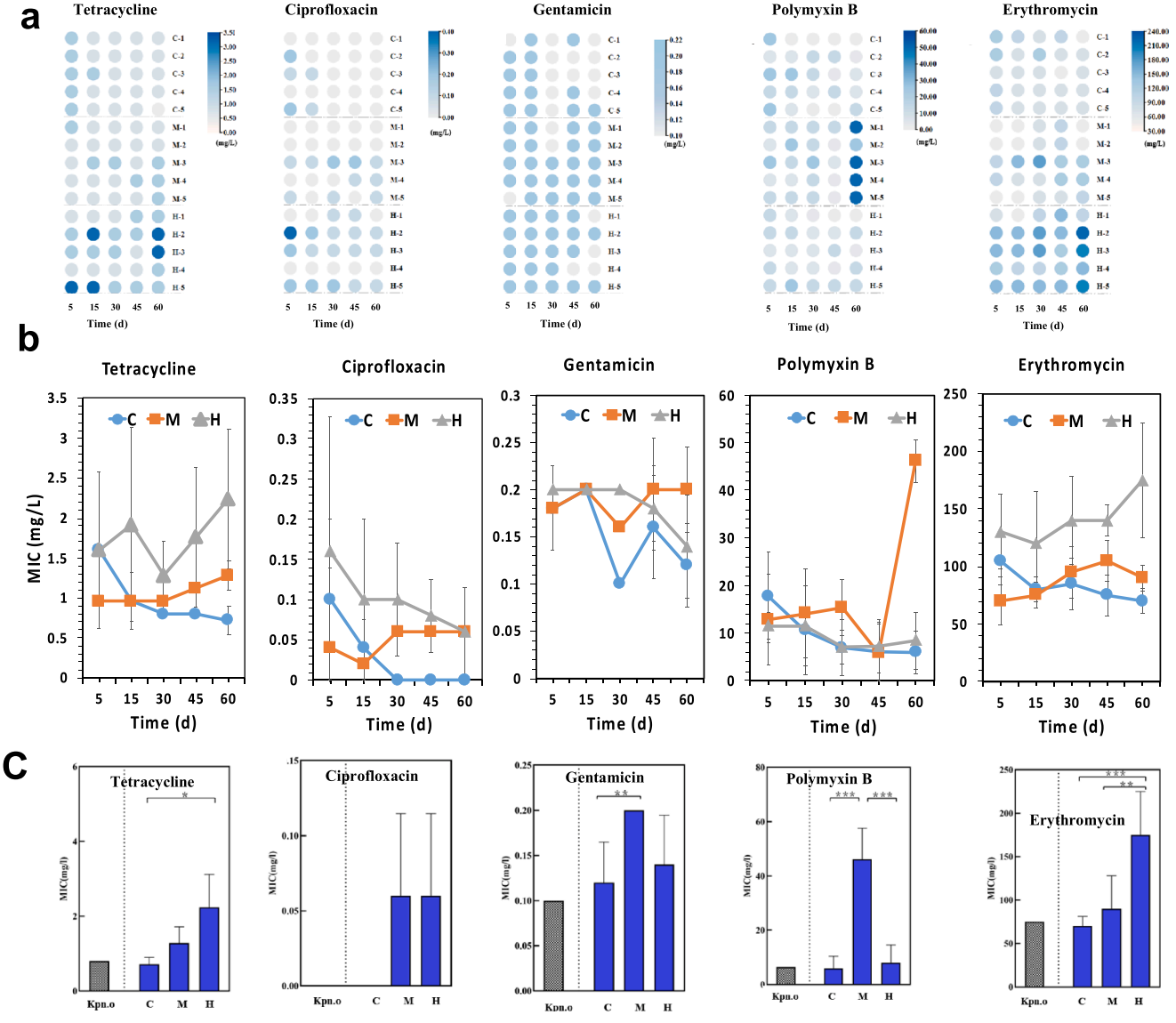


Fig. 3. Evolution of bacterial antibiotic resistance upon NaClO exposure. (a) measured MIC for all populations to tetracycline, ciprofloxacin, gentamicin, polymyxin B, and erythromycin at different treatments over time; (b) change of average MIC of antibiotics for the populations grown in three treatments over time; (c) comparison of antibiotic resistance of the ancestral population (Kpn.o) and the evolved populations from the three experimental groups after 60-days selection. C: unexposed control; M: exposed to sub-MIC level of NaClO; H: exposed to MIC level of NaClO. ANOVA test: * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

erythromycin inhibit protein synthesis by binding to the subunits of bacterial ribosomes, leading to their similar dose-response to NaClO exposure. However, similar antimicrobial mechanisms did not fully explain the observed phenomenon, as gentamicin also inhibits protein synthesis but showed a similar dose-response relationship to NaClO exposure as ciprofloxacin (inhibiting DNA synthesis) and polymyxin B (impairing bacterial outer membrane). Together, the above results indicated that both MIC and sub-MIC levels of NaClO exposure promoted the development of multidrug-resistant *K. pneumoniae*, posing a potentially significant risk to human health.

