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Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation

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ABSTRACT

Antibiotic resistance poses a major threat to public health. Overuse and misuse of antibiotics are generally recognized as the key factors contributing to antibiotic resistance. However, whether non-antibiotic, anti-microbial (NAAM) chemicals can directly induce antibiotic resistance is unclear. We aim to investigate whether the exposure to a NAAM chemical triclosan (TCS) has an impact on inducing antibiotic resistance on *Escherichia coli*. Here, we report that at a concentration of 0.2 mg/L TCS induces multi-drug resistance in wild-type *Escherichia coli* after 30-day TCS exposure. The oxidative stress induced by TCS caused genetic mutations in genes such as *fabI*, *frdD*, *marR*, *acrR* and *soxR*, and subsequent up-regulation of the transcription of genes encoding beta-lactamases and multi-drug efflux pumps, together with down-regulation of genes related to membrane permeability. The findings advance our understanding of the potential role of NAAM chemicals in the dissemination of antibiotic resistance in microbes, and highlight the need for controlling biocide applications.

1. Introduction

The dissemination of antibiotic resistance has become a major threat to public health (Taubes, 2008). Worldwide, each year about 700,000 people die from antimicrobial-resistant infections, and this mortality has been projected to reach 10 million per annum by 2050 (O'Neill, 2014).

The spread of antibiotic resistance has been attributed to the overuse and misuse of antibiotics in clinic settings, agriculture, and aquaculture (Carlet et al., 2012; Gaze and Depledge, 2017; Leonard et al., 2015; Qiao et al., 2018). Bacteria can develop antibiotic resistance by mutation, horizontal gene transfer, or vertical gene transfer under exposure to antibiotics (Blair et al., 2015). Generally, it was recognized that clinically relevant resistance occurs as a result of bacterial exposure under minimum inhibitory concentration (MIC) of antibiotics (Rice, 2009). Recently, increasing numbers of studies showed that sub-inhibitory concentrations of antibiotics (i.e. sub-MIC) also play an important role in accelerating the emergence and dissemination of antibiotic resistance by increasing mutation rates, recombination and horizontal gene transfer (Andersson and Hughes, 2014). For example, sub-MIC of antibiotics (including ampicillin, kanamycin and norfloxacin) could stimulate mutagenesis in *E. coli* and *Staphylococcus aureus*, resulting in heterogeneous increases in MICs across a variety of irrespective antibiotics (norfloxacin, ampicillin, kanamycin, tetracycline and chloramphenicol). The increased mutagenesis was associated with the overproduction of reactive oxygen species (ROS) (Kohanski et al., 2010).

On a global scale, non-antibiotic, antimicrobial (NAAM) chemicals are used in much larger quantities than antibiotics, resulting in high residual levels of NAAM chemicals in the wider environment. For example, triclosan (TCS), a common biocidal agent used in over 2000 kinds of products such as toothpaste and handwashing liquid (Dann and Hontela, 2011), is widely detected in aquatic environments at μg/L (Liu and Wong, 2013; Singer et al., 2002) to mg/L levels (Kumar et al., 2010), even up to 0.4 mg/L (Chalew and Halden, 2009). Evidence suggests there are potential links between NAAM chemicals and antibiotic resistance (Giuliano and Rybak, 2015). For instance, mupirocin-resistant (Cookson et al., 1991) and quinolone-resistant (Webber et al., 2017) mutants were reported to exhibit decreased susceptibility to TCS, while TCS-resistant were found to have increased cross-resistance to ampicillin, ciprofloxacin (Randall et al., 2004) and erythromycin (Mavri and Smole Možina, 2013). However, it remains unclear if NAAM chemicals such as TCS can directly induce antibiotic resistance. As a preventative policy, U.S. Food and Drug Administration (USFDA) has banned the addition of TCS to antibacterial soap (USFDA, 2016). However, the lack of unequivocal evidence for NAAM chemicals

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inducing antibiotic resistance has prevented such a policy being adopted in other countries.

