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# Water Disinfection Byproducts Induce Antibiotic Resistance-Role of Environmental Pollutants in Resistance Phenomena

**Dan Li**[†,‡,§], **Siyu Zeng**[‡], **Miao He**[*,†], and **April Z. Gu**[*,‡]

[†]Environmental Simulation and Pollution Control (ESPC) State Key Joint Laboratory, School of Environment, Tsinghua University, Beijing, 100084, China

[‡]Department of Civil and Environmental Engineering, Northeastern University, Boston, Massachusetts 02115, United States

[§]Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai, 200433, China

## Abstract

The spread of antibiotic resistance represents a global threat to public health, and has been traditionally attributed to extensive antibiotic uses in clinical and agricultural applications. As a result, researchers have mostly focused on clinically relevant high-level resistance enriched by antibiotics above the minimal inhibitory concentrations (MICs). Here, we report that two common water disinfection byproducts (chlorite and iodoacetic acid) had antibiotic-like effects that led to the evolution of resistant *E. coli* strains under both high (near MICs) and low (sub-MIC) exposure concentrations. The subinhibitory concentrations of DBPs selected strains with resistance higher than those evolved under above-MIC exposure concentrations. In addition, whole-genome analysis revealed distinct mutations in small sets of genes known to be involved in multiple drug and drug-specific resistance, as well as in genes not yet identified to play role in antibiotic resistance. The number and identities of genetic mutations were distinct for either the high versus low sub-MIC concentrations exposure scenarios. This study provides evidence and mechanistic insight into the sub-MIC selection of antibiotic resistance by antibiotic-like environmental pollutants such as disinfection byproducts in water, which may be important contributors to the spread of global antibiotic resistance. The results from this study open an intriguing and profound question on the

[*]**Corresponding Authors** april@coe.neu.edu (A.Z.G.). hemiao@mail.tsinghua.edu.cn (M.H.).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05113.
Description of detailed antibiotic resistance selection by water disinfection byproducts (DBPs) (Figure S1), comparison of the MICs among the original *E. coli* strain and resistant strains selected by DBPs (Figure S2), list of pathways and related genes involved in resistances to *β*-lactam (amoxicillin) and quinolones (ciprofloxacin) (Figure S3) and tables summarizing the identified genetic changes in resistant strains selected by disinfection byproducts (PDF)

Accession Codes

Whole genome sequencing data from the resistant *E. coli* strains that were selected by disinfection byproducts under both high and low (sub-MIC) exposure concentrations are publicly available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA). The submission's accession number is SRP056504. The sample accessions numbers are as follows: SRS883000 ($NaClO_2$-H-1), SRS884253 ($NaClO_2$-H-2), SRS884255 (IAA-H-1), SRS884256 (IAA-H-2), SRS884257 (IAA-H-3), SRS884258 ($NaClO_2$-L-1), SRS884259 ($NaClO_2$-L-2), SRS884260 ($NaClO_2$-L-3), SRS884261 (IAA-L-1), SRS884262 (IAA-L-2), SRS884263 (IAA-L-3), SRS1066073 (LB-control 1), and SRS1066076 (LB-control 2).

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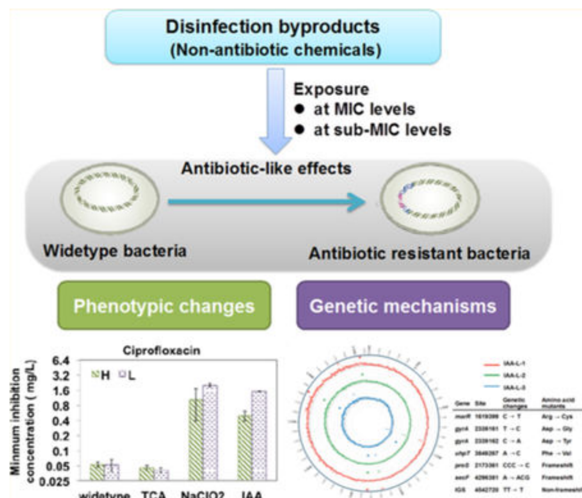
roles of large amount and various environmental contaminants play in selecting and spreading the antibiotics resistance in the environment.

## Abstract




## INTRODUCTION

The spread of antibiotic resistance is a growing public health concern worldwide resulting in significant morbidity[1] and even fatal outcomes.[1,2] Antibiotic resistance has been traditionally attributed to the overuse or misuse of antibiotics in clinical and agricultural domains for human[2] and livestock.[3] Clinically relevant high-level resistance enriched by bacterial exposure to antibiotics above the minimal inhibitory concentrations (MICs) has been the primary research focus.[4,5] However, the ability of and manifold underlying mechanisms for subinhibitory concentrations (tens to hundreds-fold below the MICs, referred to as sub-MICs) to select for bacterial resistance have recently been evidenced and discussed.[6–8] This presents profound implications because most antibiotics exist widely in natural environments at relatively low concentrations in the ng/L to $\mu$g/L ranges from both natural and anthropogenic sources.[9,10] The sub-MIC resistance selections typically occur at a high frequency because of the low fitness-cost mutations, and it favors the accumulation of broader range multiple small-step mutations in contrast to high-MIC selections that induce very specific mutations with higher resistant.[5,7,44] Recent evidence has suggested that the selection of resistant mutants by sub-MIC antibiotics operates via broadly conserved cellular functions and pathways such as those involved in bacterial SOS response[6,11] and “Reactive Oxygen Species” (ROS) response systems.[6,8] The involvement of these conserved cellular pathways and mechanisms raise important questions on the possible role of antibiotic-like environmental chemicals in the emergence and spread of antibiotic resistance on global scales.

Both the identity of chemicals exerting antibiotic effects, as well as the mechanisms behind these effects remain largely unknown. Sparse evidence of coselection of antibiotic resistance by nonantibiotic chemicals have been reported for a few types of chemicals including

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metals[12,13] and disinfectants.[14,15] The roles of metal ions, for example, in coselection of antibiotic resistance have been attributed to the shared structural and functional characteristics of prokaryotic antibiotic and metal resistance systems.[12] ROS-induced mutagenesis, oxidative stress regulators activation, and horizontal transfer of transmissible plasmids and integrons that contain both disinfectant- and antibiotic-resistant genes have been suggested to contribute to high-level (at MIC levels) antibiotic resistance selection by certain disinfectants.[14,15] The resistance selection pressures posed by the large number and diversity of other environmental chemicals, especially at relatively low concentrations (below MIC levels) in natural environments, however, have not been investigated and is a ripe area for systematic and mechanistic investigation.[4–6]

Disinfection byproducts (DBPs) are water contaminants formed during water treatment disinfection processes[16,17] and are present in nearly all water sources, posing wide exposures for human and other organisms.[17–19] Relatively increasing concentrations of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in disinfected drinking water compared to raw water sources initially raised questions as to whether DBPs were contributing to the rise of antibiotic resistance.[14,22] It had previously been thought that very low antibiotic concentrations typically present in water distribution systems would not be able to selectively cultivate antibiotic resistant bacteria.[20,21] Selection of antibiotic resistance by a number of mutagenic DBPs has been recently reported, presumably associated with identified mutations in certain genes involved in either antibiotic-specific or multidrug resistance pathways.[22,49] The role of widely present DBPs at relatively low concentrations (sub-MIC levels) on the selection and enrichment of antibiotic resistance is of great interest and importance, and the underlying mechanism remain largely unknown.

