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Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations

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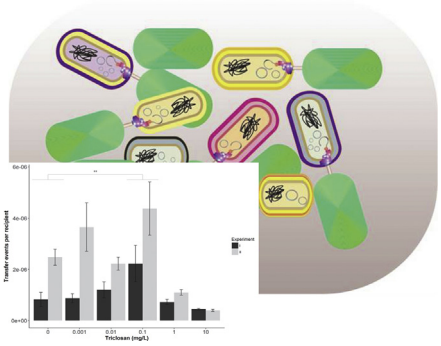
HIGHLIGHTS

- Triclosan and chlorhexidine induced horizontal gene transfer at low concentrations.
- Common antibacterial biocides may thereby promote antibiotic resistance development.
- Some, but not all tested antibiotics and biocides induced resistance gene transfer.

GRAPHICAL ABSTRACT



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ABSTRACT

There is a rising concern that antibiotics, and possibly other antimicrobial agents, can promote horizontal transfer of antibiotic resistance genes. For most types of antimicrobials their ability to induce conjugation below minimal inhibitory concentrations (MICs) is still unknown. Our aim was therefore to explore the potential of commonly used antibiotics and antibacterial biocides to induce horizontal transfer of antibiotic resistance. Effects of a wide range of sub-MIC concentrations of the antibiotics cefotaxime, ciprofloxacin, gentamicin, erythromycin, sulfamethoxazole, trimethoprim and the antibacterial biocides chlorhexidine digluconate, hexadecyltrimethylammoniumchloride and triclosan were investigated using a previously optimized culture-based assay with a complex bacterial community as a donor of mobile resistance elements and a traceable *Escherichia coli* strain as a recipient. Chlorhexidine (24.4 μg/L), triclosan (0.1 mg/L), gentamicin (0.1 mg/L) and sulfamethoxazole (1 mg/L) significantly increased the frequencies of transfer of antibiotic resistance whereas similar effects were not observed for any other tested antimicrobial compounds. This corresponds to 200 times below the MIC of the recipient for chlorhexidine, 1/20 of the MIC for triclosan, 1/16 of the MIC for sulfamethoxazole and right below the MIC for gentamicin. To our best knowledge, this is the first study showing that triclosan and chlorhexidine could stimulate the horizontal transfer of antibiotic resistance. Together with recent research showing that tetracycline is a potent inducer of conjugation, our results indicate that several antimicrobials including both common antibiotics and antibacterial biocides at low concentrations could contribute to antibiotic resistance development by facilitating the spread of antibiotic resistance between bacteria.

1. Introduction

Antibiotics and antibacterial biocides have wide applications in society, providing important means to manage undesired bacterial growth.

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At the same time, their efficacy as antimicrobials is threatened by rapid development and spread of different resistance mechanisms, both caused by mutations of the bacteria's own DNA and by genes that are transferred horizontally across different strains and species. Many, if not all, mobile antibiotic resistance genes found in pathogens today are thought to have their origin in bacteria primarily thriving in the external environment (Martinez, 2011; Wellington et al., 2013). Natural selection exerted by antibiotics favors the spread and maintenance of antibiotic resistance in the environment (Gillings, 2013; Martinez, 2009). Hence selective forces both inside and outside of the bodies of humans and domestic animals, have the ability to influence resistance development of pathogens.

While antibiotics primarily are used to affect bacteria in or on our bodies, residues also reach external environments, for example *via* sewage (Zhang and Li, 2011). Usually, concentrations of antibiotics are very low, in the ng/L range, in surface water (Carvalho and Santos, 2016; Kümmerer, 2009; Meffe and Bustamante, 2014; Scott et al., 2016; Yao et al., 2017) except for environments polluted directly by industrial discharges where they may exceed minimal inhibitory concentrations (MICs) of common pathogens (Larsson, 2014). Recent studies indicate that the presence of very low concentrations of antibiotics, clearly below known MICs, is sufficient to select for a resistance phenotype under some circumstances (Andersson and Hughes, 2014; Lundstrom et al., 2016). This creates a cause of concern also for low-level emissions (Bengtsson-Palme and Larsson, 2016).

Antibacterial biocides differ from antibiotics in the sense that they are often intentionally administered in various external environments at killing concentrations. Depending on the application and type of biocide, lower sub-inhibitory concentrations may spread far from the source, for example into waterways. Although there is some concern about resistance development to the antibacterial biocides themselves, the greatest concern with regards to resistance is their potential to co-select for resistance to antibiotics, primarily through co- and cross-selection mechanisms (Pal et al., 2017; SCENIHR, 2009; Wales and Davies, 2015; Webber et al., 2015). The concentrations of biocides needed for selection and co-selection is even less investigated than in the case for antibiotics.

In addition to providing selective advantages, certain antimicrobials may increase opportunities for resistance development by promoting mutagenesis (Chow et al., 2015; Thi et al., 2011), either favoring single nucleotide substitutions (Rensch et al., 2013) or facilitating recombination-based intracellular genetic rearrangements (Lopez and Blazquez, 2009), but also through stimulation of horizontal gene transfer (HGT) involving conjugative genetic elements (Beaber et al., 2004; Seier-Petersen et al., 2014; Waters and Salyers, 2013). In order to assure the relative importance of antimicrobials in resistance promotion in different settings and environments one of the key questions that should be addressed is what concentrations are required to drive these evolutionary processes (Gaze et al., 2013).