The objective of this study is to investigate if the TCS exposure would cause antibiotic resistance. Wild-type *Escherichia coli* was exposed to TCS ranging from a sub-MIC (0.02 and 0.2 mg/L, which are environmentally relevant concentrations) to near lethal concentration (2 mg/L, Fig. 1a). After 30-day TCS exposure, the resistance to multiple antibiotics (8 types in total) was measured, following by a combination measurement of live/dead counting, ROS production and cell membrane permeability on the flow cytometer. More importantly, the potential mechanism was revealed using genome-wide DNA and RNA sequencing. The obtained results demonstrated that TCS at sub-MIC could induce multiple antibiotic resistance via the ROS-mediated mutagenesis. Our study reveals a neglected role of NAAM chemicals for the microbial acquisition of antibiotic resistance and potentially can be used as guidance for implementing emission limits of NAAM into the external environment for regulatory bodies.

2. Methods

2.1. Bacterial strains, triclosan, antibiotics

E. coli K-12 was purchased from American Type Culture Collection (ATCC 700926). Triclosan was purchased from Sigma-Aldrich (USA). Antibiotics: amoxicillin (AMX), cephalexin (LEX), tetracycline (TET), chloramphenicol (CHL), levofloxacin (LVX), and norfloxacin (NOR) were supplied by Sigma-Aldrich (USA). Kanamycin (KAN) was supplied by Astral Scientific (Australia), and ampicillin (AMP) was purchased from Gold Biotechnology (USA). The solvent used for dissolving AMX, TET, KAN and AMP was MillQ water, while the solvent used for dissolving LEX, CHL, LVX and NOR was ethanol (≥99.5%).

2.2. Culture conditions, TCS exposure, and antibiotic-resistance determination

E. coli K-12 stock from −80 °C was cultivated on LB agar [lysogeny broth: 5 g/L yeast extract (Difco), 10 g/L NaCl and 10 g/L tryptone (Difco)] at 37 °C for 24 h to isolate a single isogenic strain. The isolate was grown in liquid LB for 12 h at 37 °C to reach a bacterial cell concentration of 10^8–10^9 CFU/mL. For TCS exposure experiments, 50 μL of the cell suspension was inoculated into 4.95 mL fresh liquid LB supplemented with different concentrations of TCS (0, 0.02, 0.2, and 2 mg/L, respectively) at 37 °C, shaken at 150 rpm, in triplicate. Every 24 h, 50 μL of the cell mixture was transferred to another 5 mL tube containing 4.95 mL fresh, liquid LB with respective concentrations of TCS. This was repeated for 30 subculture cycles. At the end of the treatment, 100 μL of each cell culture was plated on LB agar containing respective antibiotics at above MIC_{90} (Table S1) at 37 °C for 48 h to screen for antibiotic-resistant bacteria, and then the number of colonies were counted. The colonies grown on the antibiotic-supplemented plates were considered to have resistance to the corresponding antibiotic. The mutation frequency was calculated by dividing the number of antibiotic-resistant colonies by the total bacterial count, which was enumerated from the LB agar without antibiotics.

2.3. Determination of minimum inhibitory concentrations (MICs)

Following 30 days 0.2 mg/L TCS exposure, and subsequent cultivation on antibiotic selection plates, 5–8 antibiotic-resistant colonies from AMX, CHL and TET selection plates were randomly picked (namely 0.2T-AMX, 0.2T-CHL and 0.2T–TET, respectively), and incubated at 37 °C for 12 h in 2 mL of liquid LB. Using the selected strains, MIC_{90} were determined for eight antibiotics, respectively, including AMX, AMP, LEX, CHL, KAN, LVX, NOR and TET using an initial bacterial cell concentration of 10^6 CFU/mL. Then, 15 μL of this cell suspension was added to each well containing 135 μL of serially, 2-fold diluted antibiotics in a fresh 96-well plate, followed by incubation at 37 °C for 24 h. The optical density (OD_{600nm}) was measured using a plate reader Infinite® 200 PRO (Tecan, Swiss). Each strain was tested in triplicate including sterilized PBS as a blank control. Fold changes in antibiotic-MIC_{90} were also calculated by dividing MICs of all treated mutants by the MIC of the wild-type *E. coli* (Fig. 1c).