This study reports the occurrence and distinct mechanisms of antibiotic resistance selection by three water disinfection byproducts at both high (up to MIC) and low (sub-MIC, at similar order of magnitude as the highest levels detected in the environment or maximum contaminant level (MCL)) concentrations.[16–18,47,48] Three widespread DBPs, namely, trichloroacetic acid (TCA), chlorite and iodoacetic acid (IAA), which are often detected in drinking water, swimming pool and reclaimed water,[16–18,47,48] were used for the selection experiments (Figure 1). TCA and chlorite are regulated by the U.S. Environmental Protection Agency and other agencies in the world,[16] and the MCL is 0.06 mg/L for haloacetic acid (including TCA) and 1 mg/L for chlorite in drinking water.[16] IAA belongs to a new, and toxicologically significant class of DBP that was identified as part of the U.S. Nationwide Occurrence Study.[16] A total concentration of 1.6 mg/L as sum of six haloacetic acids (HAAs) has been detected in swimming pools.[47] Chlorite concentration as high as 1.1 mg/L has been reported in a water distribution system,[16] and the IAA were detected in waters from drinking water plants at levels near 1.7 $\mu$g/L.[48] In addition to the evaluation of the phenotypic changes of resistance to antibiotics by the MIC measurements, we further investigated the genetic changes and mechanisms responsible for resistance by Illumina whole-genome sequencing of selected resistant colonies (Figure 1). The results demonstrated that DBPs, at both MIC and sub-MIC levels, can select for antibiotic resistance. The whole genomic analysis identified chromosomal genetic mutations known to be involved in multiple drug and drug-specific resistance, as well as in genes not yet

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identified to play role in antibiotic resistance and expanded our understanding of the mechanisms in DBPs-induced antibiotic resistance.

## MATERIALS AND METHODS

### Strains and Selection Conditions.

First, −80 °C stock drug sensitive *E. coli* K12 (MG1655) was streaked on a Luria−Bertani (LB) agar plates and allowed to grow for 16 h. One single colony was picked and inoculated into a tube containing 10 mL of LB broth for 12 h incubation at 37 °C, which was used as isogenic "seeds" for selection experiments.

The resistance selection experiments were performed in two conditions, with gradually increasing dose concentrations up to 1 × MIC (referred as high concentration selection) and lower concentrations of DBPs (sub-MIC, 10 mg/L) (Figure 1). In the high concentration selection experiments, the concentrations of TCA, $NaClO_2$, and IAA increased from an initial value of 10 mg/ L to final MIC concentrations of 64, 400, and 350 mg/L, respectively (MIC were predetermined, see below), in a gradually step by step fashion (Supporting Information, SI, Figure S1). In the low concentration selection experiments, the concentrations of TCA, $NaClO_2$, and IAA were maintained consistently at 10 mg/L during selection cycles. All treatments and control were performed in triplicate.

The selection procedures are shown in Figure 1 as previously described.[7] Initially, 0.5 mL of isogenic *E. coli* cultures (about $10^8$ CFU) and 4.5 mL fresh LB mediums with different concentrations of DBPs were added into a 15 mL sterilized tube, then incubated at 37 °C for 24 h, and then the 0.5 mL of *E. coli* cultures from this tube was subcultured into 4.5 mL fresh LB medium with DBPs. *E. coli* selected in fresh LB medium without DBPs for 40 subculture cycles were also conducted in parallel as control. All the experimental tubes were incubated in aerated incubators without shaking, and the selection experiments were repeated for 40 subculture cycles of every 24 h.

### Isolation of Resistant Strains and Determination of Minimum Inhibitory Concentrations (MICs).

After 40 selection cycles, the *E. coli* cultures were streaked on an LB agar plates with serially dilutions and were allowed to grow for 16 h at 37 °C. Then 30−40 clones were randomly picked up from each DBP-selected culture, as well as from the untreated control culture by streaking the selected colonies onto LB-agar plates. These randomly selected colonies were grown in 3 mL of LB for 5−6 h at 37 °C, and the minimum inhibitory concentrations (MICs) of *E. coli* K12 (MG1655) were determined against six antibiotics, namely: amoxicillin, ciprofloxacin, gentamicin, polymyxin B, tetracycline, and erythromycin. In addition, MICs for the original *E. coli* K12 strain before the selection process against the six antibiotics, as well as against each of the three DBPs, were also determined. The MIC determinations were carried out in 96-well plates, as previously described,[24–26] and the initial concentrations of *E. coli* exposed to antibiotics were about $10^6$ CFU/mL. Briefly, in each 150 $\mu$L well of 96-well plates, we introduced a 5 $\mu$L of selected clone cultures, 15 $\mu$L of serially 2-fold diluted antibiotics or DBPs, and 130 $\mu$L of fresh LB

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medium, and then followed growth by measuring absorbance over 20 h. Sterilized PBS was used as blank control. The MIC was determined as the concentration of antibiotics that inhibits 90% of growth in LB. Each strain was tested at least in triplicate.

### DNA Extraction.

Two to three resistant strains from each of the DBPs-exposed culture and LB-control strains on LB plates were cultured in LB medium at 37 °C for 16 h, and the total DNA was extracted with the Universal Genomic DNA Extraction Kit (Takara, Dalian, China) according to the manufacturer's instructions. Then the DNA concentration was determined on the Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE).

### Whole-genome Sequencing and Data Analysis.

Illumina sequencing libraries were prepared from each *E. coli* genomic DNA (<50 ng) using the Nextera DNA Sample Prep Kit (Epicenter) and HiSeq2000 (Sinogene and Novogene Bioinformatics Institute, Beijing, China). The whole-genome shotgun library was sequenced across one lane (PE50 reads on Illumina Hiseq 2000, Illumina GAIIx, and Roche 454).

A reference genome for *E. coli* strain K12 was obtained from Genbank (Accession NC000913.3). Illumina reads were mapped as paired-ends to the reference genome using Bowtie. Unaligned reads were iteratively clipped from their 3′ ends by 5 bases until reads mapped or a threshold of 70 base pairs (for GAIIx) or 25 base pairs (for Hiseq) had been reached. Ambiguously mapped reads were discarded. Roche 454 reads were aligned using Newbler, proprietary alignment software from Roche, according to the manufacturer's recommendation.

Alignments were converted to pileup format using SAMtools. The percentage of mismatched bases was calculated for each base in the reference. Bases where sequenced reads differed from reference by at least 50% and had at least 5X coverage were considered genetic changes and were kept for further analysis. A two-sided *t* test was performed on the frequencies of mutations between selected samples and reference sequences. Mutations with a *p*-value result <0.01 were considered to be enriched in samples.[23,24] Genetic changes found to be enriched in antibiotic resistant samples were checked against Genbank RefSeq.

### Transcriptomic Analysis of Impact of Disinfection Byproducts on Oxidative Stress and SOS Response Pathways.

A group of transcriptional fusions of GFP that include different promoters in *E. coli* K12 MG1655 was employed in this study (Open Biosystem, Huntsville, AL). Each promoter is expressed from a low-copy plasmid, pUA66 or pUA139, which contain a kanamycin resistance gene and a fast-folding gfp-mut thus enabling real-time measurement of gene expression with high accuracy and reproducibility.[45,46] For this particular study, 43 recombinant *E. coli* strains with different promoters were selected, which control the expression of oxidative stress and SOS response related DNA damage and repair pathways in *E. coli* (Table S3).

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The recombinant *E. coli* strains were transferred from frozen stocks into LB medium supplemented with 50 $\mu$g/mL kanamycin, and incubated at 37 °C for 16–18 h. The *E. coli* were then diluted 1:100 into fresh M9 medium to a total volume of 60 $\mu$L into individual wells of black 384-well plates (Costar, U.S.A.), and then the plates were incubated at 37 °C for about 2.5 h. When the cultures reached early exponential growth (OD590 about 0.15), and 10 $\mu$L of DBPs (TCA, $NaClO_2$ and IAA), negative controls ( L-glutamic acid (LG), dextrose (Dex)) and positive control (Mitomycin C, MMC) were added per well. Then the 384-well plate was measured by the microplate reader (Synergy HTMulti-Mode, Biotech, Winooski, VT) with continuous measurement of the optical density at 590 nm (cell density, OD590) and fluorescence (GFP level, filters 485 nm, 528 nm) for 2 h at a time interval of 6 min. A more detailed methods description is available in a previous study.[47] All tests were performed in triplicates.

The data were processed according previous study analyses published by our group.[45,46] First, all data were corrected for medium control (with and without chemicals). The altered gene expression level is described as induction factor $I$ ($I$ = Pe/Pc).[47] For a given strain (gene) at each time point due to chemical exposure, induction factor ($I$) is represented by the ratio of the normalized GFP level (Pe = $(GFP/OD)_{experiment}$) in the experiments condition with chemicals exposure to that (Pc = $(GFP/OD)_{control}$) in the control condition without any chemicals exposure.[46] A conservative cutoff background noise threshold value of 0.4 ([Ln(I)]abs = 0.4) was chosen based on previous reproducibility and control tests. The significantly upregulated gene expression were analyzed based on Ln($I$) > 0.4 and $p < 0.05$.