Several earlier studies suggest effects of antibiotics on conjugal resistance transfer (al-Masaudi et al., 1991; Feld et al., 2008; Kim et al., 2014; Showsh and Andrews, 1992; Torres et al., 1991). However, the accuracy of HGT assessment is often suffering from a number of limitations. One of the major methodological problems is the obscuring effect of selection that acts upon the bacterial community at post-transfer level (Sorensen et al., 2005). Such selection might mislead the assessment of conjugal transfer induction by enriching for transconjugants with corresponding resistance phenotype (Bahl et al., 2004). Also, inability to see the effect of the antibiotic on HGT could be possibly explained by the choice of the donor/plasmid for conjugation experiments and by a limited tested concentration range of the antibiotics (Feld et al., 2008). A common experimental design for exploring the effects of antimicrobials on HGT is based on bi-parental mating system involving a single donor and a single recipient at a time, using a rather narrow range of tested concentrations near the MIC values. Recognizing the necessity for improvements, we recently established an assay for conjugal resistance transfer with reduced risks for bias caused by selection and donor/plasmid specificity limitations (Jutkina et al., 2016). This was achieved by shortening the time allowed for conjugation, using a different antibiotic agent to select for transconjugants than the one investigated for its effect on HGT and by using a diverse bacterial community of treated sewage effluent as donors. Using this setup, we showed that tetracycline (TET) promotes HGT at 10 μg/L, which corresponds to 1/150 of the MIC of the inducer for the resistance-acquiring bacterial strain (Jutkina et al., 2016).

Although an induction of HGT by certain stressors, including some antibiotics, has been demonstrated (Aminov, 2011), little is known about the ability of different antimicrobials to induce conjugation. Despite that several common biocides, including ethanol, cetrimide, chlorhexidine (CHG), sodium dodecyl sulfate, sodium hypochlorite, chlorine, chloramine and hydrogen peroxide (H_2O_2), have been investigated for their ability to affect conjugal transfer (al-Masaudi et al., 1991; Pearce et al., 1999; Seier-Petersen et al., 2014; Zhang et al., 2017), to our best knowledge, conjugation inductive properties have only been reported for ethanol (Seier-Petersen et al., 2014), chlorine, chloramine and H_2O_2 (Guo et al., 2015; Zhang et al., 2017). In addition, nano-titanium dioxide (TiO_2) a photo-catalysing nano-material that is frequently present in aqueous environments can promote conjugal transfer of resistance plasmids (Dunlop et al., 2015; Qiu et al., 2015; Qiu et al., 2012).

In this study we aimed to screen a range of extensively used antibiotics and antibacterial biocides for their ability to induce conjugal resistance transfer. Importantly, we used a wide community of donor bacteria to increase the probability of testing suitable donor-recipient pairs. Also, we tested concentrations from around the MIC to far below the MIC of the resistance-acquiring recipient strain. We show that several antibiotics and common antibacterial biocides triclosan (TCS) and chlorhexidine (CHG) stimulate HGT at sub-MIC concentrations, whereas for some antimicrobials no effects were observed. Overall, we think this study strengthens the concern for effects of antimicrobials on horizontal gene transfer as a mean for promoting antibiotic resistance.

2. Materials and methods

2.1. Chemicals and reagents used in the study

The following antimicrobials were used in the study: cefotaxime sodium salt (CTX) (Sigma-Aldrich, C7912), CHG (Sigma-Aldrich Co., St Louis, MO, USA, C9394), ciprofloxacin HCl (CIP) (AppliChem, Darmstadt, Germany, A4556), erythromycin (ERY) (Amdipharm Mercury Ltd., UK), gentamicin sulfate (GEN) (Sigma-Aldrich, 48760), hexadecyltrimethylammoniumchloride (CTAC) (Sigma-Aldrich, 52366), kanamycin (KAN) (Sigma-Aldrich, K4000), rifampicin (RIF) (Sigma-Aldrich, R3501), sulfamethoxazol (SMX) (Sigma-Aldrich, S7507), triclosan (TCS) (Sigma-Aldrich, 72779), trimethoprim (TMP) (Duchefa Biochemie, Haarlem, The Netherlands, T0154).

The experiments were performed using commercially available agar media, *i.e.* Mueller Hinton (MH) (Oxoid Ltd., Hampshire, United Kingdom, CM0337), Tryptic soy agar (TSA) (Acumedia, Neogen Corp., Lansing, MI, USA, 7100) and CHROMagar™ MH Orientation (Chromagar, Paris, France).

2.2. Preparation of the bacterial donor and the recipient for conjugation

The *gfp*-marked KAN and RIF resistant recipient strain *E. coli* CV601 (Heuer et al., 2002) was pre-grown at 30 °C shaking overnight in LB broth supplemented with KAN (50 mg/L). Following a twenty-fold dilution in fresh LB broth the culture was incubated at the same conditions for several additional hours while the donor preparation was performed. The bacterial suspension was subsequently centrifuged for 15 min at 2755 × *g*. The pellet was washed twice in PBS and finally resuspended in PBS to a cell density of approximately 10^8 CFU/mL.