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Subinhibitory Concentrations of Disinfectants Promote the Horizontal Transfer of Multidrug Resistance Genes within and across Genera

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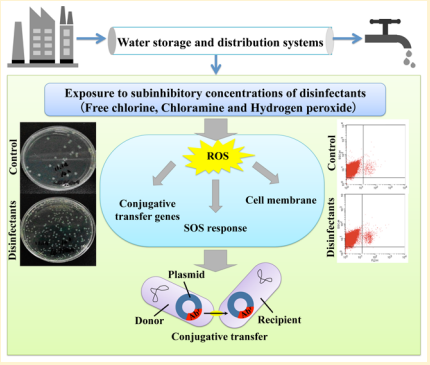
Supporting Information

ABSTRACT: The greater abundances of antibiotic resistance genes (ARGs) in point-of-use tap and reclaimed water than that in freshly treated water raise the question whether residual disinfectants in distribution systems facilitate the spread of ARGs. This study investigated three widely used disinfectants (free chlorine, chloramine, and hydrogen peroxide) on promoting ARGs transfer within *Escherichia coli* strains and across genera from *Escherichia coli* to *Salmonella typhimurium*. The results demonstrated that subinhibitory concentrations (lower than minimum inhibitory concentrations [MICs]) of these disinfectants, namely 0.1−1 mg/L Cl_2 for free chlorine, 0.1−1 mg/L Cl_2 for chloramine, and 0.24−3 mg/L H_2O_2, led to concentration-dependent increases in intragenera conjugative transfer by 3.4−6.4, 1.9−7.5, and 1.4−5.4 folds compared with controls, respectively. By comparison, the intergenera conjugative frequencies were slightly increased by approximately 1.4−2.3 folds compared with controls. However, exposure to disinfectants concentrations higher than MICs significantly suppressed conjugative transfer. This study provided evidence and insights into possible underlying mechanisms for enhanced conjugative transfer, which involved intracellular reactive oxygen species formation, SOS response, increased cell membrane permeability, and altered expressions of conjugation-relevant genes. The results suggest that certain oxidative chemicals, such as disinfectants, accelerate ARGs transfer and therefore justify motivations in evaluating disinfection alternatives for controlling antibiotic resistance. This study also triggers questions regarding the potential role of environmental chemicals in the global spread of antibiotic resistance.



INTRODUCTION

The development and spread of antibiotic resistance poses a serious public health threat on a global scale.[1,2] Bacteria acquire and disseminate antibiotic resistance via genetic mutations and horizontal transfer of antibiotic resistance genes (ARGs),[2,3] and the occurrences of antibiotic resistance have been traditionally considered to be caused by misuse or overuse of antibiotics in clinical and agricultural for humans,[2] livestock,[4] and aquatic products.[5] Horizontal transfer of ARGs is considered as one of the major drivers that accelerate the development and enrichment of antibiotic resistance in the environment,[3,6] and it is generally associated with foreign genetic elements containing ARGs, such as plasmids, transposons, and integrons.[3,7,8] Usually, horizontal transfer of ARGs occurs between closely related species of bacteria, but it also happens across bacterial genera, occurring at a relatively low frequency but posing significant clinical importance.[7,9]

Some antibiotics are constantly escaping into the environment as a result of the long-term and uncontrolled application of antibiotics, which produces an additional selection pressure for antibiotic resistance.[10] As expected, antibiotic resistant bacteria (ARB) and ARGs have been detected frequently in environmental water, including municipal wastewater,[11] reclaimed wastewater,[12] and even drinking water.[13] The effects of disinfection processes on the removal of ARB and ARGs in reclaimed water and drinking water have been investigated in the laboratory scale[14,15] and field studies.[16] However, these results cannot confirm that disinfectants are playing a key role in the control of the occurrence and dissemination of antibiotic resistance in real water systems.[17,18] It was observed that although the levels of bacteria were significantly reduced in the finished reclaimed water and drinking water after disinfection treatments, the abundances of both ARB and ARGs were much higher in the point-of-use of tap and in reclaimed water than those in the freshly treated water.[19] Thus, water distribution

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systems have the potential to facilitate the spread and enrichment of antibiotic resistance.

Conventionally, residual disinfectants, such as free chlorine, chloramine, and hydrogen peroxide (H_2O_2), are applied to control the regrowth of microorganisms in both drinking and reclaimed water distribution pipelines.[20−22] The residual disinfectants in the drinking distribution system after long-distance distribution, or in point-of-use of tap water, often decrease to subinhibitory levels or are even undetectable.[20−23] Therefore, ARB and ARGs in disinfected drinking and reclaimed water may be exposed to relatively low concentrations of disinfectants for a period ranging from a few hours to a few days.

Recent studies have explored the ability and the underlying mechanisms of subinhibitory levels (tens to hundreds of folds below the minimal inhibitory concentrations [MICs], also referred to as sub-MICs or sublethal concentrations) of antibiotics to induce resistant mutants and to stimulate the horizontal transfer of ARGs.[3,24,25] Evidences indicated that the horizontal transfer of ARGs between bacteria can be induced by subinhibitory levels of antibiotics via broadly conserved cellular response pathways such as those involved in reactive oxygen species (ROS) response systems[25] and the SOS response.[3,25] The involvements of these conserved cellular pathways and mechanisms raise important implications regarding the possible role of environmental chemicals other than antibiotics in the transmission of ARGs. A recent study of the effects of chlorine and ultraviolet irradiation (UV) disinfection processes on the horizontal transfer of ARGs indicated that the frequency of conjugative transfer was significantly increased by subinhibitory chlorine doses (up to 40 mg Cl_2 min/L), while inhibitory doses of chlorine (>80 mg Cl_2 min/L) or UV (>10 mJ/cm^2) greatly suppressed the frequencies of ARG transfers.[26] The role of widespread residual disinfectants in promoting ARG transfer, particularly at environmentally relevant low concentrations (subinhibitory levels), such as those in water storage and distribution systems, has seldom been evaluated and warrants a more systematic investigation.

In this laboratory-based study, we investigated the effects of subinhibitory levels of three widely used disinfectants, namely free chlorine, chloramine, and H_2O_2, on the conjugative transfer of ARGs within two different *Escherichia coli* (*E. coli*) strains and across genera from *E. coli* to *Salmonella typhimurium* (*S. typhimurium*). Free chlorine and chloramine are residual disinfectants that are regulated by the U.S. Environment Protection Agency (USEPA) and other agencies worldwide.[21,22] The maximum residual disinfectant level goals are 4.0 mg/L for both chlorine and chloramine in the USEPA National Primary Drinking Water Regulations (Table S1), and the drinking water sanitary standard in China requires that residual concentrations at the end of distribution systems must be greater than 0.05 mg/L chorine or 0.05 mg/L chloramine (Table S2). H_2O_2, the simplest peroxide, is one kind of ROS that is widely used as a strong oxidizer and disinfectant of skin, food, and drinking water.[27] We also systematically explored the mechanisms that underlie this process with regards to the intracellular ROS, the SOS response, the cell membrane permeability, and the expression of conjugation-relevant genes (e.g., *korA*, *korB*, *trbA*, *trbBp*, *traF*, *trfAp*, and *traJ*), outer membrane-encoding genes (e.g., *ompA*, *ompF*, and *ompC*), and oxidative stress regulatory genes (e.g., *rpoS*). To our knowledge, this is the first study to explore the effect and mechanisms of subinhibitory levels of disinfectants on the promotion of the horizontal transfer of ARGs within and across genera. The results of the present study will provide guidance for disinfection practices for water treatment and distribution systems and contribute to prevention of antibiotic resistance transfer.