# RESULTS AND DISCUSSION

## DBPs Selected Antibiotic Resistance at both MIC and sub-MIC Concentrations.

We evaluated three DBPs (TCA, $ClO^-$, IAA) in present study[16–18] (Figure 1). The MICs test was used to identify phenotypic evidence of resistance to antibiotics.[23] Genetic changes and mechanisms responsible for resistance were investigated by Illumina whole-genome sequencing of selected resistant colonies[23,24] (Figure 1).

Compared with the MIC of the original *E. coli* K12 strain before selection, the untreated control samples grown with LB medium only did not exhibit resistance to antibiotics (Figure 2). With both sub-MIC and high MIC levels exposure, two of the three tested DBPs, namely $NaClO_2$ and IAA, selected strains that exhibited clinically relevant[26] resistances to amoxicillin (penicillin group of $\beta$-lactam antibiotic) and ciprofloxacin (quinolone antibiotic) with significant increases in MICs by 4 to 8 folds (Figure 2). The resistance to other antibiotics including gentamycin, polymyxin B, tetracycline, and erythromycin, were to a lesser degree (less than 4 fold increase in MICs) (Figure S2). TCA exposure did not select any strains that had resistance to any of the six antibiotics tested. The average MICs for $NaClO_2$ and IAA selected amoxicillin-resistant strains were more than 4-fold higher than that of the original or LB-control *E. coli* (16 mg/L; Figure 2a). For ciprofloxacin, the $NaClO_2$ and IAA-selected resistant strains exhibited more than 8-fold higher MICs than that of the LB-control *E. coli* (0.053 mg/L) (Figure 2b). Surprisingly, the ciprofloxacin-resistant strains selected with low sub-MIC DBP concentrations showed significantly higher (by 2–3 folds) resistance than those selected by exposure to high concentrations (Figure 2b).

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### Resistance Mechanisms for High-Concentration Selection.

To identify the genetic changes responsible for antibiotic resistance to amoxicillin and ciprofloxacin, three randomly selected isogenic resistant clones from the evolved population under both high and low-selection conditions for the two DBPs were subjected to whole-genome sequencing so that genetic changes could be counted and identified. Sequencing of the 4 resistant clones selected by the two DBPs at high concentrations revealed 19 genetic changes in 8 genes including 11 insertions, 4 deletions and 4 single nucleotide polymorphisms (SNPs; Figure 3a, Table S2) comparing with the reference sequence (Accession NC000913.3).

Genetic changes identified in those 4 strains obtained under exposure to high DBPs concentrations up to MIC levels were categorized into three major functional groups for: (i) membrane structure and transport; (ii) transcription and translation, and (iii) unknown functions (Figure 3a). Exposure to the two DBPs at high concentrations selected resistant *E. coli* strains that acquired resistance via different sets of small numbers of mutations in genes involved in membrane proteins and structures such as *dsdX*,[27] *exbB*,[28] *frdD*,[29] *glpF*,[30] and *kup*,[31] related to transcription and translations such as *rpoS*[8] and *firmE*,[23] or with unclear function *ylbE*[32] (Figure 3a, Table S2). The two genes *rpoS* and *ylbE* had mutations common to resistant strains selected by both $NaClO_2$ and IAA (Figure 3a). The other genetic changes seemed to be chemical-specific: IAA was associated with changes in efflux pump system, and $NaClO_2$ was linked to modifications in membrane proteins and structure (Figure 3a, Table S2).

Many of the genes that demonstrated changes under the pressure of high-level DBP exposure were previously identified as playing a role in both amoxicillin and ciprofloxacin resistances. The mechanisms underlying this resistance includes outer membrane gene *exbB* that participates in a proton pathway,[28] efflux pump system gene *dsdX*, the second $_D$-Serine transporter in ion transporter (IT) superfamily,[27] and *kup* gene involved in transporting cations such as potassium ions.[31] These identified mutations likely contribute to both ampicillin and ciprofloxacin resistances because they are related to membrane-mediated multidrug resistance pathways, which yield impermeability of the membrane or overexpressed efflux pump systems[24,33] (Figure S3).

Mutations were also observed in translation- and transcription-related resistance genes. In particular, the exact same deletion at position 2,865,017 from GA → G causing a frameshift mutation in gene *rpoS* occurred repeatedly in the sequenced resistant strains selected by both IAA and $NaClO_2$ at high concentrations (Table S2). *RpoS* is a transcriptional regulator, also called stress-response sigma factor, and it is known to play role in surviving multidrug resistance and various environmental stress conditions.[5,8] In the ampicillin resistance model, it was shown that *rpoS* positively regulates the small RNA (sRNA) *sdsR*.[8] This upregulation of *rpoS* led to elevated sRNA that binded to and repressed the *mutS* mRNA.[8] As a consequence, cells became depleted for the MutS protein, a protein with a central role in the repair of replication errors, thereby leading to an increase in mutation rate.

The whole genome sequencing of resistant clones also identified mutation in 4 genes that were not previously known to be involved in antibiotic resistance, including *ylbE*, *frdD*,

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*fimE*, and *glpF* (Figure 3a, Figure S3). The gene with the highest frequency of mutation changes was *ylbE*, comprising 7 out of the 19 total genetic changes identified among the 5 sequenced clones. Changes included 5 inserts (a G insert in base 547, 835 leading to a frameshift mutation) and 2 SNPs (A → G in base 547,694 causing no amino acid change, Glu [GAA → GAG]; Table S2). Note that the 2 SNPs (A → G in base 547,694) and 2 insertions (a G insert in base 547,831 leading to a frameshift mutation) were also identified in the LB-control clones, indicating their possible association with spontaneous mutations (i.e., incubation condition and possible stress) (Table S1). The function of the gene *ylbE* remains unclear, but it has been reported to be associated with resistance to nitric oxide and L-1, 2-Propanediol.[32,34] Only $NaClO_2$ at high concentrations induced mutations in *frdD*, *fimE*, and *glpF*. The gene *frdD* is involved in the anchoring of the catalytic components of the fumarate reductase complex to the cell inner membrane, which is regulated at the transcriptional level in response to the cellular availability of the alternate electron acceptors oxygen, nitrate, and fumarate.[29] *FimE* is a surface structure regulator,[35] and *glpF* involves in trans-membrane diffusion of glycerol in *E. coli*.[30] Our results suggest that these genes may potentially play critical roles in antibiotic resistance, thus warranting further investigation.

## Resistance Mechanisms for Low-Concentration Selection.

In contrast to studies performed at high concentrations of DBPs and LB medium only (LB-control), a genomics analysis with selected resistant strains evolving under constant and lower DBP concentrations (sub-MICs) covered completely different mutations (Tables S1, S2, and S3). These mutations were localized to only 7 genes belonging to three major functional groups related to (i) transcription and translation (*gryA*, *proS*), (ii) membrane transport (*marC*, *marR*, *secF*, and *uhpT*), and (iii) intergenic spacer (IGS; Figure 3b, Figure 4, Table S3). Two types of substitution mutations (T → C in base 2,339,161 causing an Asp → Gly missense mutation, and C → A in base 2,339,162 causing an Asp → Tyr missense mutation) in *gyrA* gene (DNA gyrase) and a same deletion (CCC → C in base 2,173,361 causing a frameshift mutation) in *proS* (prolyl-tRNA synthetase) were identified in all the sequenced resistant strains selected by both $NaClO_2$ and IAA at sub-MIC concentrations (Figure 4; Table S3). Ciprofloxacin inhibited *gyrA* function by sitting in the active site of the enzyme.[24,36] Previous research also indicated that amino acid variation in *gyrA* subunit of bacteria is potentially associated with resistance to quinolone antibiotics such as ciprofloxacin.[24,33] The two mutations identified in this study are also likely to be contributing to resistance to ciprofloxacin.