3.3. Increased resistance was associated with reduced bacterial growth and increased biofilm formation ability

It has been pointed out that the resistance of bacteria to antibiotics is often associated with the fitness cost, typically observed as reduced bacterial growth rate [42]. Similarly, we observed that the growth of the evolved populations from the NaClO-exposed groups was significantly lower than that of the unexposed control, as indicated by the lower growth rate and the longer lag-time (Fig. 4a). Formation of biofilm is one of the important ways for bacteria to develop resistance to antibiotics. It has been found that antibiotic resistance of *K. pneumoniae* in biofilms is 10–1000 times higher than that in planktonic state [43]. Therefore, it was also of great significance to explore the biofilm formation ability of the evolved *K. pneumoniae* populations selected by NaClO. As shown in Fig. 4b, compared with the ancestral population (Kpn.o), the biofilm-forming ability of the evolved populations in the three experimental groups all improved, although not significantly in statistics (ANOVA, $p > 0.05$). These results indicate that the observed increase in *K. pneumoniae* resistance to NaClO and antibiotics upon NaClO exposure was associated with decreased growth capacity and improved biofilm formation ability. Similar phenomena were also found in previous studies, for example, El-Banna *et al.*, found that *P. aeruginosa* clinical isolates increased antibiotic resistance after adapting to disinfectant BAC, with 66% of the isolates showing retardation of growth and 23.5% exhibiting enhanced biofilm formation [44]. Techaruvichit et al. pointed out that after treatment with subinhibitory concentrations of five biocides, *Campylobacter jejuni* strains improved their biofilm formation ability, resulting in increased resistance to kanamycin and streptomycin [45]. These studies highlight the potential for disinfectant exposure to induce changes in bacterial behavior and antibiotic resistance, emphasizing the importance of understanding the broader impacts of antimicrobial use in various settings.

3.4. Genetic mutations in resistant mutants revealed the underlying mechanisms

In this study, the evolved *K. pneumoniae* populations with stronger resistance to both NaClO and antibiotics were selected for whole-population sequencing. As a result, the ancestor (Kpn.o), C-3 population from the control group, M-3 and M-5 populations from the sub-MIC exposure group, as well as H-2 and H-5 populations from the MIC exposure group, were selected (supplemental Fig. S1). Draft genomes were reconstructed as depicted in Fig. S2. The genome size of ancestor *Kpn.o*, C-3, M-3, M-5, H-2, and H-5 was determined to be 5342163, 5342164, 5313956, 5343238, 5342169, and 5342162 kb, respectively. Further, MCScanX software was used to analyze the collinear relationship among the genomes, comparing C-3 genome with Kpn.o, M-3, M-5, H-2, and H-5 genomes. As shown in Fig. S3a, compared to the unexposed C-3, the NaClO-exposed populations (M-3, M-5, H-2, H-5) exhibited more translocations, insertions, inversion, etc, demonstrating that NaClO exposure induced the genetic mutation of *K. pneumoniae*. This was further supported by the phylogenetic analysis of 6 studied *K. pneumoniae* genomes (Fig. S3b). Kpn.o, C-3, M-3, and M-5 genomes were closely related, whereas the H-2 and H-5 genomes were distinct from them, suggesting that higher concentration of exposure could cause more significant genome mutations.

3.5. InDel mutations

Insertion-deletion (InDel) mutations represent an evolutionary strategy employed by bacteria when facing stressors. These mutations involved the insertion or deletion of nucleotide fragments of varying sizes (less than 1 kb) into DNA sequence. Thus, InDel in the evolved *K. pneumoniae* populations upon NaClO exposure was examined using the ancestor Kpn.o as the reference. As shown in supplemental Table S1,