MATERIALS AND METHODS

Establishment of Conjugative Transfer Models. In order to evaluate the conjugative transfer of ARGs in the environment within and across genera, two conjugative transfer models were established.

In the intragenera conjugative transfer model, the donor *E. coli* S17-1 strain, containing the pCM184-*Cm* plasmid carrying ampicillin (Amp), chloromycetin (Chl), and tetracycline (Tet) resistance genes, was cultured in Luria−Bertani (LB) medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L; pH, 7.4) containing 20 mg/L Chl. The recipient *E. coli* K12 strain, containing the pUA139 plasmid carrying a kanamycin (Km) resistance gene, was cultured in LB medium containing 100 mg/L Km. Intragenera transconjugants were selected on LB plates containing 10 g/L agar, 20 mg/L Chl, and 100 mg/L Km.

In the intergenera conjugative transfer model, the donor *E. coli* S17-1 strain, containing pBHR1 plasmid carrying the Km and Chl resistance genes, was cultured in LB medium supplemented with 20 mg/L Chl. The recipient *S. typhimurium* TA1535 strain, carrying the Amp resistance gene in its chromosome, was cultured in LB medium supplemented with 100 mg/L Amp. Intergenera transconjugants were selected on LB agar plates that were supplemented with 20 mg/L Chl and 100 mg/L Amp.

In order to optimize the conjugative transfer models, the cell density (10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 colony-forming units (CFU)/mL), donor/recipient ratios (0.5:1, 1:1, 1.5:1, 2:1, and 3:1), mating times (4, 8, 12, and 24 h), and temperatures (25 and 37 °C) were optimized by a single-factor experiment.

Specific descriptions of all the strains and plasmids used in the present study are provided in Table S3. All of the donor and recipient bacteria were incubated at 37 °C for 16−18 h, with shaking at 180 rpm. Then, these bacterial cultures were prepared and subjected to a conjugation experiment, as well as assays that evaluated cell membrane permeability, oxidative stress via intracellular ROS production, transcriptomic analysis, and the mRNA expression of integron genes.

Conjugation Experiments. In order to eliminate the influence of the culture medium, overnight cultures of the donor and recipient bacteria were centrifuged for 5 min at 10,000 × g and 4 °C. Following centrifugation, the supernatants were removed, and the bacteria were resuspended in different volumes of phosphate-buffered saline (PBS, pH = 7.2) to obtain different bacterial concentrations. Then, the donor and recipient bacteria were mixed and exposed to different sub-MIC (subinhibitory) concentrations of disinfectants, including free chlorine (0, 0.1, 0.3, 0.5, 1, and 5 mg/L as Cl_2), chloramine (0, 0.1, 0.3, 0.5, 1, and 5 mg/L as Cl_2), and H_2O_2 (0, 0.24, 1.2, 3, 6, and 30 mg/L). For comparison, conjugation experiments were also performed with inhibitory concentrations (>90% growth inhibition), namely 10 mg/L for free chlorine, 10 mg/L for chloramine, and 60 mg/L for H_2O_2. The MICs (90% growth inhibition)[28] of the donor and recipient bacteria against three disinfectants were predetermined based on the concentration-inhibition curves of donor (*E. coli* S17-1) and recipient (*E. coli* K12 and *S. typhimurium*) strains following

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Environ. Sci. Technol. 2017, 51, 570−580

treatment with free chlorine, chloramine, and H_2O_2, respectively (Figure S1). The Hach pocket colorimeter II kit (cat. no. 59530-00, Hach, Loveland, CO, USA) was used to determine the concentrations of free chlorine and chloramine. These tested concentrations of disinfectants were selected with consideration of the residual disinfectant requirements of drinking water regulations (Tables S1 and S2),[21,22] as well as the concentrations commonly occurring in drinking water and reclaimed water storage and distribution systems in previous reports.[20,29] Sodium hypochlorite served as free chlorine in the experiments. Chloramine was prepared by reacting ammonium chloride and free chlorine according to a previous method.[26]

In order to simulate water distribution processes, the mixtures were incubated in dark at 25 °C for 30 min exposure, and then sodium thiosulfate ($Na_2S_2O_3$, 3.5%) was added to each sample for neutralization. After the subsequent incubation at a certain temperature for several hours that are based on the results of the single factor experiments, the mixtures were appropriately diluted in PBS and plated on LB agar plates containing the appropriate antibiotics to determine and calculate the numbers of donors, recipients, and transconjugants as described previously. Samples that were not exposed to the disinfectants were processed as controls. All of the conjugation experiments were conducted at least in triplicate.

Evaluation of the Effects of Disinfectants on Cell Membrane Permeability Using Flow Cytometry. A flow cytometry (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) approach was employed to evaluate the membrane permeability of the donor and recipient bacteria, as well as control cells, upon exposure to the disinfectants based on DNA-intercalating fluorescent dye, propidium iodide (PI) staining that correlates fluorescence intensity with cell membrane permeability.[8,30,31] The detailed detection and data analysis processes were conducted according to previous studies (Text S1).[8,31]

Assessment of Oxidative Stress Induced by the Disinfectants. To explore whether oxidative stress plays an important role in promoting disinfectant-induced conjugative transfer, intracellular ROS formation was determined using the fluorescent reporter dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen, Carlsbad, USA) and a microplate reader (Synergy HTMulti-Mode, BioTek, Winooski, VT, USA) as described previously.[32] Briefly, bacterial suspensions (approximately 10^6−10^7 CFU/mL) were stained with DCFH-DA (at a final concentration of 10 μM) for 20 min at 37 °C in the dark with gentle shaking. After washing twice with PBS, the bacteria were treated with different disinfectants as described above. Then, all the treated samples were transferred into a 96-well plate (200 μL per well), and the fluorescence (488 nm/525 nm) intensity (FI) was measured using the microplate reader. The ROS production level for each disinfectant treatment was normalized to that of the control samples. All of the experiments were conducted at least in triplicate. The relative fold increases in ROS productions were calculated using eq 1.

$$\text{relative ROS production (folds)} = \frac{\text{FI of treated samples}}{\text{FI of control samples}} \quad (1)$$

To test if the ROS formation enhances the frequency of conjugative transfer within and across genera, the intragenera conjugative models were treated with 1 mg/L free chlorine, 0.1 mg/L chloramine, and 3 mg/L H_2O_2, as well as the intergenera conjugative models being supplemented with 0.5 mg/L free chlorine, 0.3 mg/L chloramine, and 1.2 mg/L H_2O_2. Meanwhile thiourea (CH_4N_2S, TU) (TCI, Tokyo, Japan), a scavenger of ROS, was added to each culture at 100 μM final concentrations. Then, the process of conjugative transfer was conducted, and the numbers of transconjugants were counted.