Both $NaClO_2$ and IAA at sub-MIC concentrations induced a genetic deletion in *proS* (CCC → C in base 2,173,361) and consequently caused a frameshift mutation. The gene *proS* is involved in encoding proline-tRNA synthetase (ProRS), which plays a critical role in amino acid editing and is capable of misactivating and editing Ala via both the pre- and post-transfer editing pathways.[37] While the genetic changes in *proS* gene in bacteria have never been directly shown to be involved in antibiotic resistance, recent studies described two mutations in the cytoplasmic prolyl-tRNA synthetase (*cPRS*) gene that conferred resistance to halofuginone,[38] as well its association to pyrazolopyrimidinedione (PPD) resistance.[39] In conjunction with this finding, our results indicate for the first time that a genetic change in *proS* in bacteria may be involved in antibiotic resistance.

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Genomic mutations were also detected in four genes encoding membrane proteins among the resistance strains selected by the two DBPs at sub-MIC concentrations. These included two multiple antibiotic resistance genes (*marR* and *marC*) and two membrane transporter genes whose role in amoxicillin and ciprofloxacin resistance are not yet known (*uhpT* and *secF*) (Figure 3b, Figure 4, Table S3). The same substitution mutation of a missense A → C in base position 3,846,267 in *uhpT* (causing a Phe → Val amino acid substitution) was identified in all the sequenced resistance clones selected by the two low-level DBPs (Figure 4; Table S3). Gene *uhpT* encodes a sugar phosphate transporter and is a component of the major super family (MSF) transporter complex in *E. coli* previously reported to export other antibiotics (fosfomycin), but has not been reported to be involved in amoxicillin and ciprofloxacin resistance.[40] One clone evolved after exposure to sub-MIC $NaClO_2$ resulting a missense mutation in the inner membrane protein gene *marC*,aA → G in base 4,277,878 leading to an Asp → Gly mutation; (Figure 3b, Figure 4, Table S3). One resistance clone selected by IAA had a C → T mutation in base 1,619,399 of *E. coli* genome that causes a missense mutation of Arg → Cys in *marR*. *MarR* represses the multiple antibiotic resistance (mar) operon and was previously identified to be involved in resistance to both high and sub-MIC concentrations of the quinolones antibiotics such as ciprofloxacin in *E. coli*.[7,24,41] Only sub-MIC concentrations of IAA selected stains with insertions in *secF* gene (A → ACG in base 4,296,381) leading to a frameshift mutation; these insertions have been identified to be a part of the Sec protein translocase complex in *E. coli* that confers $Na^+$-dependent protein translocation.[42] There has been no report regarding the possible involvement of *secF* in antibiotic resistance yet.

Deletions (TT → T in base 4,542,720 causing a nonframeshift mutation) in intergenic spacer (IGS) were induced by both $NaClO_2$ and IAA at sub-MIC concentrations (Figure 4, Table S3). IGSs comprise both noncoding and nonfunctional regions, including decaying pseudogenes at varying stages of recognizability, as well as functional elements such as genes for sRNAs and regulatory control elements.[43] The noncoding regions can result from reductive evolution and genome decay, i.e., through the inactivation of previously functional regions. However, they may contain any of several classes of regulatory control elements that have been maintained over the history of the symbiosis.[43] The functional elements present in intergenic regions of bacteria have not been well studied, and there has been no report regarding the possible involvement of IGS in antibiotic resistance.

The ubiquitous presence of disinfection byproducts (DBPs) in drinking water treatment units, water distributing systems, and swimming pools have resulted in widespread exposure for humans and other organisms.[17,19] Here, we report for the first time that two DBPs can act like antibiotics and lead to resistance under exposure to both high (at MICs) and low (sub-MICs) concentrations. Additionally, through whole-genome sequencing, this investigation revealed distinct mutations and implied mechanisms of resistance that evolved under low versus high exposure concentrations. It has been hypothesized that sub-MIC antibiotic resistance selection typically occurs at a high frequency because of the low fitness-cost mutations. Resistance occurring in this way favors the accumulation of a broader range multiple small-step mutations in contrast to high-MIC selections that induce very specific mutations with higher resistance.[5,44] However, our results showed that the selection by sub-MIC concentrations of the DBPs led to mutations in antibiotic-specific genetic targets (i.e.,

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*gyrA* and ciprofloxacin) and consequently resulted in much higher levels of resistance than those which evolved under higher concentrations in multidrug resistance pathways. Only certain DBPs at tested concentrations could select for resistance to particular antibiotics, suggesting chemical structure-specific actions or varying minimal selection concentrations (MSC)[2,5] among different DBPs.[22] In addition, in this proof-of-concept study, we selected the low DBP exposure concentration based on their maximum detected levels reported in the literature. Further study on larger number and wider range of DBPs classes, and at lower levels as those often observed in drinking water distribution systems in the U.S. would be of interest.

Previous studies indicated that sub-MIC antibiotics can select resistant mutants by affecting the mutation frequency, cellular SOS regulation, and oxidative stress response pathways, which consequently impact the rates of mutagenesis, horizontal gene transfer (HGT), or recombination in genes involved in various antibiotic resistance pathways (Figure 5a).[5–8,22,44,49] Oxidative stress has been linked to antibiotic resistance via ROS-mediated induction of multidrug efflux systems,[6,8] interference on tRNA synthetase, and ROS-induced DNA mutagenesis.[6,8,11] The two DBPs studied here have been shown to cause oxidative stress and DNA damage.[16,18] Our transcriptional analysis also confirmed that that low levels of these two DBPs ($NaClO_2$ and IAA) induced significant expression changes in oxidative stress response genes (i.e., *soxR*, *soxS*, *marR*, and *sodC* genes) and SOS response related DNA damage and repair genes (i.e., *recA*, *lexA*) (Table S4).[45,46] Therefore, our results suggest that these water disinfection byproducts, at both sub-MIC and high near-MIC concentrations, likely promoted antibiotic resistance via oxidative stress and SOS response related pathways-mediated mutations. Particularly, sub-MIC exposure to oxidative-DBPs seemed to enrich for repeated and frequent mutations in genes involve in chromosome replication such as *proS* (prolyl-tRNA synthetase) and *gyrA* (DNA gyrase).

This study, along with several previous reports on observations of enrichment of antibiotics resistance by heavy metals and disinfectants open an intriguing and profound question on the roles of varying concentrations and mixtures of environmental contaminants in selecting and spreading antibiotics resistance in the environment (Figure 5b). Most likely, the widespread presence of antibiotic resistant strains in the environment may have arisen in response to various selective pressures. The results of this study provide mechanistic evidence supporting the idea that the selection of resistance by antibiotic-like environmental pollutants could have been overlooked as potential contributors to the widespread emergence of bacterial resistance on a global scale. The conventional view that clinical and veterinary antibiotics usages are the most significant contributors to the antibiotic resistance phenomena should be re-evaluated. Future studies with greater numbers and larger variety of emerging environmental contaminants and mixtures are needed, in order to determine how antibiotic-like contaminants select and spread antibiotic resistant bacteria. In addition, the relationships among the antibiotic-like effects, exposure dose, properties, and structures of the chemicals should be explored (Figure 5b). Finally, a detailed delineation of the complex inter-relationships between more environmental contaminants and antibiotics will be critical to provide a cohesive and rigorous understanding of the persistence and proliferation of antibiotic resistance in the environment.