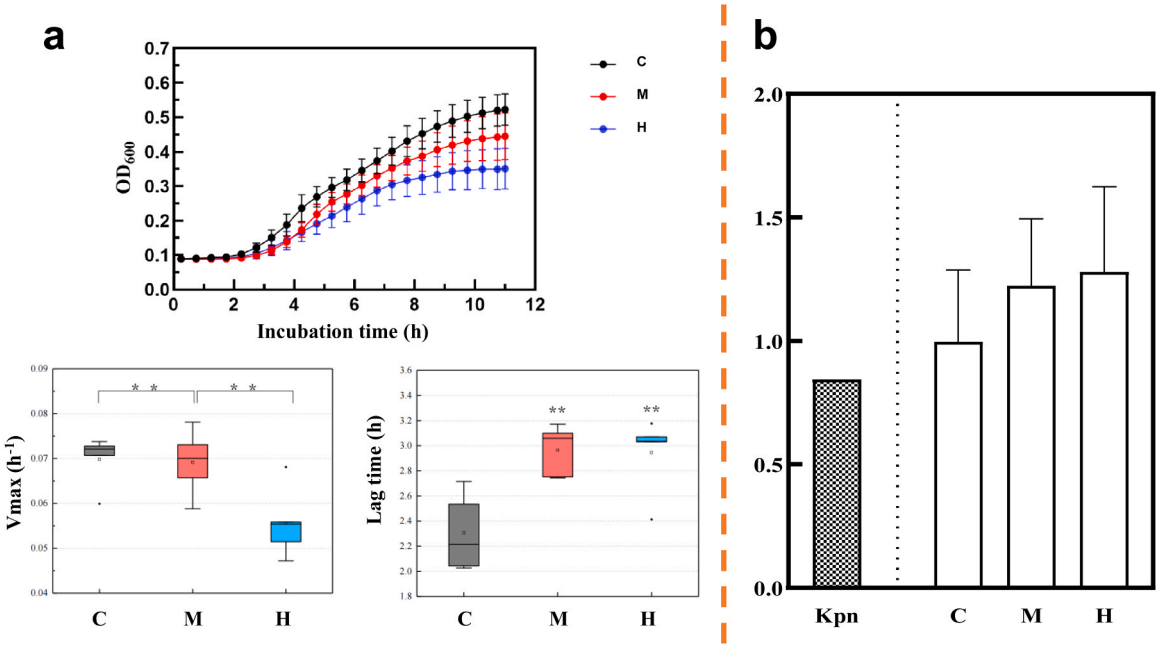


Fig. 4. Comparison of (a) the growth and (b) biofilm formation capability of the populations selected by LB-media and NaClO at sub-MIC and MIC levels. The growth of the population was indicated by the growth curves, the maximum growth rate, and the growth lag time. C: unexposed control; M: sub-MIC level exposure to NaClO; H: MIC level exposure to NaClO. ANOVA test: * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

compared to the Kpn.o, the evolved population cultivated in NaClO-free LB media (C-3) had 1 InDel mutation in the non-coding region. For the populations evolved upon sub-MIC level exposure (M-3 and M-5), there were 4 InDel in non-coding regions of M-3 genome and 2 InDel in non-coding regions of M-5 genome. For the resistant mutants exposed to MIC exposure level, the H-2 genome displayed 9 InDel mutations in non-coding region and 6 InDel mutations in coding region, while the H-5 genome had 5 InDel in non-coding region and 3 InDel in coding region. Consistently increasing InDel mutation number from the unexposed control to the high concentration of exposure indicates NaClO induces genetic mutations in *K. pneumoniae.* Notably, there were 2 identical InDel mutations present in the genomes of H-2 and H-5, both occurring in genes *mutS* (inserting 2 bases) and *mfpsA* (inserting 1 base). The *mutS* gene encodes a mismatch repair protein critical for maintaining DNA replication fidelity and combating the adverse effects of genomic damage. Mutations in *mutS* have been extensively reported to significantly contribute to the emergence of mutational resistance to various antibiotic classes in bacteria [46]. For example, Willems et al. found that *Enterococcus faecium* with mutation in *mutS* gene is prone to the development of resistance to oxazolidinone antibiotics. [47] Similarly, in *Acinetobacter baumannii*, mutations in *mutS* also increased the rate of mutation to rifampicin resistance by ~50-fold. [48] The DNA mismatched repair system is crucial in avoiding mutations and maintaining replicative fidelity, bacteria with defects in this system have a reduced ability to repair DNA damage, making them more susceptible to accumulating mutations in facing stressors. Thus, it was plausible that the observed *mutS* mutation resulting from high concentrations of NaClO exposure may contribute to the development of resistance to multiple antibiotics. The *mfpsA* gene, which codes mannosylfructose-phosphate synthase, is involved in the biosynthesis of disaccharide mannosylfructose, an important osmolyte in *Agrobacterium tumefaciens* that helps bacterium respond to osmotic stress [49,50]. While the specific

