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Induction of bacterial antibiotic resistance by mutagenic halogenated nitrogenous disinfection byproducts

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ABSTRACT

Halogenated nitrogenous disinfection byproducts (N-DBPs) raise concerns regarding their mutagenicity and carcinogenicity threatening public health. However, environmental consequence of their mutagenicity has received less attention. In this study, the effect of halogenated N-DBPs on bacterial antibiotic resistance (BAR) was investigated. After exposure to bromoacetamide (BAcAm), trichloroacetonitrile (TCAN) or tribromonitromethane (TBNM), the resistance of *Pseudomonas aeruginosa* PAO1 to both individual and multiple antibiotics (ciprofloxacin, gentamicin, polymyxin B, rifampin, tetracycline, ciprofloxacin + gentamicin and ciprofloxacin + tetracycline) was increased, which was predominantly ascribed to the overexpression of efflux pumps. The mechanism of this effect was demonstrated to be mutagenesis through sequencing and analyzing antibiotic resistance genes. The same induction phenomena also appeared in *Escherichia coli*, suggesting this effect may be universal to waterborne pathogens. Therefore, more attention should be given to halogenated N-DBPs, as they could increase not only genotoxicological risks but also epidemiological risks of drinking water.

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1. Introduction

Emerging halogenated nitrogenous disinfection byproducts (N-DBPs), such as haloacetamides (HAcAms), haloacetonitriles (HANs) and halonitromethanes (HNMs), have drawn much attention in recent years (Shah and Mitch, 2011). N-DBPs are a group of unintended byproducts formed by chemical reactions between disinfectants and components of dissolved organic matter (DOM), especially dissolved organic nitrogen (DON), during chlorination or chloramination (Pehlivanoglu-Mantas and Sedlak, 2006). N-DBPs are usually present at low μg/L or even ng/L levels in drinking water, which are much lower than concentrations of currently regulated disinfection byproducts (DBPs) (Richardson et al., 2007). Nevertheless, N-DBPs still receive increasing concern due to the following reasons. First, drinking water sources are vulnerable to contamination from algae and municipal wastewater effluents, which are the key sources of N-DBPs precursors. This impairment is likely more prevalent with population growth and further exploitation of source waters (Fang et al., 2010). Second, alternative disinfectants, particularly chloramines, are increasingly replacing chlorine to reduce the formation of regulated THMs and HAAs; meanwhile their application may promote formation of N-DBPs (Choi and Richardson, 2004; Huang et al., 2012; Krasner et al., 2012; Plewa et al., 2008a, 2008b; Richardson et al., 2007; Shah et al., 2011; Yang et al., 2010). Finally, many N-DBPs exhibit dramatically elevated cytotoxicity and genotoxicity relative to most regulated DBPs species (Muellner et al., 2007; Plewa et al., 2008a, 2004a).

Many studies on toxicity of halogenated N-DBPs have been reported, covering a wide range of test systems. *In vitro* toxicity assays have demonstrated HAcAms to exhibit chronic cytotoxicity and genotoxicity in Chinese hamster ovary (CHO) cells (Plewa et al., 2008a) and normal rat kidney cells (Yang et al., 2014); *in vivo* tests also confirmed toxic effects of HAcAms on mice (Zhang et al., 2013). Mutagenicity and carcinogenicity of HANs have been extensively investigated in prokaryotic cells (e.g. *Salmonella typhimurium* (Mortelmans et al., 1986) and *Escherichia coli* (Le Curieux et al., 1995)), in eukaryotic cells *in vitro* (e.g. CHO cells (Muellner et al., 2007) and HepG2 cells (Zhang et al., 2012)), and *in vivo* (e.g. male Sprague–Dawley rats (Ahmed et al., 1991) and male F344 rats (Lin et al., 1992)). The mutagenicity of HNMs was initially evaluated in *S. typhimurium* (Giller et al., 1995; Kundu et al., 2004), and later in CHO cells, NCM460 cells and TK6 cells *in vitro* (DeAngelo et al.,

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2007; Liviac et al., 2009; Plewa et al., 2004a). However, most previous studies on halogenated N-DBPs mainly focused on their potential health effects on humans, very little is known about the undesirable consequence of their mutagenicity on bacteria, which has important environmental significance.

Specifically, we hypothesized that the mutagenicity of halogenated N-DBPs might increase bacterial antibiotic resistance (BAR). It is well known that chromosomal mutation is the fundamental pathway for bacteria to acquire antibiotic resistance (Dwyer et al., 2009). Halogenated N-DBPs could lead mutation frequency of bacteria to increase due to mutagenesis stated above. Theoretically, the acquisition of antibiotic resistance by bacteria would be promoted by exposure to mutagenic N-DBPs. This is especially the case for drinking water distribution systems, where DBPs and bacteria in suspension or biofilm can be in close contact for a period of time, ranging from several days to years.

Recently, bacterial antibiotic resistance in drinking water systems has become an active research area because it might threaten both environmental safety and human health. Drinking water has been recognized as a reservoir for antibiotic resistant bacteria and antibiotic resistance genes (ARGs) (Xi et al., 2009). Actually, previous study has reported that levels of antibiotic resistant bacteria (ARB) and ARGs in tap water were greater than that in finished water (Xi et al., 2009). And *tet (M)* and *tet (O)* genes were detected in the prechlorination and postchlorination treated water in a drinking water treatment plant, while none was detected in source water (Pruden et al., 2006). However, both of these studies did not supply an explicit and sufficient elaboration. While, other studies have focused on the impacts of horizontal gene transfer on BAR (Karatzas et al., 2007; Zhang et al., 2009). Little is known about whether mutagenesis would lead to BAR in drinking water due to DBPs, especially halogenated N-DBPs.