2.4. Hereditary stability test

Antibiotic-resistant mutants (0.2T-AMX, 0.2T-CHL and 0.2T-TET) used for MIC profiling were cultivated in 5 mL liquid LB without antibiotics or TCS at 37 °C and 150 rpm. After 24 h, 1% of each liquid cell culture was transferred to fresh 5 mL treatment-free liquid LB and incubated under the same conditions. After five cycles, the MIC_{90} of eight antibiotics were tested respectively using the same method described previously, and the fold changes in MICs were determined. Each sample was tested in triplicate. MIC_{90} fold changes were determined for the cell cultures at day 0 and day 5 of incubation (Fig. 1d, e and f).

2.5. Live and dead cells percentages

The inhibitory effect of TCS on *E. coli* K-12 was investigated by staining with BacLight™ Bacterial Viability Kit (Invitrogen, USA). The LIVE/DEAD cell ratio of TCS-treated (0, 0.02, 0.2, and 2 mg/L TCS for 2 h) *E. coli* was then dual stained with propidium iodide (final concentration: 30 μM) and SYTO 9 (final concentration: 5 μM) in the dark at room temperature for 30 min. The fluorescence was quantified by applying 500 μL (10^6 CFU/mL) of the stained samples to a CYTOFLEX flow cytometer (BD Biosciences, USA) with 488 nm excitation, and emissions were measured above 630 nm for red (PI) fluorescence and at 520 nm for green (SYTO 9) fluorescence. Untreated and heat-treated (2 h at 80 °C) cells were used as controls for intact and damaged cells, respectively.

2.6. Detection of reactive oxygen species (ROS)

To explore whether oxidative stress plays a role in promoting TCS-induced mutation, intracellular ROS formation was determined using the dye 2′,7′-dichlorofluorescein diacetate (Abcam, UK), which can be oxidized by ROS into fluorescent compound, 2′, 7′–dichlorofluorescein, and measured with an Accuri C6 cytometer (BD Biosciences, USA). Briefly, bacterial cell suspensions (approximately 10^6–10^7 CFU/mL) were incubated with 2′,7′-dichlorofluorescein diacetate (at a final concentration of 20 μM) for 30 min at 37 °C, shaken at 100 rpm in the dark. The bacterial cells were then directly treated with TCS (0.02, 0.2, and 2 mg/L) for 2 h at 37 °C, shaken at 100 rpm in the dark. A *tert*-butyl hydrogen peroxide-treated sample (50 μM) was used as a positive control, and three non-TCS-treated samples as negative controls. The samples were then scanned by an Accuri C6 cytometer, and the 2′, 7′–dichlorofluorescein fluorescence (excitation at 488 nm/emission at 525 nm) was measured to deduce the ROS production level (Fig. 1h). The relative ROS production level after dosage with 0.2 mg/L TCS for 2 h was determined for wild-type *E. coli* and 0.2T-AMX-, 0.2T-CHL- and 0.2T-TET-resistant strains which had been originally incubated with 0.2 mg/L TCS, 30 days (Fig. 1i).

2.7. DNA extraction, Illumina sequencing and data processing

MIC-profiled colonies from 0.2T–AMX-, 0.2T-CHL- and 0.2T–TET-resistant strains, and untreated *E. coli* K-12 were cultured in duplicate in 10 mL liquid LB at 37 °C for 16 h (shaken at 150 rpm) to reach 10^7–10^8 CFU/mL. Bacteria were then collected by 10 min centrifugation at 8000 × *g*, and genomic DNA was extracted using FastDNA™ SPIN Kit for Soil (MP, USA) following the manufacturer's instructions. The NexteraXT DNA Sample preparation kit (Illumina, USA) was used to prepare a whole-genome shotgun library which was sequenced by