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## Supplementary Material

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## REFERENCES

(1). Taubes G The bacteria fight back. Science 2008, 321, 356–361. [PubMed: 18635788]

(2). Witte W Medical consequences of antibiotic use in agriculture. Science 1998, 279, 996–997. [PubMed: 9490487]

(3). Zhu YG; Johnson TA; Su JQ; Qiao M; Guo GX; Stedtfeld RD; Hashsham SA; Tiedje JM Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. U. S. A 2013, 110, 3435–3440. [PubMed: 23401528]

(4). Wright GD Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol 2010, 13, 589–594. [PubMed: 20850375]

(5). Andersson DI; Hughes D Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol 2014, 12, 465–478. [PubMed: 24861036]

(6). Kohanski MA; DePristo MA; Collins JJ Sublethal antibiotic treatment leads to multidrug resistance via radical-Induced mutagenesis. Mol. Cell 2010, 37, 311–320. [PubMed: 20159551]

(7). Gullberg E; Cao S; Berg OG; Ilbačk C; Sandegren L; Hughes D; Andersson DI Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011, 7 (7), e1002158. [PubMed: 21811410]

(8). Gutierrez A; Laureti L; Crussard S; Abida H; Rodríguez-Rojas A; Blaźquez J; Baharoglu Z; Mazel D; Darfeuille F; Vogel J; Matic I beta-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat. Commun 2013, 4, 1610. [PubMed: 23511474]

(9). Kummerer K Antibiotics in the aquatic environment - A review - Part I. Chemosphere 2009, 75, 417–434. [PubMed: 19185900]

(10). Senta I; Terzic S; Ahel M Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment. Water Res 2013, 47 (2), 705–714. [PubMed: 23186859]

(11). Miller C; Thomsen LE; Gaggero C; Mosseri R; Ingmer H; Cohen SN SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science 2004, 305 (5690), 1629–1631. [PubMed: 15308764]

(12). Baker-Austin C; Wright MS; Stepanauskas R; McArthur JV Co-selection of antibiotic and metal resistance. Trends Microbiol 2006, 14 (4), 176–182. [PubMed: 16537105]

(13). Berg J; Thorsen MK; Holm PE; Jensen J; Nybroe O; Brandt KK Cu exposure under field conditions co-selects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. Environ. Sci. Technol 2010, 44 (22), 8724– 8728. [PubMed: 20964403]

(14). Chapman JS Disinfectant resistance mechanisms, cross-resistance, and co-resistance. Int. Biodeterior. Biodegrad 2003, 51, 271–276.

(15). Bouzada ML; Silva VL; Moreira FA; Silva GA; Diniz CG Antimicrobial resistance and disinfectants susceptibility of persistent bacteria in a tertiary care hospital. J. Microbiol. Antimicrob 2010, 2, 105– 112.

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(16). Richardson SD; Plewa MJ; Wagner ED; Schoeny R; Demarini DM Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. Mutat. Res., Rev. Mutat. Res 2007, 636 (1–3), 178–242.

(17). Chowdhury S; Alhooshani K; Karanfil T Disinfection byproducts in swimming pool: occurrences, implications and future needs. Water Res 2014, 53, 68–109. [PubMed: 24509344]

(18). Nieuwenhuijsen MJ; Martinez D; Grellier J; Bennett J; Best N; Iszatt N; Vrijheid M; Toledano MB Chlorination disinfection by-products in drinking water and congenital anomalies: review and meta-analyses. Environ. Health. Persp 2009, 117 (10), 1486–1493.

(19). Guilherme S; Rodriguez MJ Occurrence of regulated and non-regulated disinfection by-products in small drinking water systems. Chemosphere 2014, 117, 425–432. [PubMed: 25194329]

(20). Ye Z; Weinberg HS; Meyer MT Trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry. Anal. Chem 2007, 79 (3), 1135–1144. [PubMed: 17263346]

(21). Watkinson AJ; Murby EJ; Kolpin DW; Costanzo SD The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Sci. Total Environ 2009, 407 (8), 2711–2723. [PubMed: 19138787]

(22). Lv L; Jiang T; Zhang SH; Yu X Exposure to mutagenic disinfection byproducts leads to increase of antibiotic resistance in Pseudomonas aeruginosa. Environ. Sci. Technol 2014, 48 (14), 8188–8195. [PubMed: 24933271]

(23). Toprak E; Veres A; Michel JB; Chait R; Hartl DL; Kishony R Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nat. Genet 2011, 44 (1), 101– 105. [PubMed: 22179135]

(24). Zhang Q; Lambert G; Liao D; Kim H; Robin K; Tung CK; Pourmand N; Austin RH Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments. Science 2011, 333 (6050), 1764–1767. [PubMed: 21940899]

(25). World Health Organization Guidelines for Drinking-water Quality, 4th ed.; World Health Organization: Geneva, 2011; ISBN 9789241548151.

(26). Clinical and laboratory standards institute (CLSI). Performance standards for antimicrobial susceptibility testing; Institute antimicrobial susceptibility testing standards M02-A11 and M07-A9 Clinical and Laboratory Standards Institute: Wayne, PA, 2012.

(27). Anfora AT; Welch RA DsdX is the second D-serine transporter in uropathogenic Escherichia coli clinical isolate CFT073. J. Bacteriol 2006, 188 (18), 6622–6628. [PubMed: 16952954]

(28). Baker KR; Postle K Mutations in *Escherichia coli* exbB transmembrane domains identify scaffolding and signal transduction functions and exclude participation in a proton pathway. J. Bacteriol 2013, 195 (12), 2898–2911. [PubMed: 23603742]

(29). Westenberg DJ; Gunsalus RP; Ackrell BAC; Sices H; Cecchini G. *Escherichia coli* fumarate reductase frdC and frdD mutants Identification of amino-acid-residues involved in catalytic activity with quinones. J. Biol. Chem 1993, 268 (2), 815–822. [PubMed: 8419359]

(30). Chen LY Glycerol modulates water permeation through Escherichia coli aquaglyceroporin GlpF. Biochim. Biophys. Acta, Biomembr 2013, 1828, 1786–1793.

(31). Liu Y; Ho KK; Su J; Gong H; Chang AC; Lu S Potassium transport of *Salmonella* is important for type III secretion and pathogenesis. Microbiology 2013, 159 (8), 1705–1719. [PubMed: 23728623]

(32). Justino MC; Vicente JB; Teixeira M; Saraiva LM New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. J. Biol. Chem 2005, 280 (4), 2636–2643. [PubMed: 15546870]

(33). Ruiz J Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J. Antimicrob. Chemoth 2003, 51 (5), 1109–1117.

(34). Lee DH; Palsson BO Adaptive evolution of Escherichia coli K-12 MG1655 during growth on a nonnative carbon source, L-1, 2-Propanediol. Appl. Environ. Microb 2010, 76 (13), 6327–6327.

(35). Blomfield IC; Mcclain MS; Princ JA; Calie PJ; Eisenstein BI Type-1 fimbriation and fime mutants of *Escherichia coli* K-12. J. Bacteriol 1991, 173 (17), 5298–5307. [PubMed: 1679429]

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(36). Bryskier A Fluoroquinolones: mechanisms of action and resistance. Int. J. Antimicrob. Agents 1993, 2, 151–83. [PubMed: 18611533]

(37). Wong FC; Beuning PJ; Nagan M; Shiba K; Musier-Forsyth K Functional role of the prokaryotic proline-tRNA synthetase insertion domain in amino acid editing. Biochemistry 2002, 41 (22), 7108–7115. [PubMed: 12033945]

(38). Herman JD; Rice DP; Ribacke U; Silterra J; Deik AA; Moss EL; Broadbent KM; Neafsey DE; Desai MM; Clish CB; Mazitschek R; Wirth DF A genomic and evolutionary approach reveals non-genetic drug resistance in malaria. Genome Biol 2014, 15 (11), 511. [PubMed: 25395010]

(39). Montgomery JI; Smith JF; Tomaras AP; Zaniewski R; McPherson CJ; McAllister LA; Hartman-Neumann S; Arcari JT; Lescoe M; Gutierrez J; Yuan Y; Limberakis C; Miller AA Discovery and characterization of a novel class of pyrazolopyrimidinedione tRNA synthesis inhibitors. J. Antibiot 2015, 68, 361. [PubMed: 25464974]

(40). Nilsson AI; Berg OG; Aspevall O; Kahlmeter G; Andersson DI Biological costs and mechanisms of fosfomycin resistance in Escherichia coli. Antimicrob. Agents Chemother 2003, 47 (9), 2850–2858. [PubMed: 12936984]

(41). Alekshun MN; Levy SB The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol 1999, 7 (10), 410–413. [PubMed: 10498949]

(42). Pradel N; Delmas J; Wu LF; Santini CL; Bonnet R Sec-and Tat-dependent translocation of beta-Lactamases across the Escherichia coli inner membrane. Antimicrob. Agents Chemother 2009, 53 (1), 242–248. [PubMed: 18981261]

(43). Degnan PH; Ochman H; Moran NA Sequence conservation and functional constraint on intergenic spacers in reduced genomes of the obligate symbiont buchnera. PLoS Genet 2011, 7 (9), e1002252 (2011)..

