High levels of antibiotic tolerance and persistence are induced by the commercial anti-microbial triclosan

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Antimicrobial tolerance and bacterial persistence are confounding factors in the treatment of recurrent and chronic infections. Here we report that the anti-microbial additive triclosan renders *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* tolerant to a panel of bactericidal antibiotics. Triclosan treatment increased antimicrobial tolerance up to 10,000 fold, amplified persister populations by several orders of magnitude, and in one instance, rendered *E. coli* refractile to the cell wall active antibiotic, ampicillin.

The prophylactic use of antibiotics in consumer goods ranging from animal feed to personal care products is widely believed to be a major contributor to the epidemic increase in antibiotic resistant pathogens[1-3]. While the inverse relationship between antibiotic use and antibiotic efficacy is largely attributable to the selection of heritable traits, non-heritable traits such as antibiotic tolerance and persistence are also likely to be involved[4].

In contrast to genetically resistant bacteria, which grow in the presence of an antibiotic, tolerant bacteria are able to survive antibiotic challenge for longer periods of time than their more sensitive counterparts[5]. Persister cells are the small sub-set of an otherwise-sensitive population (~1 in 10^6) that exhibit levels of tolerance sufficient to protect them from otherwise lethal concentrations of antimicrobial compounds[6]. Increases in antibiotic tolerance and persistence are confounding factors in the treatment of chronic *P. aeruginosa*[7] and *S. aureus*[8] infections and are thought to contribute to the refractile nature of medically relevant biofilms. Reduced growth rate is associated with increased antibiotic tolerance[9] and is a defining trait of persister cells.

Banned from consumer soaps effective September 2017 by the US Food and Drug Administration, the antimicrobial triclosan remains approved for use in products ranging from toothpaste to cleansers employed in healthcare settings[10]. In contrast to bactericidal antibiotics, which kill pathogens outright, triclosan is a bacteriostatic drug that inhibits growth by targeting enoyl-acyl carrier protein reductase to interfere with early steps in fatty acid synthesis[11].

Based on its ability to inhibit growth, we wondered if physiologically relevant levels of triclosan might provide protection against bactericidal antibiotics. To address this possibility we examined the relative sensitivity of *E. coli* (MG1655) and *S. aureus* (FPR3757 an USA-300 MRSA strain) cultured in minimal inhibitory concentrations (MIC) of triclosan to a panel of bactericidal antibiotics. Triclosan MICs for *E. coli* and MRSA were 200 ng/mL and 100 ng/mL, respectively under our growth conditions, equivalent to the urine concentration of individuals using triclosan-containing consumer products[12,13]. In all cases, triclosan was added 30 minutes prior to the addition of the specified bactericidal antibiotic and both antibiotics maintained in the culture for the remainder of the experiment.

We utilized two approaches to evaluate the impact of triclosan on bacterial tolerance and persistence: a qualitative, end-point plating efficiency assay and a quantitative kinetic kill-curve. Plating efficiency assesses relative antimicrobial tolerance and the kinetic kill-curve distinguishes between changes in total antibiotic tolerance and changes in persister frequency[4].

Triclosan had a dramatic protective effect on *E. coli* in the end point assay, increasing survival by several orders of magnitude in the presence of three bactericidal antibiotics and providing

nearly complete protection against a fourth (Figure 1). *E. coli* treated with triclosan exhibited a 1000-fold increase in survival in the presence of 50 μg/mL (~5x MIC) kanamycin, an inhibitor of peptide bond formation, and a 10,000-fold increase in survival in the presence of streptomycin (50 μg /mL: ~2x MIC), an inhibitor of tRNA-ribosome interaction, and ciprofloxacin (100 ng/mL: ~3x MIC) a gyrase inhibitor. (Figure 1). Strikingly, triclosan rendered *E. coli* almost completely refractile to treatment with the cell wall active antibiotic ampicillin (100 μg /mL; ~10x MIC). Viable cell numbers were essentially identical in triclosan and triclosan-ampicillin treated cultures at 2 hours, and 10% of cells in triclosan-ampicillin cultures were viable at 20 hours, suggesting triclosan qualitatively increased persister frequency.