Evaluation of the mRNA Expression of Genes Responsible for Conjugative Transfer. Total RNA was isolated from the disinfectant-treated bacterial samples using RNAiso Plus (cat. no. D9108A, TaKaRa, Dalian, China). Then, the RNA was transcribed into cDNA using a reverse transcription kit (cat. no. 2680A, TaKaRa). The expression of horizontal transfer global regulator genes (*korA*, *korB*, and *trbA*), conjugation-related genes (*trbBp*, *trfAp*, *traF*, and *traJ*), outer membrane protein-encoding genes (*ompA*, *ompF*, and *ompC*), and an oxidative stress regulatory gene (*rpoS*) were quantified using a real-time polymerase chain reaction (PCR), and 16S rRNA was used as an internal control. Real-time PCR was carried out by using SYBR Green I (cat. no. DRR420A, TaKaRa, Dalian, China) in a real-time PCR instrument (CFX 96, Bio-Rad, Hercules, CA, USA).

The primers used in present study are shown in Table S4. The real-time PCR mixtures consisted of 5 μL of 2 × SYBR Premix *Ex Taq* (TaKaRa, Dalian, China), 0.2 μL of each primer (10 μM final concentrations), 1 μL of cDNA template, and 3.6 μL of distilled H_2O. The thermocycling profile for the amplifications was 95 °C for 30 s, followed by 40 cycles of 95 °C for 45 s, 60 °C for 45 s, a melting curve analysis at 95 °C for 15 s, and, finally, annealing at 60 °C for 1 min. Each experiment was conducted at least in duplicate.

Transcriptomic Analysis of the Effects of the Disinfectants on the SOS Response Pathways. The effects of the disinfectants on the SOS response pathways, which were previously shown to contribute to antibiotic resistance,[3,24,25] were evaluated using a green fluorescent protein (GFP)-transformed *E. coli* K12 MG1655 library (Open Biosystems, Huntsville, AL, USA) as described previously.[28,33] Each promoter was expressed from a low-copy plasmid, pUA66 or pUA139, which contains a Km resistance gene and a fast-folding form of GFP, thus enabling real-time measurement of the gene expression. For this particular study, 19 recombinant *E. coli* strains with different promoters, which control the expression of the SOS response pathways, were selected (Table S5). The detection processes and data analysis were performed according to previous studies (Text S2).[28,33]

Statistical Analysis. Each experiment was conducted independently at least in triplicate. The SPSS 16.0 (SPSS, Chicago, USA) was performed for all data analysis. Significant differences were statistically assessed using Analysis of variance (ANOVA) and Independent-sample *t* test. A value of $P < 0.05$ was considered to be significant, and a value of $P < 0.01$ was considered to be very significant.

RESULTS AND DISCUSSION

Optimization of Experimental Models for Conjugative Transfer within and across Genera. Conjugative transfer models for evaluating intra- and intergenera transfer were optimized, including the donor/recipient ratio, bacterial concentrations, mating times, and temperature. As shown in Figure 1a, the efficiency of spontaneous conjugative transfer was approximately 10^{-5} when the bacterial concentrations ranged from 10^6 to 10^8 CFU/mL. This result is consistent with

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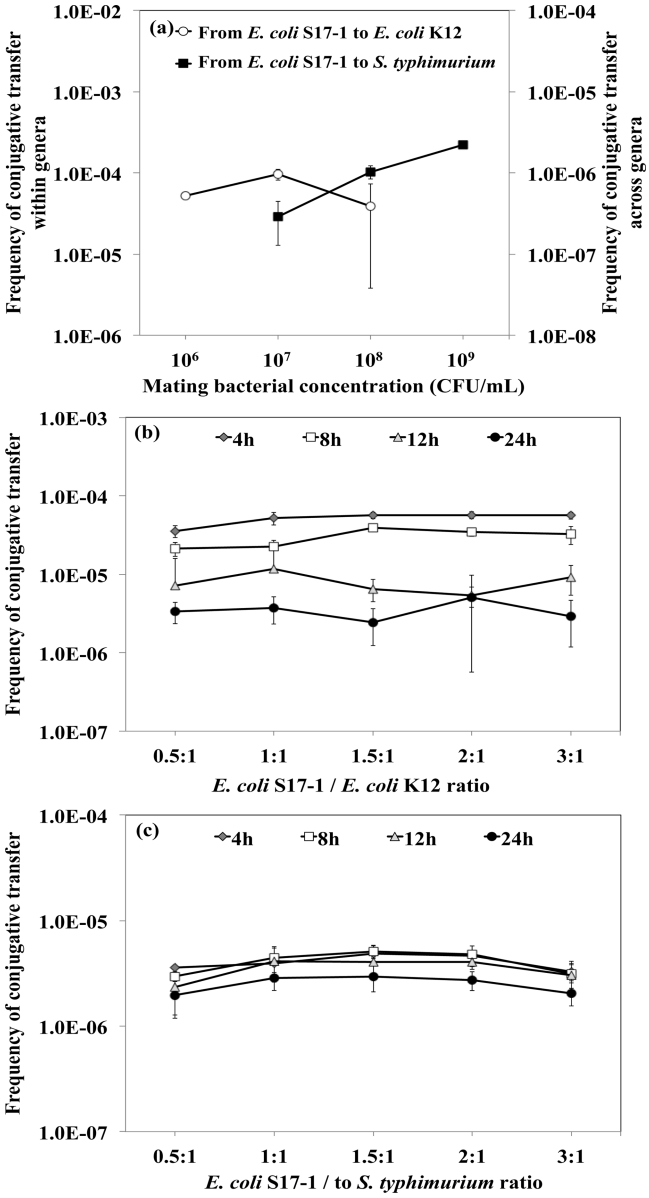

Figure 1. Effects of initial bacterial concentration, donor/recipient ratio, and mating time on the conjugative transfer within genera (from *E. coli* S17-1 to *E. coli* K12) and across genera (from *E. coli* S17-1 to *S. typhimurium*). (a): The effect of mating bacterial concentration on the conjugative transfer within and across genera at 4-h mating time. The mating bacterial concentration had no significant effect on intra- and intergenera transfer (ANOVA, $P > 0.05$). (b): The effect of donor/recipient ratio and mating time on the conjugative transfer from *E. coli* S17-1 to *E. coli* K12. The mating time had significant effects on intragenera transfer (ANOVA, $P < 0.05$), and the donor/recipient ratio had no significant effects (ANOVA, $P > 0.05$). (c): The effect of donor/recipient ratio and mating time on the conjugative transfer from *E. coli* S17-1 to *S. typhimurium*. The mating time and the donor/recipient ratio had significant effects on intergenera transfer (ANOVA, $P < 0.05$).

previous studies where the bacterial concentrations applied in conjugative transfer studies generally ranged from 10^6 to 10^9 CFU/mL.[9,26,34] Very few transconjugants were detected when the bacterial concentration was less than 10^5 CFU/mL. As conjugation requires cell-to-cell contact to form a pilus or pores that are needed for plasmid transfer, higher bacterial concentrations lead to higher frequencies of cell-to-cell contacts.[26] Previous studies observed bacterial concentrations ranging from 10^2 to 10^5 CFU/mL in reclaimed water[35] and less than 10^2 CFU/mL in drinking water.[19,22,36] However, the potential numbers of donor and recipient bacteria in biofilms in the storage and distribution systems of drinking and reclaimed water are rather high, which may imply higher frequency of conjugative transfer.[37,38] Because the goal of this study was to elucidate the potential and mechanism of horizontal transfer of ARGs, we selected the experimental conditions that yielded the highest transfer efficiency for further evaluations.