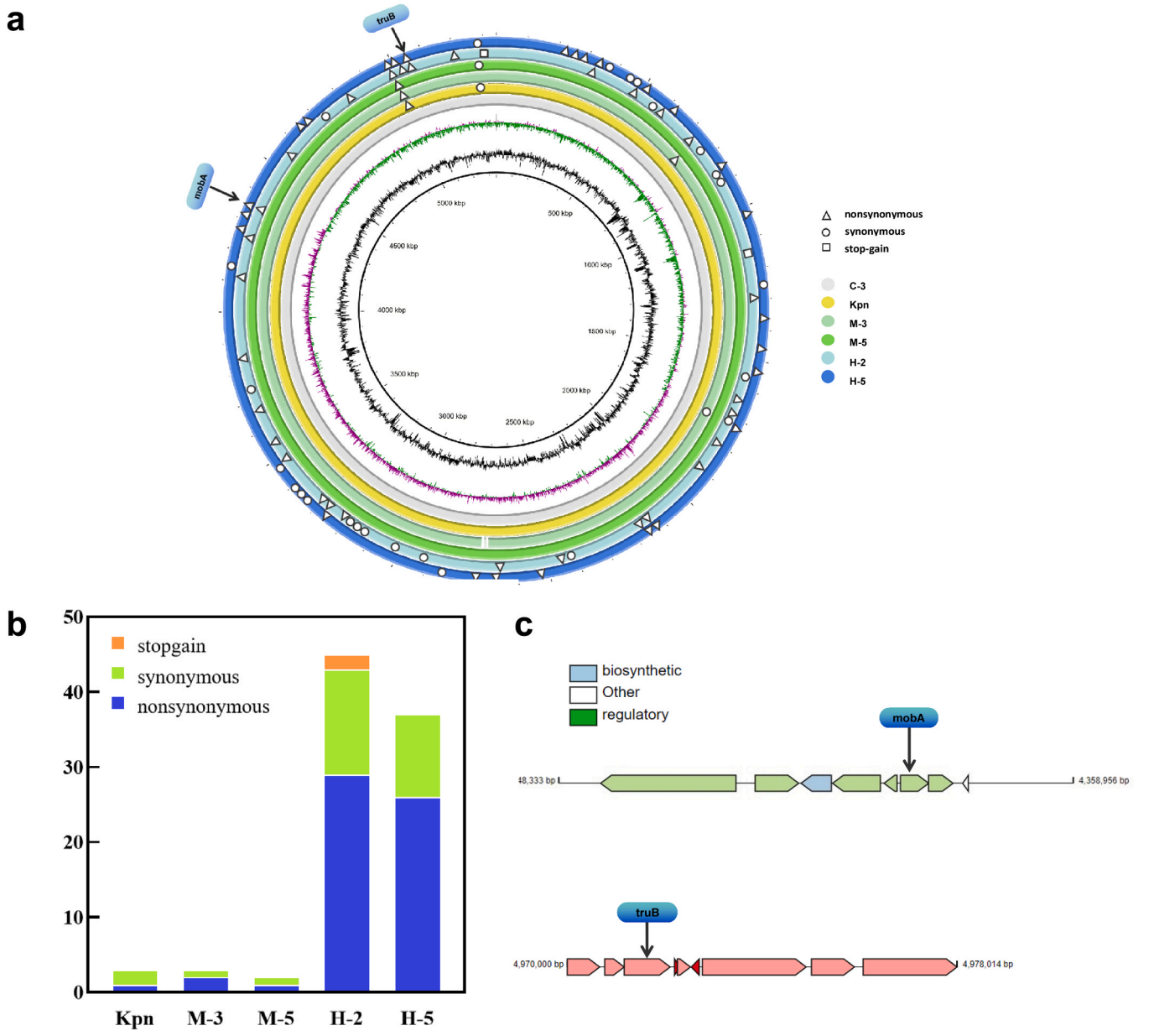


Fig. 5. (a) cSNP mutation in the genome of bacterial clones, △ represents non-synonymous mutation, ○ represents synonymous mutation, □ represents nonsense mutation, and the circle graph from inside to outside shows genome size marker, GC content, GC skew (green represents GC skew+, purple represents GC skew−), K. pneumoniae.o, C-3, M-3, M-5, H-2, H-5 chromosome genome; (b) counted number of cSNP mutation; (c) Location diagram of *mobA* and *truB* gene on Kpn.o chromosome genome.

involvement of *mfpsA* in antibiotic resistance is not as extensively studied, research by Boinett et al. has highlighted its importance for the survival of *A. baumannii* in sub-inhibitory concentrations of colistin [51]. The finding of our study further underscores the potential role of *mfpsA* in modulating *K. pneumoniae* resistance to NaClO and antibiotics, suggesting its multifaceted involvement in bacterial stress responses and adaptation mechanisms.