To further assess the protective effect of Triclosan, we next performed a kinetic kill curve, in which we measured colony forming units (CFU) over a 20-hour time, focusing on ciprofloxacin, the broad spectrum antibiotic used to treat *E. coli* related UTIs. Consistent with the results of the end point assay, triclosan substantially protected *E. coli* from ciprofloxacin-induced cell death throughout the duration of the time course. Protection was particularly pronounced at the 2-hour time point, where the slope of the kill curve for pre-treated cells diverges substantially from that of untreated cells (Figure 2). A divergent slope is diagnostic for the presence of persister cells in the pre-treated population[13].

Persister population size was proportional to the concentration of the bacteriocidal antibiotic: 10% of triclosan treated cells cultured at 100 ng/mL ciprofloxacin remained viable at 2 hours, while only 0.1% cultured at the more clinically relevant 1,000 ng/mL ciprofloxacin were viable at the same time point. For perspective, 0.1% of the population is equivalent to 1 in 10^3 cells,

1000-fold higher than the expected frequency of persisters in an untreated population[4]. At the 20 hour time point, 90,000 cells per mL were viable in 100 ng/mL ciprofloxacin and 30 cells per mL in 1000ng/mL ciprofloxacin. In contrast, we observed only 20 cells/ml after 20 hours of growth in 100 ng/mL ciprofloxacin alone. Cells cultured in 1,000 ng/mL ciprofloxacin alone had no observable colonies (<10 cells per mL).

Remarkably, triclosan also protected MRSA cells from high concentration of the cell wall inhibitor vancomycin over the course of a 20-hour experiment (Figure 2C). MRSA treated with 100 ng/ml of triclosan were essentially refractile to 50 ng/ml vancomycin (10x the MIC) at 4 hours and exhibited a viable cell count 200-times that of untreated cells at 8 hours. Although not statistically significant, at 20 hours the viable cell count was several times higher in the presence of both triclosan and vancomycin than vancomycin alone (360,000 +/- 200,000 cells/ml versus 130,000 +/- 80,000 cells/ml), consistent with induction of a persistent state.

Together these data highlight an unexpected and potentially important role for triclosan as a contributor to antibiotic tolerance and persistence in both community and health care settings. We have yet to identify the mechanism underlying triclosan-induced tolerance, however, defects in fatty acid synthesis induce accumulation of the alarmone ppGpp, which has itself been implicated in tolerance and persistence[14-16]. While the impact of triclosan and other bacteriostatic drugs on antibiotic efficacy in the clinic will require further analysis, our findings reinforce the need for substantial caution—as well as consideration of unintended consequences—in evaluating the costs and benefit of antimicrobial additives in consumer products.

Methods

Materials and Strains

Triclosan, ampicillin, kanamycin, streptomycin, ciprofloxacin, and vancomycin were purchased from Sigma-Aldrich. Stock solutions were made in water for ampicillin (100 mg/mL), kanamycin (50 mg/mL), streptomycin (100 mg/mL) and ciprofloxacin (10 mg/mL). Triclosan was dissolved in ethanol (10 mg/mL), and vancomycin was dissolved in DMSO (100 mg/mL). *E. coli* MG1655 and *S. aureus* FPR3575 were both lab strains. *E. coli* was grown in Luria-Bertani broth (LB) and *S. aureus* was grown in tryptic soy broth (TSB). Growth temperature was 37°C for all experiments.

Determination of Minimum Inhibitory Concentration (MIC)

To determine the MIC for the panel of antibiotics utilized in this study, *E. coli* and *S. aureus* were grown to OD-600 = 0.1 in LB or TSB respectively. Cells were tehn back-diluted 1000-fold and transferred to a 96-well plate containing 2-fold dilutions of respective antibiotics and cultured at 37° for 16 additional hours with vigorous shaking in a BioTek Eon plate reader. MIC was calculated as the lowest antibiotic concentration preventing development of detectable turbidity at OD-600.