(44). Hughes D; Andersson DI Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr. Opin. Microbiol 2012, 15 (5), 555–560. [PubMed: 22878455]

(45). Onnis-Hayden A; Weng H; He M; Hansen S; Ilyin V; Lewis K; Gu AZ Prokaryotic real-time gene expression profiling for toxicity assessment. Environ. Sci. Technol 2009, 43 (12), 4574–4581. [PubMed: 19603679]

(46). Gou N; Gu AZ A new transcriptional effect level index (TELI) for toxicogenomics-based toxicity assessment. Environ. Sci. Technol 2011, 45 (12), 5410–5417. [PubMed: 21612275]

(47). Tang HL; Ristau RJ; Xie YF Disinfection By-Products in Swimming Pool Water: Formation, Modeling, and Control. ACS Symp. Ser 2015, 1190, 381–403.

(48). Richardson SD; Fasano F; Ellington JJ; Crumley FG; Buettner KM; Evans JJ; Blount BC; Silva LK; Waite TJ; Luther GW; Mckague AB; Miltner RJ; Wagner ED; Plewa MJ Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. Environ. Sci. Technol 2008, 42 (22), 8330–8338. [PubMed: 19068814]

(49). Lv L; Yu X; Xu Q; Ye C Induction of bacterial antibiotic resistance by mutagenic halogenated nitrogenous disinfection byproducts. Environ. Pollut 2015, 205, 291–298. [PubMed: 26114900]

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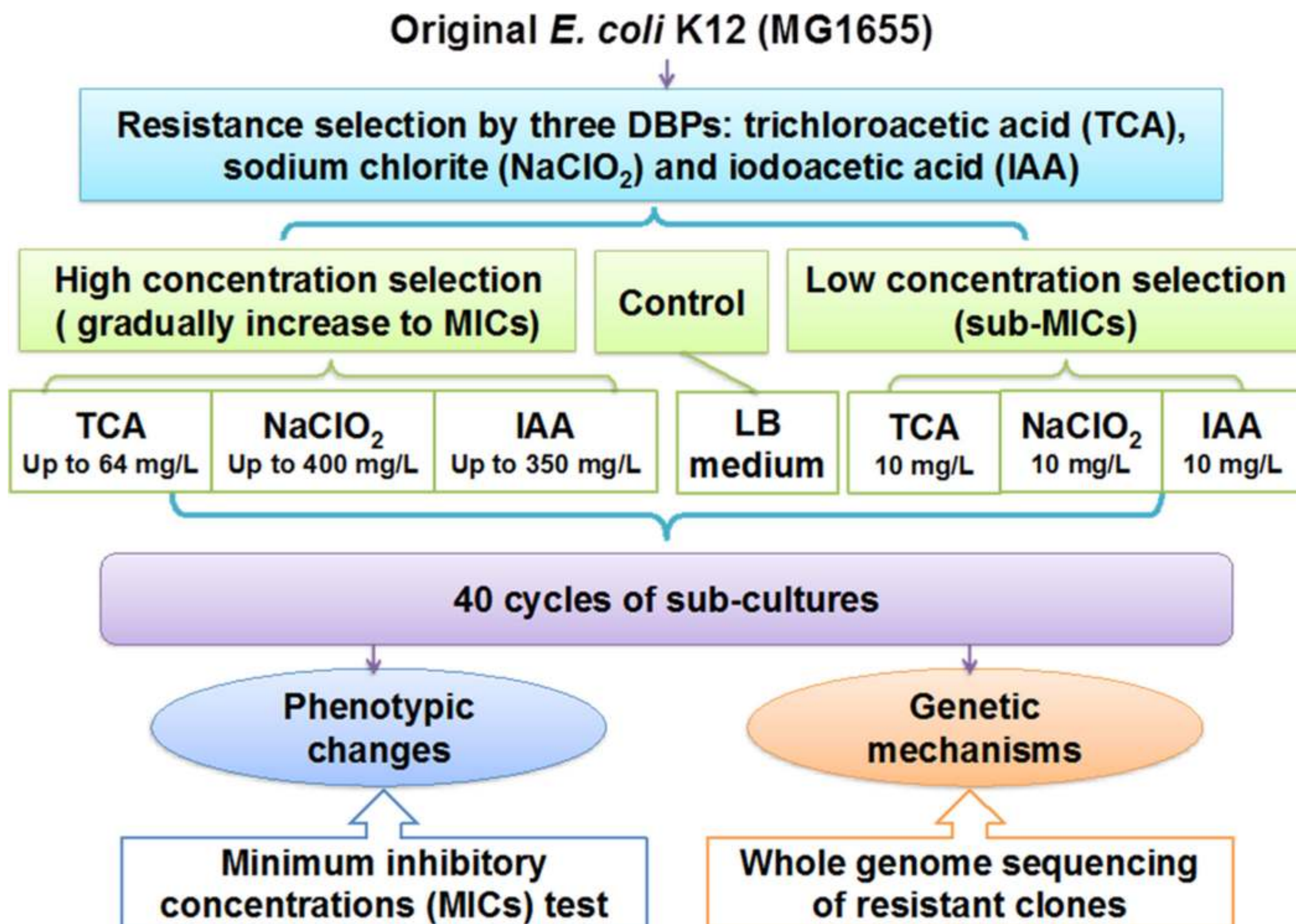


**Figure 1.**
Antibiotic resistance selection by water disinfection byproducts (DBPs). Flow diagram of antibiotic resistance selection upon exposure to three common water DBPs widely present in drinking water and other water sources, at both high (gradual increase to MIC) and low (sub-MIC) concentrations, and subsequent resistance phenotypic and genomic characterization.

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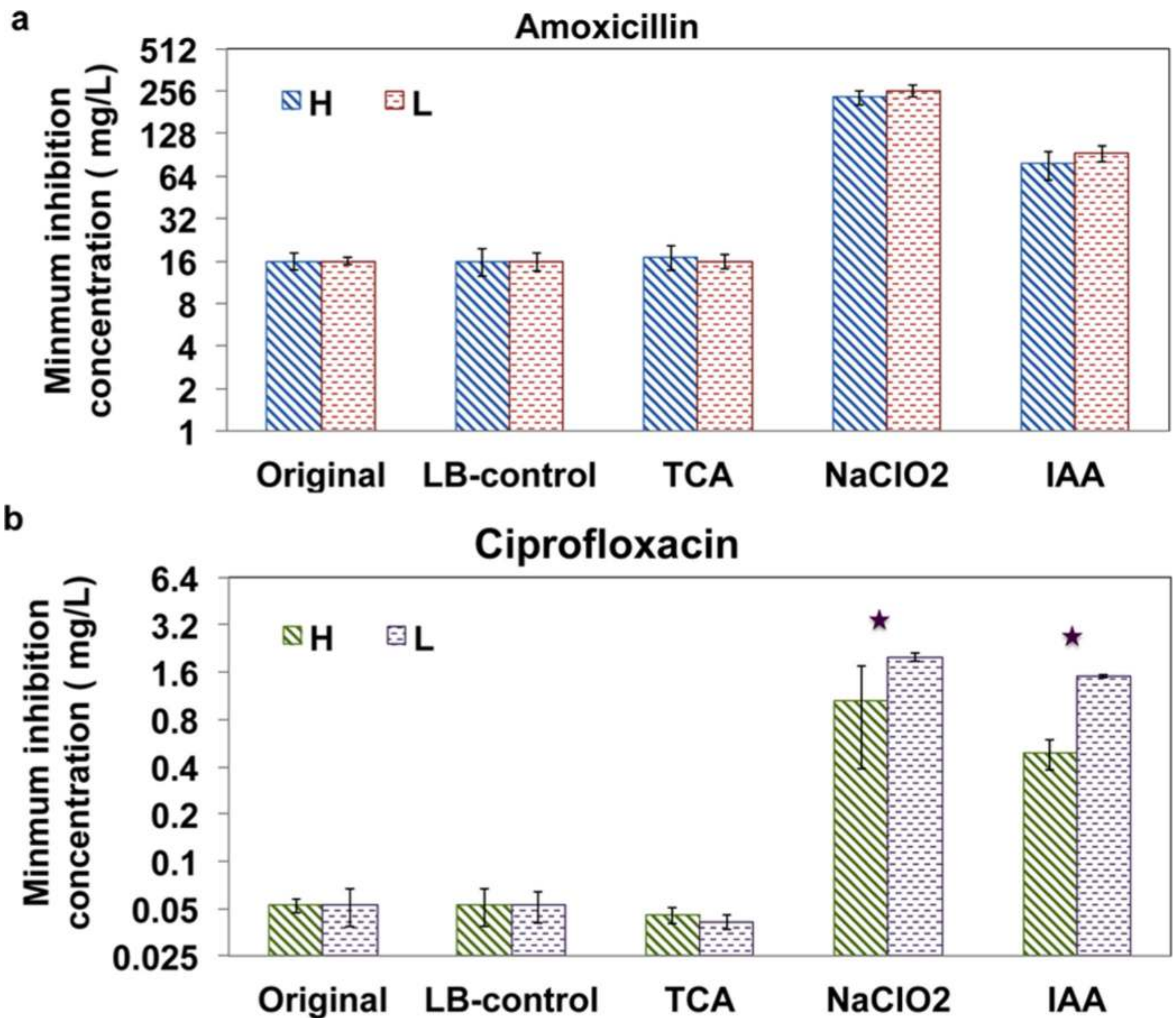