The frequency of intragenera conjugative transfer ranged from 1.96×10^{-6} to 5.12×10^{-6} was slightly influenced by the donor/recipient ratios (from 0.5:1 to 3:1), and the highest frequency of intergenera conjugative transfer occurred when the donor/recipient ratio was 1.5:1 in our study (Figure 1c). Mating time significantly influenced the frequency of intra- and intergenera conjugative transfer, especially for the model from *E. coli* S17-1 to *E. coli* K12, which decreased when the mating time was greater than 4 h (Figure 1b and 1c). Previous studies found that the conjugative transfer of the RP4 plasmid required 6−8 h of mating to obtain the optimal efficiencies,[9,26] which was longer than that of the pCM184-*Cm* and pBHR1 plasmids in the present study. This may be because the RP4 plasmid (60,099 bp) is much larger than pCM184-*Cm* (7625 bp) and pBHR1 (5300 bp).

The mating temperature (25 and 37 °C) did not have a significant effect on the conjugative transfer efficiency (data not shown). Therefore, the intra- and intergenera conjugative models were optimized when the bacterial concentration ranged from 10^8 to 10^9 CFU/mL, with a donor/recipient ratio of 1.5:1, and 4 h of mating in PBS at 25 °C. The optimized models were applied in all of the following experiments to investigate the effects of subinhibitory concentrations of the disinfectants on the conjugative transfer of antibiotic resistance plasmids within and across genera.

Subinhibitory Concentrations of Disinfectants Promote the Conjugative Transfer of ARGs within and across Genera. To test the hypothesis that subinhibitory levels of disinfectants increase horizontal gene transfer, we evaluated both intra- and intergenera conjugate transfer following in exposure to a range of sub-MIC concentrations of chlorine, chloramine, and H_2O_2. The results showed that certain subinhibitory concentrations (lower than MICs) of these disinfectants, namely 0.1−1 mg/L Cl_2 for free chlorine, 0.1−1 mg/L Cl_2 for chloramine, and 0.24−3 mg/L H_2O_2, led to concentration-dependent increases in intragenera conjugative transfer by 3.4−6.4, 1.9−7.5, and 1.4−5.4 folds compared with controls, respectively (Figure 2). These low levels of disinfectants were below the maximum levels of residual disinfectants in drinking water (Tables S1 and S2), which represent the levels that occur in drinking water and reclaimed water storage and distribution systems.[20,29]

By comparison, the intergenera conjugative frequencies from *E. coli* S17-1 to *S. typhimurium* were only slightly increased by approximately 1.4−2.3 folds following treatment with subinhibitory concentrations of these three disinfectants (Figure 2). For both the intra- and intergenera conjugative transfer experiments, the frequencies of conjugative transfer were suppressed when the concentrations of the disinfectants increased to inhibitory levels (Figure S1 and Figure 2). These results are mainly due to the inactivation of both the donor and recipient bacteria treated with high levels of disinfectants (Figure S1), which is in agreement with previous studies.[9,26]

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Environ. Sci. Technol. 2017, 51, 570−580

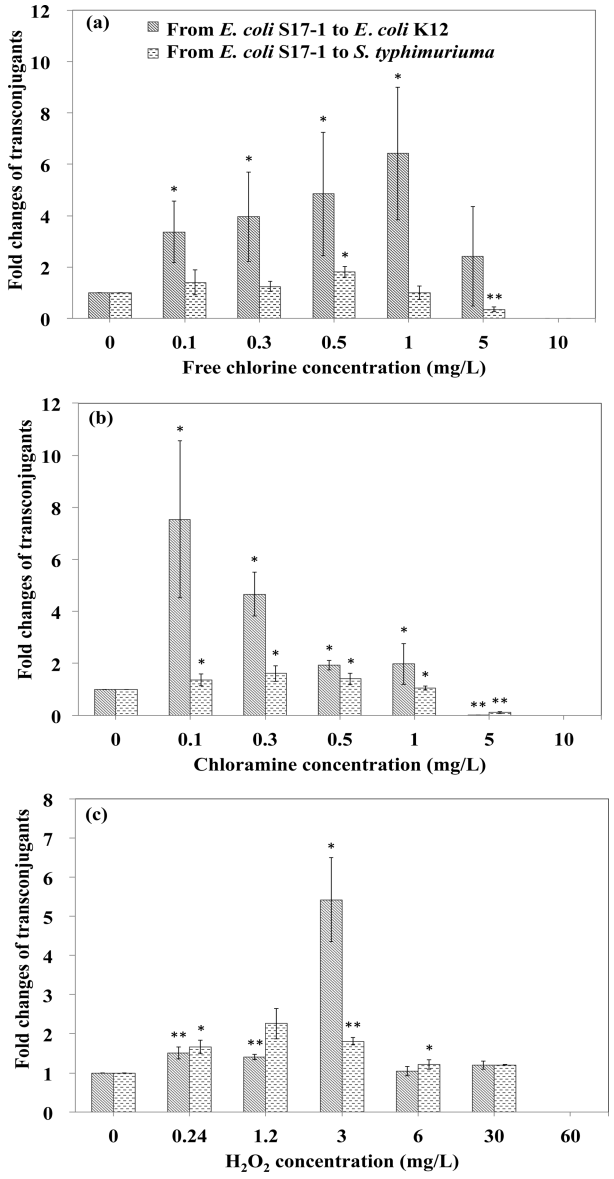

Figure 2. Effects of free chlorine (a), chloramine (b), and H_2O_2 (c) on the conjugative transfer within and across genera. No transconjugants were detected by exposure to 10 mg/L free chlorine, 10 mg/L chloramine, and 60 mg/L H_2O_2. All disinfectants had significant effects on the intra- and intergenera conjugative transfer of ARGs (ANOVA, $P < 0.05$); significant differences between individual disinfectant treated groups and the control (0 mg/L of disinfectants) were tested with Independent-sample *t* test and shown with * ($P < 0.05$), ** ($P < 0.01$).