3.6. SNP mutations

As same to InDel mutation, SNP mutations in the genomes of evolved mutants (C-3, M-3, M-5, H-2, H-5) were also examined in this study. As shown in Table S1, three SNP mutations comprising 1 non-synonymous mutation and 2 synonymous mutations were found in C-3, indicating that the aforementioned selection pressure from LB medium also led to the mutation of the bacterial genome. In line with InDel mutation, we also found that the SNP mutations in the concentration group (H-2, H-5) were significantly more than those in the low concentration group (M-3, M-5). Specifically, when only 4 and 1 SNP mutations were found in the genomes of M-3 and M-5, respectively, a much higher number of SNP mutations, comprising 52 and 43 mutations, were identified in the genomes of H-2 and H-5 genomes, respectively. These SNP mutations include genes known to be involved in antibiotic resistance such as *wcaJ* [52], *mobA* [53], *rfaC* [54], *pat* [55], *csbC* [56], *csgA* [57], *secA* [58], *bcsA* [59], *srmB* [60], as well as the genes not yet identified to play a role in antibiotic/disinfectant resistance, including *yrbK*, *purD, argE, infB, truB, andAa, zipA, ccml, ygbM, astE,ybiU,pcaR_1, phnC, phnE_2, cimH, rhaA, proX, sepB, yejM, gipR_1,nhoA, fimD_1, yvoA_3, and phoA* (Table S2). Given their importance, particular attention was focused on the SNP mutations occurring in the coding region of the genome (cSNP) (Fig. 5a and b). The cSNP mutations in populations exposed to MIC-level NaClO were likewise significantly higher than that in the low-concentration exposure group and the ancestor (Fig. 4b). The main class of cSNP mutations was non-synonymous mutations, which changed the protein sequence and potentially affected protein function. Of particular note, two identical non-synonymous cSNP were identified in both H-2 and H-5 chromosomes. These mutations occurred in genes *mobA* (SNP: G→A; amino acid: Gly→Glu) and *truB* (SNP: C→T; amino acid: Arg→Cys), respectively (Fig. 5a and c). The *mobA* gene encodes a mobilization protein that has been reported to be induced by certain antimicrobials [53,61]. Gene *truB* encodes a pseuduridine synthase involved in catalyzing the pseuduridylation of U55 in tRNA [62]. While previous studies have highlighted the role of *truB* in optimizing cell survival at high temperatures through tRNA modification, [63] its involvement in disinfectant and antibiotic resistance has not been reported before. Thus, our study suggests a potential new role for the *truB* gene in mediating resistance mechanisms against both disinfectants and antibiotics, expanding our understanding of bacterial adaptation under selective pressures such as NaClO exposure.

The analysis of non-synonymous SNPs in the evolved populations was further enriched by utilizing different databases for annotation, providing insights into the functional implications of these mutations (Fig. S4 and Tables S3-S6). The GO database annotation revealed that there were 54 non-synonymous SNPs in all evolved strains, among which 28 genes were involved in cell components, 38 in molecular functions, and 40 in biological processes (Fig. S4). According to KEGG Pathway analysis (Table S3), it was found that 20 non-synonymous SNPs of H-2 were involved in metabolic pathways, spanning four categories of Cellular Processes, Environmental Information Processing, Genetic Information Processing, and Metabolism. Similarly, the non-synonymous SNPS of H-5 involved 16 metabolic pathways, which were divided into two categories of environmental information processing and metabolism. Interestingly, only the MIC level of NaClO exposure caused mutations in genes related to bacterial metabolic pathways, and the major metabolic pathways they participated in included ABC transporters, Metabolic pathways, Biosynthesis of antibiotics, Biosynthesis of secondary metabolites, and Microbial metabolism in diverse environments. Through TCDB annotation of the non-synonymous SNPs (Table S4), it was found that there were 1, 1, 11, and 11 membrane transporter genes in M-3, M-5, H-2, and H-5, respectively. Among these, 4 belonged to the ABC superfamily known for its crucial role in bacterial cell vitality, virulence, pathogenicity, and multiple drug resistance, [64, 65]. The ABC superfamily proteins use energy generated by hydrolysis of ATP to reverse concentration gradient transport to remove drugs from cells, contributing to multiple drug resistance. Additionally, 1 FUP family protein identified belongs to the outer membrane pili guiding porer protein, which is associated with the processes related to bacterial pili. Pili are essential for various functions, including adherence to surfaces and host cells, biofilm formation, and pathogenicity. The presence of mutations in this protein could influence the bacterial life cycle and pathogenicity. Furthermore, there were 4 members of the MFS family, which plays essential role in bacterial uptake of various toxic compounds including antibiotics, fungicides, and other drugs [66]. In this study, CARD database annotation in Table S5 showed that only H-2 non-synonymous mutation had an Antibiotic Resistance Ontology (ARO) of CRP, a global regulator that represses MdtEF multidrug efflux pump expression. Finally, by comparison with the VFDB database (Table S6), 1, 5, and 6 virulence factors were found in the non-synonymous SNP of M-3, H-2, and H-5 genomes, respectively. These mutations need to be focused on because they are likely to affect the ability of *K. pneumoniae* to cause disease. It is important to highlight that NaClO exposure caused mutations in virulence genes, especially *bopD* and *PIA*, which are known to be associated with bacterial biofilm formation. This finding provides a clear explanation for the observed enhancement in biofilm formation under NaClO exposure conditions.

4. Conclusion

This study convincingly demonstrated that exposure to sub-MIC and MIC levels of NaClO co-selects for resistance in *K. pneumoniae* against both NaClO itself and a range of antibiotics, including erythromycin, polymyxin B, gentamicin, tetracycline, and ciprofloxacin. Such increased resistance was associated with decreased growth rate and increased biofilm formation capacity, indicating the existence of fitness cost from the evolved resistance. Through whole-population sequencing, this study identified several chromosomal genetic mutations, some of which are known to be involved in antibiotic resistance, while others represent novel candidates deserving further exploration. Notably, genes such as *mobA*, *truB*, *mutS*, and *mfpsA* emerged as potential key players in antibiotic resistance induced by NaClO exposure, underscoring the need for in-depth investigations into their mechanisms of action. Given the widespread use of NaClO-based disinfectants amid the COVID-19 epidemic, the findings of this study provided important evidence that the disinfectants may select more multidrug-resistant bacteria, potentially harming human health.