**Figure 2.**

Comparison of the minimum inhibition concentrations (MICs) for amoxicillin (a) and ciprofloxacin (b) among the original *E. coli* K12 (MG1655), strains selected by LB medium (LB-control) and three different DBPs (TCA, $NaClO_2$ and IAA). H: resistant strains selected at high DBP exposure concentrations that gradually increased up to MIC levels; L: resistant strains selected at low DBP exposure concentrations (sub-MIC, 10 mg/L). Results shown are an average of triplicates with error bar indicating standard deviation. The asterisks indicate statistically significant ($p < 0.05$) increases of MICs in ciprofloxacin resistant strains selected at low DBPs exposure concentration compared with those evolved under high DBPs concentrations.

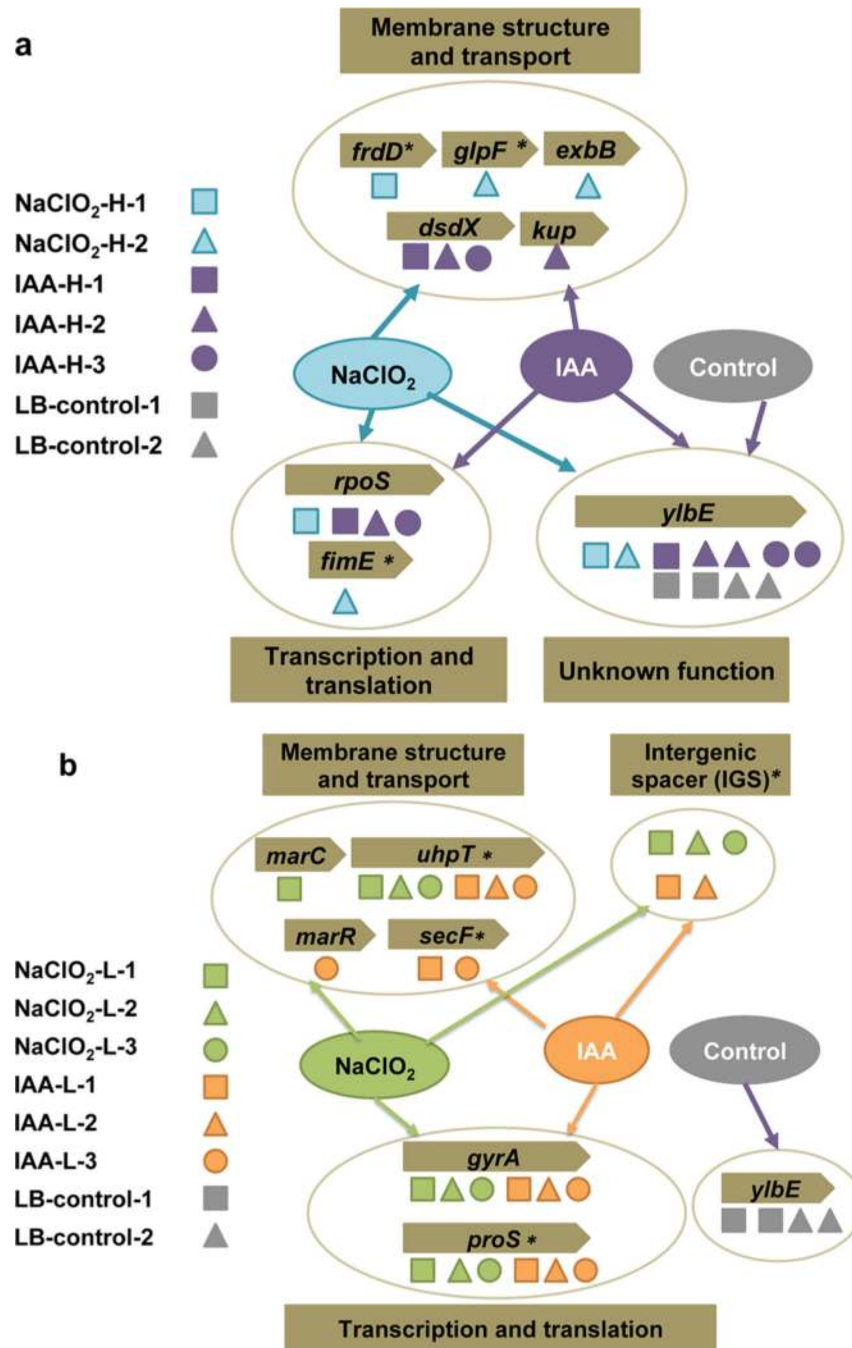


**Figure 3.**

Comparison of genetic changes identified in DBPs-selected antibiotic resistant strains under either high or low exposure concentrations. Three individual resistant clones from each selection condition are shown by three different symbols and are color-coded to differentiate $NaClO_2$, IAA, and LB-selected strains. (a) Genetic changes identified in antibiotic resistant strains evolved under exposure to high DBPs concentrations up to MIC levels were categorized into three major functional groups for: (i) membrane structure and transport; (ii) transcription and translation, and (iii) unknown functions. (b) Genetic changes identified in



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antibiotic resistant strains evolved under constant exposure to low DBPs concentrations (sub-MIC, 10 mg/L) were categorized into three major functional groups for: (i) membrane structure and transport, (ii) transcription and translation, (iii) intergenic spacer (IGS), and (iv) unknown functions. Two randomly selected LB-control clones have four spontaneous mutations on *ylbE* gene with unclear function. Mutations identified in this study that have not been reported to play role in antibiotic resistance to amoxicillin or ciprofloxacin were marked with *. The detailed information on these identified genetic changes was summarized in Tables S1, S2, and S3.