These previous studies showed that decreased conjugative transfer was due to the reduced numbers of donor and recipient bacteria following exposure to inhibitory levels of nanoparticles of aluminum oxide[9] or an ionic liquid.[8]

Mechanisms by Which Subinhibitory Disinfectants Promote Intra- and Intergenera Conjugative Transfer. Disinfectants, including free chlorine, chloramine, and H_2O_2, can produce radicals and stimulate the formation of intracellular ROS, which are highly reactive molecules interference with the normal functions of bacteria during aerobic respiration.[27] ROS can directly damage cell membranes and DNA and then induce the SOS response, which is a global regulatory response protecting cells from DNA damage.[27,39] Previous studies revealed that subinhibitory concentrations of antibiotics induced horizontal transfer of ARGs through conserved ROS and SOS response pathways.[3,25] Reports also found that nanomaterials and ionic liquids stimulated conjugative transfer by increasing cell membrane permeability and affecting the expression of conjugation-related genes.[8,9] In this study, we hypothesized that the generation of intracellular ROS by subinhibitory concentrations of the disinfectants can promote intra- and intergenera conjugative transfer by inducing the SOS response, increasing cell membrane permeability, and altering the expression of conjugation-related genes.

1. Effects of Subinhibitory Concentrations of Disinfectants on ROS Formation and SOS Response. To determine the effects of the disinfectants on the levels of ROS formation in donor and recipient bacteria, intracellular ROS were measured using DCFH-DA.[32] The radical levels in both the donor and recipient bacteria significantly increased with the increasing concentrations of disinfectants compared with the control samples (Figure 3 a, b, and c). H_2O_2 exhibited the highest oxidative capacity, while free chlorine caused greater oxidative stress than chloramine.

To determine whether the observed increase in ROS formation in both the donor and recipient bacteria was related to intra- and intergenera conjugative transfer, the correlation between conjugative transfer and ROS production was determined (Figure S2). Interestingly, it is notable that high levels (>10 folds increases) of ROS formation suppressed conjugative transfer, while medium levels (approximately 1.5−5 folds increases) of ROS formation promoted conjugative transfer (Figure 3 and Figure S2). This may be due to that the high levels of ROS damaged bacterial components, while moderate ROS levels probably affected membrane permeability that plays a vital role in the transfer of plasmids encoding multiple antibiotic resistances and increased the expression of genes that are involved in conjugative transfer.[9,25,27]

The addition of thiourea, which is widely considered as a scavenger of ROS, significantly reduced the conjugative transfer frequency (Figure S3). This result further indicated that the level of ROS production induced by disinfectants is one of important parameters for ARGs transfer.

Previous studies indicated that antibiotics could stimulate horizontal transfer of ARGs, which is mediated by the induction of SOS response.[3,25] For example, antibiotic-stimulated SOS induction can promote the transmission of ARGs, as exemplified by the spread of integrative conjugative elements throughout populations of *Vibrio cholerae*.[25] The three disinfectants studied here have been shown to cause oxidative stress and DNA damage.[27,40] Our transcriptional analysis using the promoter insertion library also demonstrated that subinhibitory levels of these three disinfectants induced significant expression changes in SOS response-related DNA damage and repair genes (i.e., *recA*, *polB*, *uvrD*, *umuD*, *ssb*, and *ada*) (Table S5).[33]

We also quantified *rpoS* gene expression using a quantitative real-time PCR, and the results indicated that *rpoS* gene expression was significantly induced by these three disinfectants compared with the control samples (Figure 3 d, e, and f). RpoS is a stress-response sigma factor, and it is known to play a role in promoting survival in response to exposure to multidrug resistance and various environmental stress conditions.[3,41] A previous study found that RpoS positively regulates the small RNA (sRNA) SdsR, and the up-regulation of *rpoS* expression led to elevated levels of sRNA that bound to and repressed the

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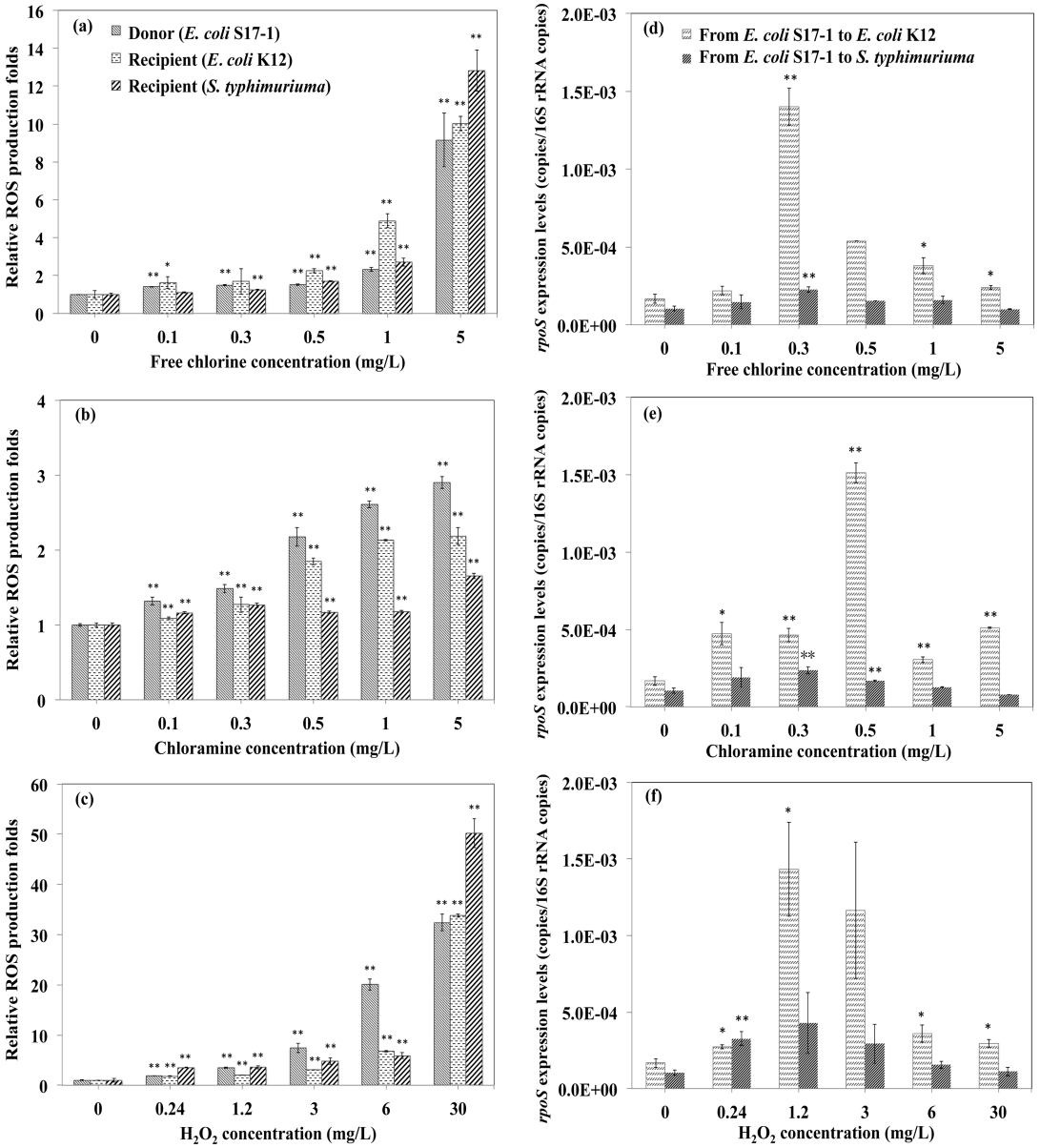