CRediT authorship contribution statement

Yi Luo: Conceptualization, Project administration, Supervision, Writing – review & editing. **Zeyou Chen:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Yulin Zhang:** Data curation, Formal analysis, Methodology. **Daqing Mao:** Resources, Writing – review & editing. **Xiaolong Wang:** Funding acquisition, 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.

Data Availability

No data was used for the research described in the article.

Acknowledgment

This work was partly funded by National Key R&D Program of China (2020YFC1806904 and 2022YFC3704600), National Natural Science Foundation of China (42277386, 42107456, 31870351), and Key Projects of the National Natural Science Foundation of China (41831287).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.134102.

References

[1] House, W., 2015. National action plan for combating antibiotic-resistant bacteria. The White House, *Washington, DC*.
[2] Lewis, D., 2021. COVID-19 rarely spreads through surfaces. So why are we still deep cleaning. Nature 590 (7844), 26–28.
[3] EPA, United States Environmental Protection Agency. List N: Disinfectants for Coronavirus (COVID-19). (accessed November 29, 2020). 2020.
[4] Dewey, H.M., Jones, J.M., Keating, M.R., Budhathoki-Uprety, J., 2022. Increased use of disinfectants during the COVID-19 pandemic and its potential impacts on health and safety. ACS Chem Health Saf 29 (1), 27–38.
[5] Kim, M., Weigand, M.R., Oh, S., Hatt, J.K., Krishnan, R., Tezel, U., et al., 2018. Widely used benzalkonium chloride disinfectants can promote antibiotic resistance. Appl Environ Microbiol 84 (17) e01201-18.
[6] Lu, J., Jin, M., Nguyen, S.H., Mao, L., Li, J., Coin, L.J., et al., 2018. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. Environ Int 118, 257–265.
[7] Russell, A., 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect Dis 3 (12), 794–803.
[8] Elekhnawy, E., Sonbol, F., Abdelaziz, A., Elbanna, T., 2020. Potential impact of biocide adaptation on selection of antibiotic resistance in bacterial isolates. Future J Pharm Sci 6 (1), 97.
[9] Gilbert, P., McBain, A.J., 2003. Potential Impact of Increased Use of Biocides in Consumer Products on Prevalence of Antibiotic Resistance. Clin Microbiol Rev 16 (2), 189–208.
[10] Ortega Morente, E., Fernández-Fuentes, M.A., Grande Burgos, M.J., Abriouel, H., Pérez Pulido, R., Gálvez, A., 2013. Biocide tolerance in bacteria. Int J Food Microbiol 162 (1), 13–25.
[11] Mc Cay, P.H., Ocampo-Sosa, A.A., Fleming, G.T.A., 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. Microbiology 156 (1), 30–38.
[12] Hou, A.-m, Yang, D., Miao, J., Shi, D.-y, Yin, J., Yang, Z.-w, et al., 2019. Chlorine injury enhances antibiotic resistance in Pseudomonas aeruginosa through over expression of drug efflux pumps. Water Res 156, 366–371.
[13] Cole, E.C., Addison, R.M., Rubino, J.R., Leese, K.E., Dulaney, P.D., Newell, M.S., et al., 2003. Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of antibacterial product users and nonusers. J Appl Microbiol 95 (4), 664–676.
[14] Kücken, D., Feucht, H.-H., Kaulfers, P.-M., 2000. Association of qacE and qacE Δ1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. FEMS Microbiol Lett 183 (1), 95–98.
[15] Lear, J.C., Maillard, J.Y., Dettmar, P.W., Goddard, P.A., Russell, A.D., 2006. Chloroxylenol- and triclosan-tolerant bacteria from industrial sources—susceptibility to antibiotics and other biocides. *International Biodeterioration &*. Biodegradation 57 (1), 51–56.
[16] Antibiotic Susceptibility in Benzalkonium Chloride-Resistant and -Susceptible Listeria monocytogenes Strains. Foodborne Pathogens and Disease 2014, 11, (7), 517–519.
[17] Giuliano, C.A., Rybak, M.J., 2015. Efficacy of triclosan as an antimicrobial hand soap and its potential impact on antimicrobial resistance: a focused review. Pharm: J Hum Pharmacol Drug Ther 35 (3), 328–336.
[18] Chuanchuen, R., Beinlich, K., Hoang, T.T., Becher, A., Karkhoff-Schweizer, R.R., Schweizer, H.P., 2001. Cross-resistance between triclosan and antibiotics in Pseudomonas aeruginosa is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother 45 (2), 428–432.
[19] Bagley, S.T., Seidler, R.J., Talbot Jr, H.W., Morrow, J., 1978. Isolation of Klebsielleae from within living wood. Appl Environ Microbiol 36 (1), 178–185.
[20] Brown, C., Seidler, R.J., 1973. Potential pathogens in the environment: Klebsiella pneumoniae, a taxonomic and ecological enigma. Appl Microbiol 25 (6), 900–904.
[21] Edberg, S.C., Piscitelli, V., Cartter, M., 1986. Phenotypic characteristics of coliform and noncoliform bacteria from a public water supply compared with regional and national clinical species. Appl Environ Microbiol 52 (3), 474–478.
[22] Matsen, J.M., Spindler, J.A., Blosser, R.O., 1974. Characterization of Klebsiella isolates from natural receiving waters and comparison with human isolates. Appl Microbiol 28 (4), 672–678.