Assays for antibiotic Tolerance and Persistence

To assay tolerance and persistence, *E. coli* and *S. aureus* were grown to an OD 600 = 0.2 in LB or TSB respectively. Cells were then back-diluted into media containing triclosan at indicated concentrations to an OD-600 = 0.1, cultured for an additional 30 minutes, before being challenged with bactericidal antibiotics. For dot plating, 10 μL of a 10-fold dilution series was

plated on antibiotic free LB-agar or TSB-agar as appropriate. For determination of colony forming units (CFU), 100 μL of a 10-fold dilution series was spread on antibiotic free LB-agar or TSB-agar plates. Cells were incubated for ~12 hours at 37° prior to quantification. CFUs were normalized to CFUs at t0 to correct for the ~2-fold increase in cell number in untreated cultures during the 30 minute pre-treatment period. Relative persistence is defined as the CFU's of the triclosan treated sample divided by the CFU's of the non-treated sample.

Statistical Analysis

All values are expressed as the mean ± standard deviation of n=3 replicates. Data was analyzed using a one-tailed Student's t-test. Statistical significance was determined when $p<.05$.

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Author Contributions

C.S.W. and P.A.L designed the experiments. C.S.W. performed the experiments. C.S.W. and P.A.L. analyzed the data and prepared the manuscript.

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Figures

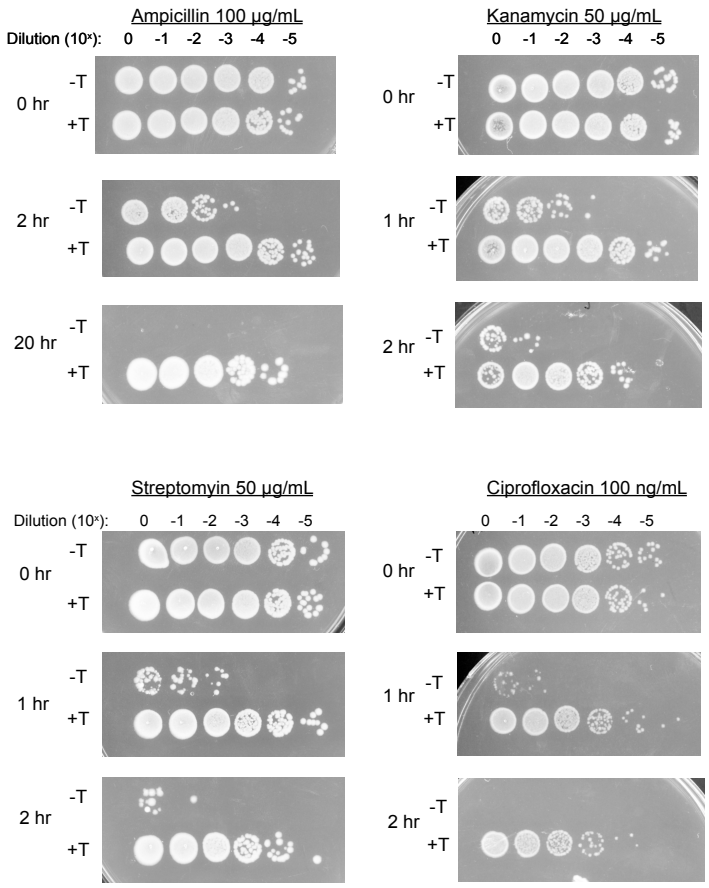

Figure 1: Triclosan induces tolerance to multiple antibiotics. *E. coli* (MG1655) were cultured to OD600 = 0.2, split and cultured for an additional 30 minutes with (+T) or without 200ng/ml triclosan (-T). Indicated bactericidal antibiotics were then added and cells cultured for an additional 2 to 20 hours prior to dilution plating. Each experiment was replicated three independent times with only representative data shown.