The current study aimed to explore the impacts of halogenated N-DBPs on the development of bacterial antibiotic resistance and the corresponding mechanism. DBPs are related to chemical safety of drinking water, while BAR is related to microbiological safety. It is expected that this study could establish a linkage between these two issues. In addition, this research is hoped to enrich understanding of the environmental consequence of mutagenic halogenated N-DBPs, and provide a novel interpretation on the generation of antibiotic resistance in drinking water systems.

2. Material and methods

2.1. Strains and media

The opportunistic pathogen *Pseudomonas aeruginosa* PAO1 and *E. coli* K12 used for antibiotic resistance test, were generous gifts from Dr. Feng Guo (Xiamen University, China) and Professor Chang-ping Yu (Institute of Urban Environment, Chinese Academy of Sciences, China), respectively. The engineered bacterium *S. typhimurium* TA1535/pSK1002 used for umu test, was donated by Professor Wenjun Liu (Tsinghua University, China). LB broth and LB agar for cultivation of PAO1 and *E. coli* K12 were purchased from Qingdao Hope Bio-Technology (China). TGA medium for the umu test was prepared according to ISO 13829 (ISO13829, 2000).

2.2. DBPs and antibiotics

Three emerging halogenated N-DBPs were selected as tested DBPs, i.e., bromoacetamide (BAcAm), trichloroacetonitrile (TCAN) and tribromonitromethane (TBNM). Both BAcAm (C_2H_4BrNO, CAS 683-57-8, 98%) and TCAN (C_2Cl_3N, CAS 545-06-2, neat) were purchased from Sigma–Aldrich (USA), and TBNM (CBr_3NO_2, CAS 464-10-8, 98.9%) was obtained from CanSyn Chem. Corp. (Canada). Antibiotics ampicillin (Amp), gentamicin (Gen), polymyxin B (Pol) and tetracycline (Tet) were obtained from Solarbio (China), whereas ciprofloxacin (Cip) and rifampin (Rif) were purchased from LKT Laboratories (USA) and Sigma–Aldrich (USA), respectively.

2.3. Mutagenicity test

The umu test without S9 activation was applied to examine mutagenicity of the three N-DBPs according to ISO 13829 (ISO13829, 2000). The umu test is based on the expression of DNA SOS repair mechanism and assessment of β-galactosidase activity in *S. typhimurium*. The mutagenicity is considered to be positive if the induction ratio (IR) is higher than 2.0, which is defined as the ratio of β-galactosidase activity of sample-treated bacteria to that of control. However, IR cannot be evaluated if the growth factor (G) is lower than 0.5, which is the growth rate of sample-treated bacteria to that of control. DMSO and methanol were used as solvent controls, as they dissolved TBNM and TCAN, respectively. The positive control used in this study was 4-nitroquinoline-N-oxide (4-NQO). Since the solution volume suggested in ISO 13829 might result in overflow from microplate wells, 0.8 fold volume was implemented in our protocol. N-DBPs and controls results were calculated by averaging triplicates.

2.4. Exposure protocol and BAR determination

PAO1 was incubated for 15 h, followed by a 1:500 dilution into 5 mL LB broth containing N-DBPs or vehicle as control. The tested N-DBPs concentrations were 2, 4, 8, 12, 16 ppm of BAcAm, 1, 2, 4, 6, 8 ppm of TBNM and 20, 40, 80, 120, 160 ppm of TCAN. The suspensions were then shaked for 24 h in an incubator at 37 °C. The exposed culture obtained was diluted 10 times with a 0.9% NaCl solution before plated on LB agar with Cip (0.234 μg/mL), Gen (13.2 μg/mL), Pol (3.16 μg/mL), Rif (29.6 μg/mL) or Tet (33.0 μg/mL) for individual antibiotic resistance determination. The exposed culture's multiple resistance to Cip + Gen and Cip + Tet was tested similarly, using antibiotic combinations of Cip (0.156 μg/mL) plus Gen (7.89 μg/mL) and Cip (0.234 μg/mL) plus Tet (33.0 μg/mL). To measure the total number of culturable bacteria, the exposed culture was sequentially diluted with a 0.9% NaCl solution and spread onto LB agar. All of the coated plates had three parallel repetitions. After incubation for 48 h at 37 °C, the colonies on each plate were counted. The effect of BAcAm on the antibiotic resistance of *E. coli* K12 was determined as same as described above, while the selective plates contained Amp (14.1 μg/mL), Gen (9.60 μg/mL) or Tet (6.40 μg/mL).

The colonies in the antibiotic-containing plates were supposed to gain the corresponding antibiotic resistance. The “mutation frequency” was calculated as the amount of antibiotic resistance bacteria divided by the total number of culturable bacteria, the latter was enumerated from the colony count on the LB agar and dilution ratio. To clearly represent the effect of halogenated N-DBPs compared with control, “fold change of mutation frequency” was introduced, which was calculated as the exposure mutation frequency divided by the control one. Each treatment condition was measured in triplicate for calculating the average of fold change of mutation frequency.

2.5. Contribution of efflux pump to antibiotic resistance

Whether efflux mechanisms contribute to the antibiotic resistance induced by N-DBPs was evaluated with efflux pump inhibitor

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