[23] Seidler, R.J., Knittel, M.D., Brown, C., 1975. Potential pathogens in the environment: cultural reactions and nucleic acid studies on Klebsiella pneumoniae from clinical and environmental sources. Appl Microbiol 29 (6), 819–825.
[24] Ashurst, J.V., Dawson, A. , Klebsiella pneumonia. 2018.
[25] Jernigan, J.A., Hatfield, K.M., Wolford, H., Nelson, R.E., Olubajo, B., Reddy, S.C., et al., 2020. Multidrug-resistant bacterial infections in US hospitalized patients, 2012–2017. N Engl J Med 382 (14), 1309–1319.
[26] Wiegand, I., Hilpert, K., Hancock, R.E., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3 (2), 163–175.
[27] Merritt, J.H., Kadouri, D.E., O'Toole, G.A., 2005. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol.
[28] Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H., Phillippy, A.M., 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res 27 (5), 722–736.
[29] Hunt, M., Silva, N.D., Otto, T.D., Parkhill, J., Keane, J.A., Harris, S.R., 2015. Circlator: automated circularization of genome assemblies using long sequencing reads. Genome Biol 16 (1).
[30] Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al., 2014. Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement. PLoS ONE 9 (11), e112963.
[31] Liu, Z., Klümper, U., Liu, Y., Yang, Y., Wei, Q., Lin, J.-G., et al., 2019. Metagenomic and metatranscriptomic analyses reveal activity and hosts of antibiotic resistance genes in activated sludge. Environ Int 129, 208–220.
[32] McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., et al., 2013. The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother 57 (7), 3348–3357.
[33] Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21 (18), 3674–3676.
[34] Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59 (3), 307–321.
[35] Wang, Y., Tang, H., DeBarry, J.D., Tan, X., Li, J., Wang, X., et al., 2012. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res 40 (7) e49-e49.
[36] Kram, K.E., Finkel, S.E., 2015. Rich Medium Composition Affects Escherichia coli Survival, Glycation, and Mutation Frequency during Long-Term Batch Culture. Appl Environ Microbiol 81 (13), 4442–4450.
[37] Méchin, L., Dubois-Brissonnet, F., Heyd, B., Leveau, J.Y., 1999. Adaptation of Pseudomonas aeruginosa ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. J Appl Microbiol 86 (5), 859–866.
[38] Heir, E., Sundheim, G., Holck, A.L., 1999. The qacG gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. J Appl Microbiol 86 (3), 378–388.
[39] Langsrud, S., Sundheim, G., Borgmann-Strahsen, R., 2003. Intrinsic and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp. J Appl Microbiol 95 (4), 874–882.
[40] Pál, C., Papp, B., Lázár, V., 2015. Collateral sensitivity of antibiotic-resistant microbes. Trends Microbiol 23 (7), 401–407.
[41] Hall, M.D., Handley, M.D., Gottesman, M.M., 2009. Is resistance useless? Multidrug resistance and collateral sensitivity. Trends Pharmacol Sci 30 (10), 546–556.
[42] Andersson, D.I., Hughes, D., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8 (4), 260–271.
[43] Mohamed, S., Mohamed, M., Khalil, M., Azmy, M., Mabrouk, M., 2018. Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant Klebsiella pneumoniae. J Appl Microbiol 125 (1), 84–95.
[44] El-Banna, T., El-Aziz, A., Sonbol, F., El-Ekhnawy, E., 2019. Adaptation of Pseudomonas aeruginosa clinical isolates to benzalkonium chloride retards its growth and enhances biofilm production. Mol Biol Rep 46 (3), 3437–3443.
[45] Techaruvichit, P., Takahashi, H., Kuda, T., Miya, S., Keeratipibul, S., Kimura, B., 2016. Adaptation of Campylobacter jejuni to biocides used in the food industry affects biofilm structure, adhesion strength, and cross-resistance to clinical antimicrobial compounds. Biofouling 32 (7), 827–839.
[46] Woodford, N., Ellington, M.J., 2007. The emergence of antibiotic resistance by mutation. Clin Microbiol Infect 13 (1), 5–18.
[47] Willems, R.J., Top, J., Smith, D.J., Roper, D.I., North, S.E., Woodford, N., 2003. Mutations in the DNA Mismatch Repair Proteins MutS and MutL of Oxazolidinone-Resistant or -Susceptible Enterococcus faecium. Antimicrob Agents Chemother 47 (10), 3061–3066.
[48] Young, D.M., Ornston, L.N., 2001. Functions of the mismatch repair gene mutS from Acinetobacter sp. strain ADP1. J Bacteriol 183 (23), 6822–6831.
[49] Torres, L.L., Salerno, G.L., 2007. A metabolic pathway leading to mannosylfructose biosynthesis in Agrobacterium tumefaciens uncovers a family of mannosyltransferases. Proc Natl Acad Sci USA 104 (36), 14318–14323.
[50] Smith, L.T., Smith, G.M., Madkour, M.A., 1990. Osmoregulation in Agrobacterium tumefaciens: accumulation of a novel disaccharide is controlled by osmotic strength and glycine betaine. J Bacteriol 172 (12), 6849–6855.
[51] Boinett, C.J., Cain, A.K., Hawkey, J., Do Hoang, N.T., Khanh, N.N.T., Thanh, D.P., et al., 2019. Clinical and laboratory-induced colistin-resistance mechanisms in Acinetobacter baumannii. Micro Genom 5 (2).