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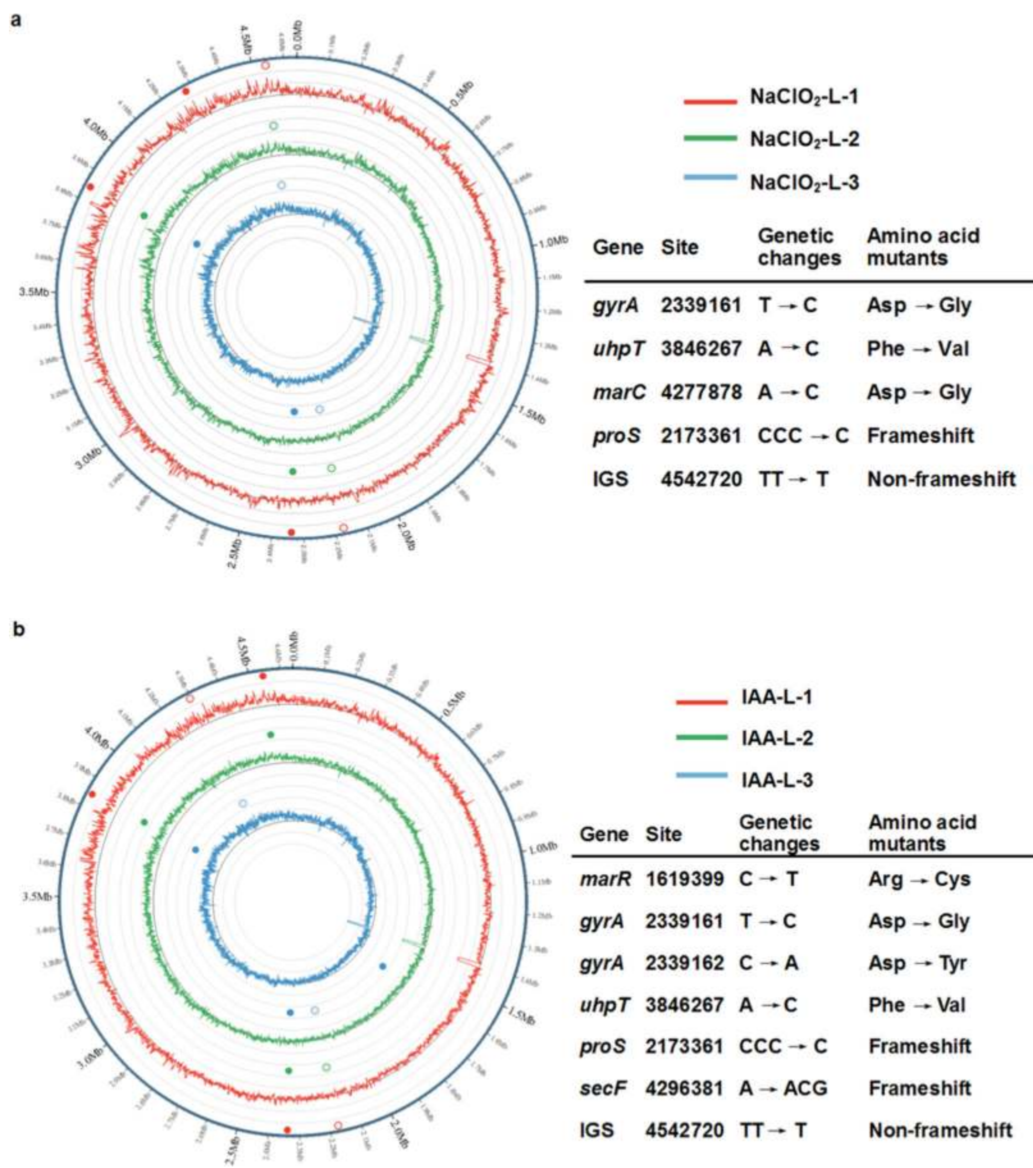


**Figure 4.**

Genetic changes identified in resistant strains selected under exposure to low (sub-MIC) DBPs concentrations. The whole genomic analysis was performed for 6 individual resistant clones, including three strains evolved under $NaClO_2$ (10 mg/L) exposure (a) and three strains evolved under IAA (10 mg/L) exposure (b). Sequencing coverage for each isolate is plotted, according to color, on concentric tracks. Each genetic change, represented by circles colored according to isolate, is marked at the appropriate genomic position. The detailed information on these genetic changes is summarized in Table S3.



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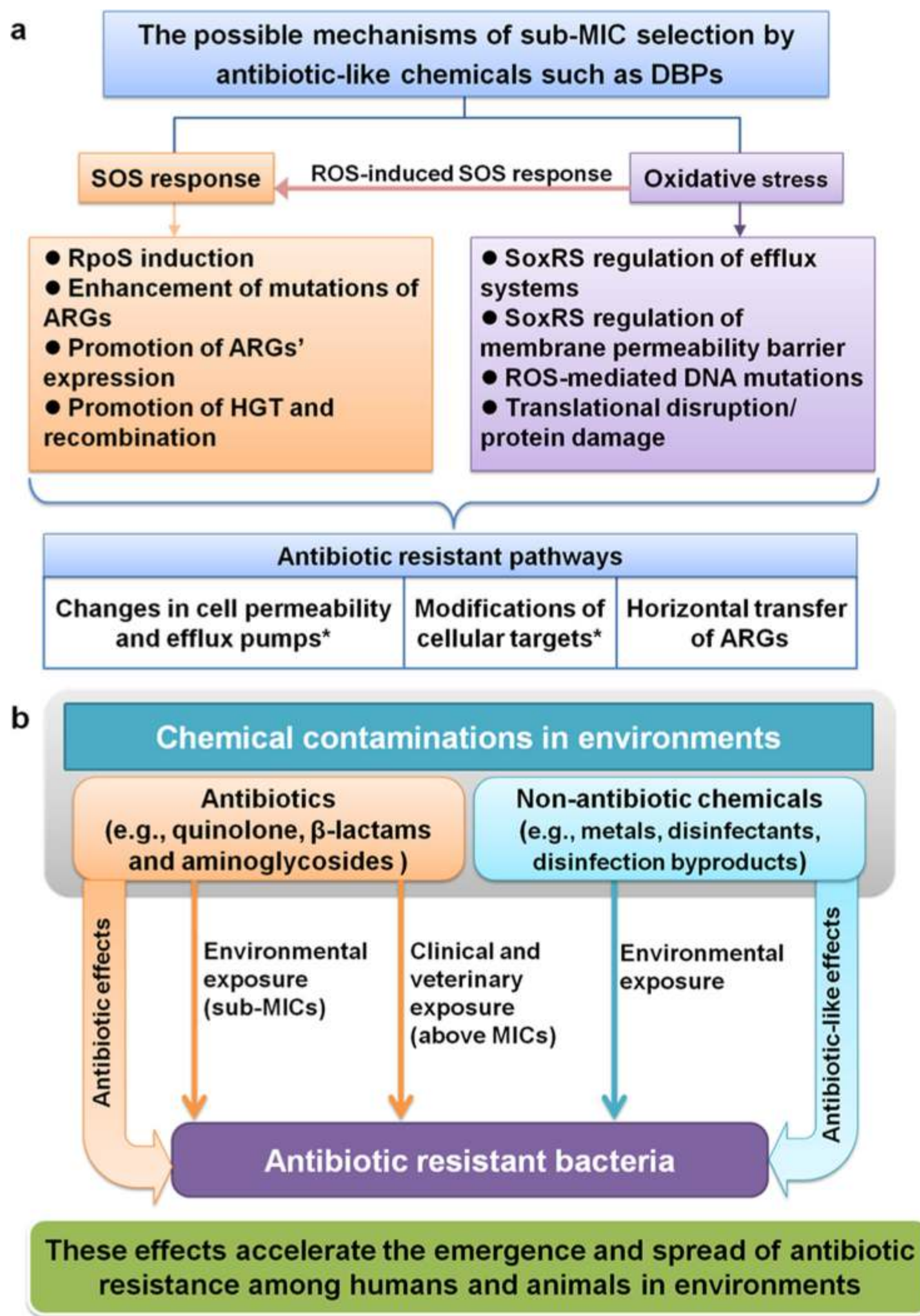


**Figure 5.**

Putative mechanisms of sub-MIC resistance selection and contribution of antibiotic-like environmental chemicals to emergence and spread of antibiotic resistance. (a) Possible mechanisms of antibiotic resistance selection under subinhibitory doses by antibiotic-like chemicals such as disinfection byproducts. Previous studies on antibiotics (e.g., quinolone, *β*-lactams, and aminoglycosides) resistance selection at subminimal inhibitory concentration (sub-MIC) levels indicated involvements of cellular SOS regulation and oxidative stress response pathways, which consequently impact the rates of mutagenesis, horizontal gene



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transfer (HGT), or recombination in genes involved in various antibiotic resistance pathways (5). The asterisks indicated the relevant antibiotic resistant pathways identified in the DBP-selected resistant strains reported by this study. (b) The hypothesis of contributions of antibiotic-like chemicals to emergence and spread of antibiotic resistance on global scales. In addition to antibiotics, greater numbers and larger variety of widely present antibiotic-like environmental contaminants, such as metals or DBPs, individually or as mixtures, can potentially select and enrich for de novo antibiotic resistance.