Figure 3. Reactive oxygen species (ROS) production (folds) in donor and recipient bacteria induced by free chlorine (a), chloramine (b), and H_2O_2 (c). All disinfectants had significant effects on the production of ROS in the donor and recipient bacteria (ANOVA, $P < 0.05$); significant differences in ROS production between individual disinfectant treated groups and the control (0 mg/L of disinfectants) were tested with Independent-sample *t* test and shown with * ($P < 0.05$) and ** ($P < 0.01$). The mRNA expression levels of oxidative stress-regulatory gene *rpoS* in the mating pairs within genera (from *E. coli* S17-1 to *E. coli* K12) and across genera (from *E. coli* S17-1 to *S. typhimurium*) stimulated by free chlorine (d), chloramine (e), and H_2O_2 (f). All disinfectants had significant effects on the *rpoS* gene expression in intra- and intergenera transfer (ANOVA, $P < 0.05$); significant differences in the *rpoS* gene expression levels between individual disinfectant treated groups and the control (0 mg/L of disinfectants) were tested with Independent-sample *t* test and shown with * ($P < 0.05$) and ** ($P < 0.01$).

translation of *mutS* mRNA.[41] As a result, cells became depleted for the MutS protein, which has a central role in the repair of replication errors. ROS formation and oxidative stress can induce the RpoS regulon (Figure 3) and subsequently improve the rates of intrachromosomal recombination,[3] which may cause more severe cell damage, such as the increased membrane permeability and the altered expression of some genes. Therefore, the present results suggest that subinhibitory concentrations of these three disinfectants likely stimulated the conjugative transfer of ARGs via ROS formation-mediated SOS induction.

2. Effects of Subinhibitory Concentrations of Disinfectants on Cell Membrane Permeability. The changes in membrane permeability of both the donor and recipient bacteria treated with the disinfectants, as well as untreated bacteria, were evaluated with PI staining followed by flow cytometry. The results showed a concentration-dependent increase in the percentage of PI-positive cells (indicating increased membrane permeability) with increasing subinhibitory concentrations of the disinfectants (Table S6 and Figure S4). However, when the levels of disinfectants were greater than inhibitory concentration, for example, 10 mg/L for free chlorine, 10 mg/L for chloramine, and 60 mg/L for H_2O_2, conjugative transfer was repressed, likely due to the fatal membrane damage and cytotoxicity (Table S6). No significant differences in cell permeability were observed between donor and recipient cells

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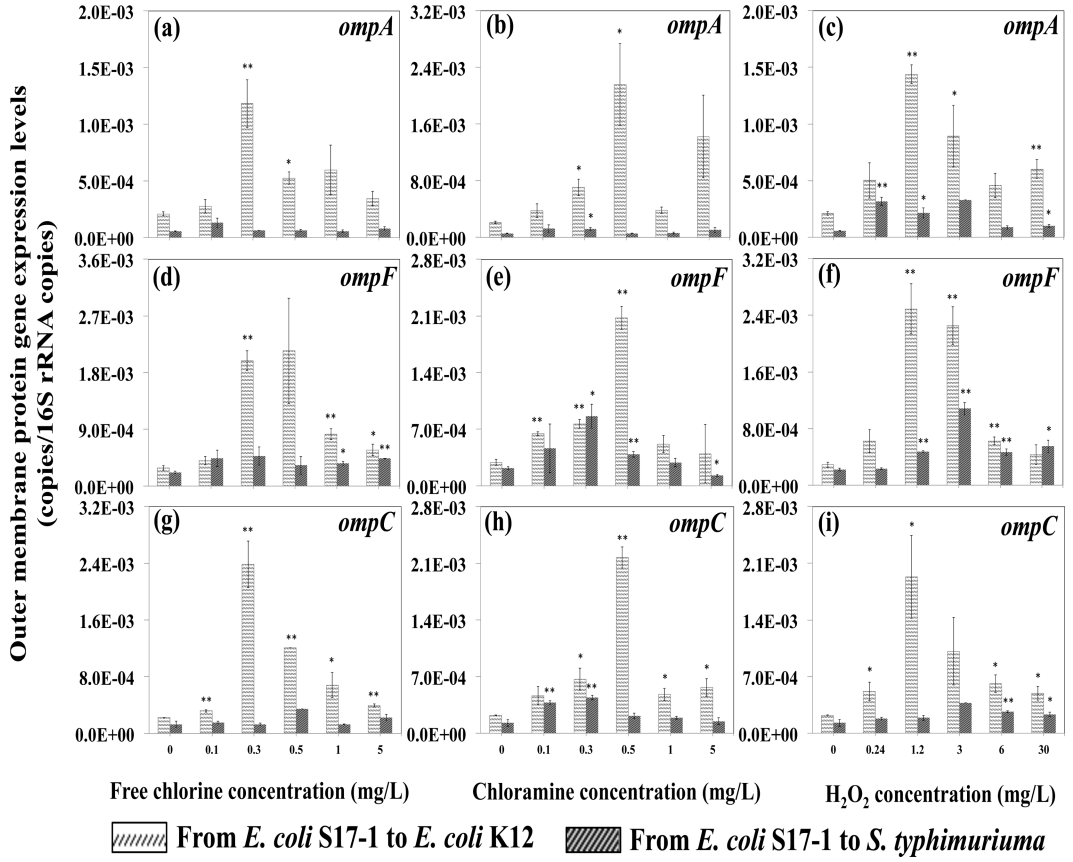

Figure 4. Effects of free chlorine (a, d, g), chloramine (b, e, h), and H_2O_2 (c, f, i) on the mRNA expression levels of outer membrane protein genes (*ompA, ompF*, and *ompC*) in the mating pairs within genera (from *E. coli* S17-1 to *E. coli* K12) and across genera (from *E. coli* S17-1 to *S. typhimurium*). Significant differences in the gene expression levels between individual disinfectant treated groups and the control (0 mg/L of disinfectants) were tested with Independent-sample *t* test and shown with * ($P < 0.05$) and ** ($P < 0.01$).

when the cells were exposed to the same disinfectants for both intra- and intergenera conjugative transfers (Table S6 and Figure S4).