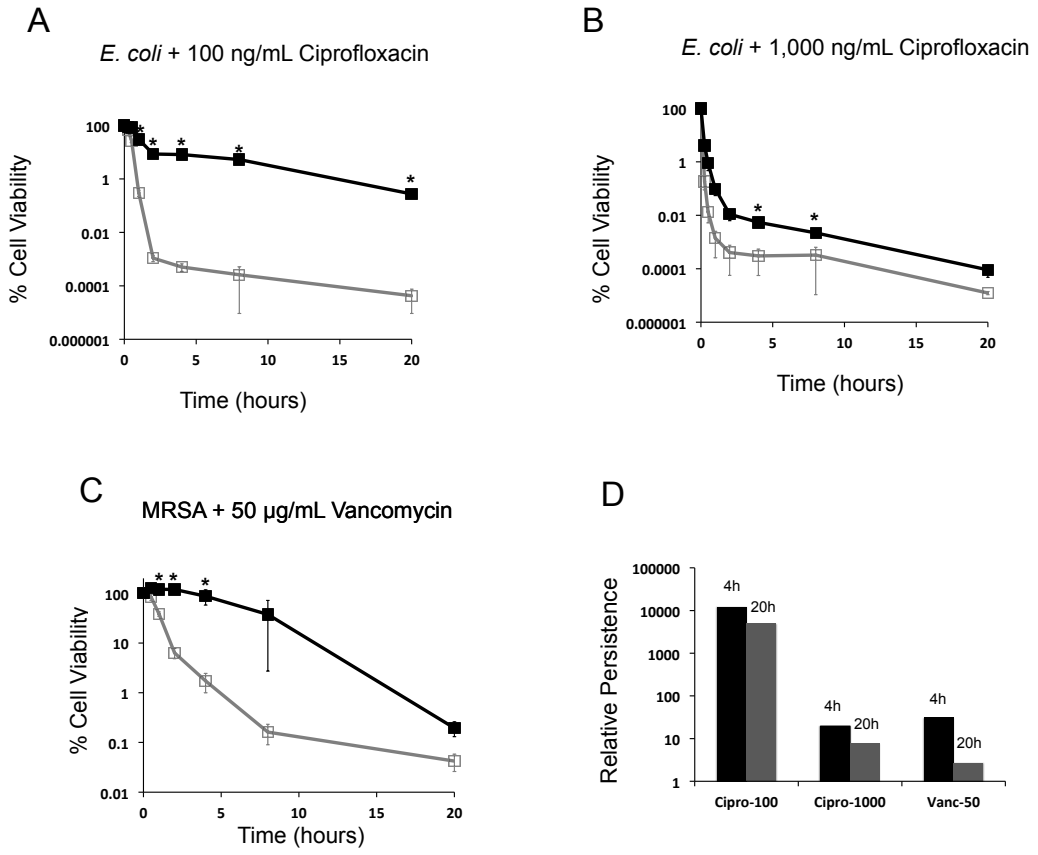

Figure 2: Kinetic analysis of triclosan-induced persistence. *E. coli* (MG1655) and MRSA (FPR3757) cells were cultured to OD600 = 0.2, split and cultured for an additional 30 minutes with (black line, closed squares) or without triclosan (grey line, open squares). At t=0, 100 ng/mL (A) or 1 µg/mL ciprofloxacin (B) was added to *E. coli* cultures and 50ng/ml vancomycin was added to MRSA cultures (C). Relative persistence in the presence of Triclosan (CFU+T/CFU-T) was calculated for the 4 and 20 hour time points (D). Values are the mean of three independent biological replicates with error bars indicating one standard deviation. Asterisks represent significant difference between the triclosan treated and non-treated using a Student's t-test with $p < 0.05$.