[52] Pal, S., Verma, J., Mallick, S., Rastogi, S.K., Kumar, A., Ghosh, A.S., 2019. Absence of the glycosyltransferase WcaJ in Klebsiella pneumoniae ATCC13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. Microbiology 165 (8), 891–904.
[53] Peed, L., Parker, A.C., Smith, C.J., 2010. Genetic and functional analyses of the mob operon on conjugative transposon CTn 341 from Bacteroides spp. J Bacteriol 192 (18), 4643–4650.
[54] Moriguchi, K., Zoolkefli, F., Abe, M., Kiyokawa, K., Yamamoto, S., Suzuki, K., 2019. Targeting antibiotic resistance genes is a better approach to block acquisition of antibiotic resistance than blocking conjugal transfer by recipient cells: a genome-wide screening in Escherichia coli. Front Microbiol 10, 2939.
[55] Christ, B., Hochstrasser, R., Guyer, L., Francisco, R., Aubry, S., Hörtensteiner, S., et al., 2017. Non-specific activities of the major herbicide-resistance gene BAR. Nat Plants 3 (12), 937–945.
[56] Schneider, O., Zehl, M., Ueoka, R., Rückert, C., Buche, T., Jiang, Y., et al., 2022. N-Succinyltransferase Encoded by a Cryptic Siderophore Biosynthesis Gene Cluster in Streptomyces Modifies Structurally Distinct Antibiotics. mBio 13 (5), e01789-22.
[57] Anchana, S.R., Girija, S.A.S., Gunasekaran, S., Priyadharsini, V.J., 2021. Detection of csgA gene in carbapenem-resistant Acinetobacter baumannii strains and targeting with Ocimum sanctum biocompounds. Iran J Basic Med Sci 24 (5), 690–698.
[58] Fortin, Y., Phoenix, P., Drapeau, G.R., 1990. Mutations conferring resistance to azide in Escherichia coli occur primarily in the secA gene. J Bacteriol 172 (11), 6607–6610.
[59] Omadjela, O., Narahari, A., Strumillo, J., Mélida, H., Mazur, O., Bulone, V., et al., 2013. BcsA and BcsB form the catalytically active core of bacterial cellulose synthase sufficient for in vitro cellulose synthesis. Proc Natl Acad Sci USA 110 (44), 17856–17861.
[60] Richardson, M.A., Kuhstoss, S., Huber, M.L., Ford, L., Godfrey, O., Turner, J.R., et al., 1990. Cloning of spiramycin biosynthetic genes and their use in constructing Streptomyces ambofaciens mutants defective in spiramycin biosynthesis. J Bacteriol 172 (7), 3790–3798.
[61] Guo, W. ; Hao, H. ; Dai, M. ; Wang, Y. ; Huang, L. ; Peng, D. ; ; ; ; et al. , Development of quinoxaline 1, 4-dioxides resistance in Escherichia coli and molecular change under resistance selection. 2012.
[62] Nurse, K., Wrzesinski, J., Bakin, A., Lane, B.G., Ofengand, J., 1995. Purification, cloning, and properties of the tRNA psi 55 synthase from Escherichia coli. Rna 1 (1), 102.
[63] Kinghorn, S.M., O'Byrne, C.P., Booth, I.R., Stansfield, I., 2002. Physiological analysis of the role of truB in Escherichia coli: a role for tRNA modification in extreme temperature resistance. Microbiology 148 (11), 3511–3520.
[64] Fath, M.J., Kolter, R., 1993. ABC transporters: bacterial exporters. Microbiol Rev 57 (4), 995–1017.
[65] Davidson, A.L., Dassa, E., Orelle, C., Chen, J., 2008. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev 72 (2), 317–364.
[66] Marger, M.D., Saier Jr, M.H., 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci 18 (1), 13–20.