The bacterial outer membrane is an important barrier against horizontal gene transfer from donor to recipient bacteria. During conjugation, plasmid DNA crosses the cell membrane of the donor, and then it is transferred to the recipient.[42] Several previous studies demonstrated that many chemicals, such as antibiotics,[43] chloramine,[26] nanomaterials,[9] and an ionic liquid,[8] affect bacterial membrane permeability by changing the membrane composition and structure. In addition, a number of researchers have proposed that some environmental pollutants damage cell membranes by means of inducing oxidative stress and ROS, as well as enhancing the expression of regulatory genes via the error-prone SOS response.[3,25]

3. Effects of Subinhibitory Concentrations of Disinfectants on the Expression of Outer Membrane Protein-Encoding Genes. Bacteria adapt their membrane permeability by modulating the expression of outer membrane proteins (OMPs). Several classical OMPs, including OmpA (34 kDa), OmpF (35 kDa), and OmpC (36 kDa), are responsible for the membrane permeability of donor and recipient bacteria, and they play important roles in pore formation and horizontal gene transfer.[44] As shown in Figure 4, the mRNA expression of the *ompA*, *ompF,* and *ompC* genes significantly increased by approximately 1.8−7.8 folds for intragenera and 1.1−3.9 folds for intergenera conjugative transfer following exposure to the three disinfectants, respectively. A previous study confirmed that the *ompA* gene is primarily regulated at the post-transcriptional level,[45] and the post-transcriptional levels of *ompA* expression in both *E. coli* and *S. typhimurium* were enhanced in the present study (Figure 4 and Figure S5). Similarly, Wang et al.[46] revealed that *ompA* mRNA expression was up-regulated in *E. coli* HB101 and *S. typhimurium* following treatment with an ionic liquid (1-ethyl-3-methylimidazolium chloride).

Gram-negative bacteria are capable of adapting to environmental stress, such as osmolality, pH, temperature, and starvation, by changing the composition of OmpA, OmpC, and OmpF in the outer membrane.[47] It has been demonstrated that OmpA and OmpC are associated with the membrane transport of genetic information between the inside of the cell and the external environment.[48] The increased formation of ROS in the donors and recipients, which was induced by the disinfectants, was suspected to influence the OMPs in the cell membrane. The enhanced expression of membrane proteins plays a vital role in forming outer membrane pores and augmenting membrane permeability, which may pose a potential risk for antibiotic resistance dissemination via the horizontal transfer of ARGs. Interestingly, the *omp* genes and *rpoS* seem to be induced following similar patterns, which may due to the ROS production in the whole conjugative process.

4. Effects of Subinhibitory Concentrations of the Disinfectants on the Expression of Conjugative Transfer-Related Genes. *E. coli* S17-1, carrying the transfer gene of the broad-host-range RP4 plasmid integrated into its chromo-

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Environ. Sci. Technol. 2017, 51, 570−580

some,[49] was used as the donor strain in present study. Conjugative transfer requires the formation of conjugation bridges between the donor and recipient bacteria, which requires the regulations of the global regulator genes, mating pair formation (Mpf) system genes, and plasmid transfer and replication (Dtr) system genes.[49,50] In the present study, we first determined whether subinhibitory levels of disinfectants enhanced conjugative transfer by regulating the expression of conjugation-related genes.

The results showed that three disinfectants at subinhibitory concentrations statistically increased conjugative transfer within and across genera and significantly repressed the mRNA expression of global regulatory genes (*korA*, *korB*, and *trbA*) that are involved in conjugative transfer (Figure 5 and Figure S6). Particularly, compared with the untreated control samples, the mRNA expression levels of the global regulatory gene *korA* decreased by approximately 93.8%, 43.3%, and 93.8% following treatment with 1 mg/L free chlorine, 0.1 mg/L chloramine, and 3 mg/L H_2O_2, respectively, during intragenera conjugative transfer (Figure 5 and Figure S6). The expression levels of the global regulator genes *korB* and *trbA* also decreased significantly during both intra- and intergenera conjugative transfer (Figure 5 and Figure S6). The decreased expression of global regulator genes plays an important role in activating the Mpf system, which serves as the secretion machinery for the conjugative transfer of plasmids.[51] The repression of the *korA* and *korB* genes significantly induces the expression of the *trfAp* promoter, and the repression of the *korB* and *trbA* genes significantly induces the expression of the *trbBp* promoter, which promotes the conjugative transfer of plasmid DNA.[52]

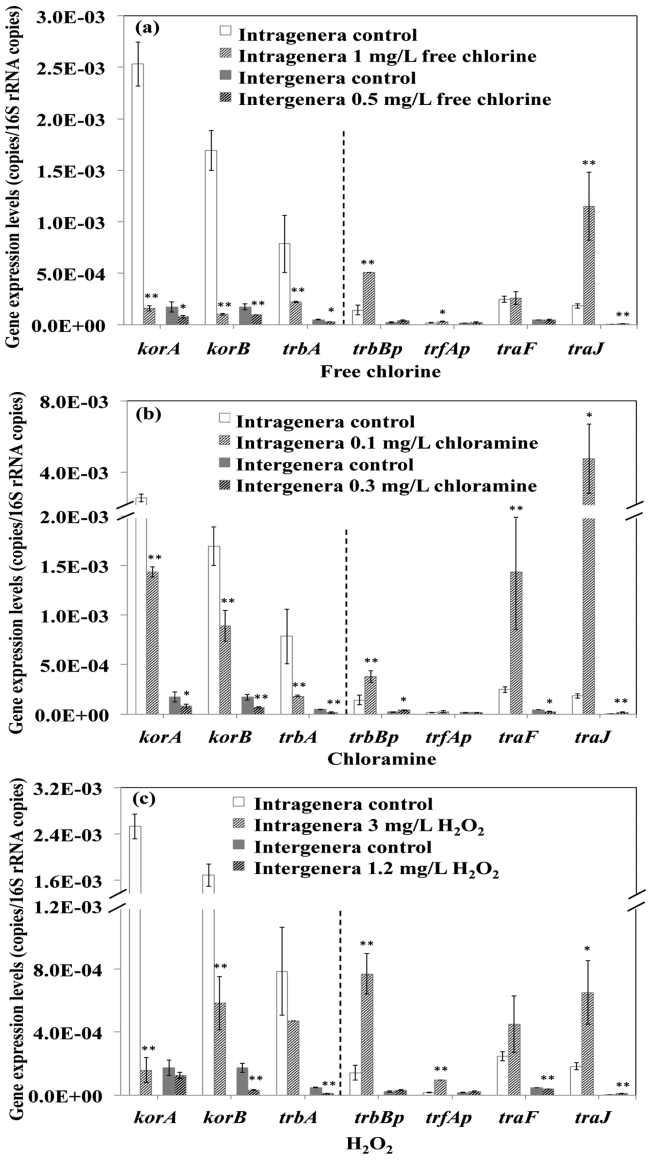

Figure 5. Effects of free chlorine (a), chloramine (b), and H_2O_2 (c) on the mRNA expression levels of conjugation-relevant genes (*korA, korB, trbA, trbBp, traF, trfAp*, and *traJ*) in the mating pairs within genera (from *E. coli* S17-1 to *E. coli* K12) and across genera (from *E. coli* S17-1 to *S. typhimurium*). Significant differences in the gene expression levels between individual disinfectant treated groups and the control (0 mg/L of disinfectants) were tested with Independent-sample *t* test and shown with * ($P < 0.05$) and ** ($P < 0.01$).

Additionally, increasing the mRNA expression of Mpf genes (*trbBp* and *traF*) facilitates the formation of conjugants.[53] The expression of *trbBp* gene following exposure to these three disinfectants increased significantly during intragenera conjugative transfer; however, it only increased slightly during intergenera conjugative transfer (Figure 5 and Figure S7). We also observed that the expression of *traF* during intragenera mating increased following exposure to subinhibitory concentrations of the disinfectants (Figure 5). This was more pronounced for the chloramine treatment, which increased *traF* expression by up to 5.8 folds compared with the control groups. Additionally, a previous study showed that for intraspecific *E. coli* mating, the *traF* gene is essential for forming the mating bridge that serves as a membrane-associated channel for the transmission of single-stranded DNA.[54]

Previous studies proved that the expression of Dtr system genes is positively correlated to conjugative transfer.[50,46] Our study confirmed that subinhibitory levels of the disinfectants promoted horizontal transfer by enhancing of the expression *trfAp* and *traJ* genes (Figure 5 and Figure S7). The *traJ* gene is responsible for the formation of the relaxosome, which triggers a specific nick in circular plasmids.[55] Subinhibitory of these three disinfectants significantly increased *traJ* gene expression by 3.6−25.9 folds during intragenera conjugative transfer; however, they induced less increases (2.4−5.6 folds) on *traJ* expression during intergenera conjugative transfer (Figure 5). The significantly higher expression level of the Mpf (*trbBp* and *traF*) and Dtr (*trfAp* and *traJ*) genes during intraspecies conjugative transfer explains why the intragenera conjugative transfer was much higher than the intergenera conjugative transfer.

IMPLICATIONS

Disinfectants, such as free chlorine, chloramine, and hydrogen peroxide, which are widely used to control the abundance and regrowth of microorganisms in water distribution systems and in swimming pools, pose widespread and persistent exposure for both microorganisms and humans.[20,56] To our knowledge, the present study is the first to demonstrate that subinhibitory concentrations of these commonly used disinfectants promote the spread of ARGs mediated by the conjugative transfer of plasmids between *E. coli* strains, as well as between *E. coli* and *S. typhimurium* strains, two opportunistic pathogens that widely exist in the environment. The results imply that subinhibitory concentrations of these disinfectants in water systems increase the risk of antibiotic resistance, and they also suggest that the

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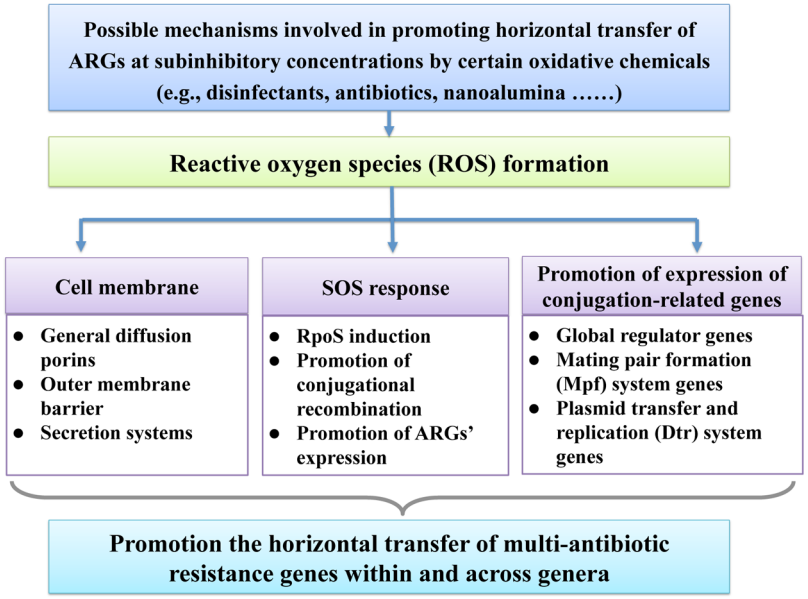

Figure 6. Schematic of possible mechanisms involved in promoting horizontal transfer of antibiotic resistance genes by certain oxidative chemicals that lead to ROS formation.

application of these disinfectants should be carefully evaluated. Further research on improving the disinfection methods to control ARB and ARGs is urgently needed.

Importantly, this investigation reveals potential mechanisms involved in promoting conjugative transfer following exposure to subinhibitory concentrations of oxidative chemicals such as these disinfectants. These mechanisms include intracellular ROS formation, SOS response, increase in cell membrane permeability, and regulation of conjugation-relevant genes that comprise global regulator genes, Mpf system genes, and Dtr system genes (Figure 6). The results of this study, consistent with previous studies, suggest that environmental chemicals can stimulate horizontal transfer by producing ROS and affecting the cellular SOS response pathways, which consequently impact horizontal transfer and recombination of ARGs (Figure 6).[3,24,25] The results imply that chemicals, such as disinfectants and other chemicals, via mechanisms involving oxidative stress, SOS response, and membrane permeability changes under subcytotoxic conditions, can potentially stimulate the spread of ARGs, thereby causing serious threats to public health.

This study, as well as previous studies[8,9] that reported increased horizontal transfer of ARGs by nonantibiotic chemicals, raises an intriguing and profound question regarding the roles of varying concentrations and mixtures of environmental chemicals in the dissemination of antibiotic resistance in the environment (Figure 6). Subinhibitory levels of disinfectants exist not only in drinking and reclaimed water systems[20−22,29] and swimming pools[56] but also in food production processes and healthcare facilities.[27] Our results suggest that relatively low levels of disinfectants and oxidants in environments and foods can enhance the spread of ARGs, thereby contributing to the emergence and transmission of antibiotic-resistant, disease-causing pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03132.

Text S1, evaluation of the cell membrane permeability using flow cytometry; Text S2, transcriptomic analysis of impact of disinfectants on the SOS response pathways; tables of the residual disinfectants in drinking water regulated by U.S EPA (Table S1) and China (Table S2), descriptions of the strains, plasmids, and primer sequences used in this study (Tables S3 and S4), and expression changes in genes involved in the SOS (Table S5), and cell membrane permeability by disinfectants (Table S6); and figures of inactivation curves of donor and recipient bacteria by three disinfectants (Figure S1), correlation between conjugative transfer and intracellular ROS formation (Figure S2), effects of scavenging ROS on conjugative transfer of multidrug resistance genes (Figure S3), cell membrane permeability of bacteria treated with the disinfectants (Figure S4), and mRNA expression levels of the OMPs genes (Figure S5) and of genes involved in conjugative transfer exposure to the disinfectants (Figures S6 and S7) (PDF)

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Notes

The authors declare no competing financial interest.

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