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 triclosan increased antibiotic tolerance up to 10,000 fold and amplified persister populations in both *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*. Triclosan-mediated protection was dependent on the alarmone ppGpp but independent of growth rate, arguing against a simplistic model in which slow growth is sufficient to confer antibiotic tolerance.

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.

The ability of triclosan to protect cells from bactericidal antibiotics appears to be independent of its impact on bacterial growth rate. Instead, genetic data support a model in which triclosan-mediated tolerance stems from accumulation of the alarmone ppGpp in response to defects in fatty acid synthesis[15]. ppGpp has been repeatedly implicated in antibiotic tolerance and persistence in several organisms including *E. coli* and *S. aureus* [14-16]. To determine the contribution of ppGpp accumulation to triclosan mediated tolerance, we compared the relative viability of wild type and ppGpp^0 (*spoT*::*cat* **Δ***relA*) *E. coli* cells after 2 hours antibiotic challenge in the presence or absence of triclosan. Triclosan was unable to substantially protect ppGpp^0 cells from either ampicillin or ciprofloxacin (Figure 2E). For kanamycin and

114 streptomycin, the ppGpp^0 cells showed no viable cells after 2 hours for both the triclosan-treated
115 and untreated cells. In contrast to previous studies which relied on either carbon starvation or the
116 addition of serine hydroxamate to induce accumulation of high concentrations of ppGpp [100x
117 above baseline][16], defects in fatty acid synthesis have at best a modest impact on ppGpp
118 levels[~5x over baseline][17]. Thus even relatively low levels of the alarmone appear to be
119 sufficient to protect cells from a panel of antimicrobials.
120
121 Triclosan inhibited growth in the presence and absence of the alarmone, arguing against a
122 simplistic model in which reductions in growth rate are inherently protective (Figure 2F).
123 Specifically how modest increases in [ppGpp] might confer tolerance to different antibiotics thus
124 remains an open question. We favor the idea that rather than exerting a global effect, ppGpp
125 mediates changes in individual biosynthetic pathways that render them tolerant of their cognate
126 antimicrobial. For example, ppGpp-dependent down regulation of ribosomal RNA synthesis
127 significantly curtails translation, conferring resistance to the translational inhibitors kanamycin
128 and streptomycin. Similarly, increases in [ppGpp] are reported to curtail DNA replication—both
129 elongation and initiation—providing a straightforward explanation for ppGpp-mediated
130 ciprofloxacin resistance.
131
132 Regardless of mechanism, these data highlight an unexpected and potentially important role for
133 triclosan as a contributor to antibiotic tolerance and persistence in both community and health
134 care settings. While the impact of triclosan on persistence and tolerance *in vivo* remains an open
135 question, the high concentration of the compound in the urine of individuals using triclosan-
136 containing products raises the possibility that it might interfere with treatment of bladder and

kidney associated infections. Along with previous work identifying a connection between triclosan exposure and antibiotic resistance[18,19], 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. The ppGpp0 strain was created by transducing spoT::cat (the kind gift of Dr. Jue D. Wang) and relA::ka*n*[20] into MG1655 using the bacteriophage P1*vir*. To test kanamycin tolerance of the ppGpp0, we first cured the kanamycin cassette using pCP20, and the pCP20 plasmid was then removed through growth of multiple generations at 42°C[21]. *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 then 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.

Growth Curve

MG1655 and MG1655 ppGpp^0 cells were grown to an OD600=0.1-0.2 in LB before back-diluting to an OD600=0.02 into LB with or without 200 ng/mL triclosan. Growth was monitored every 30 minutes at OD600.

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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192 **Author Contributions**

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

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Figures

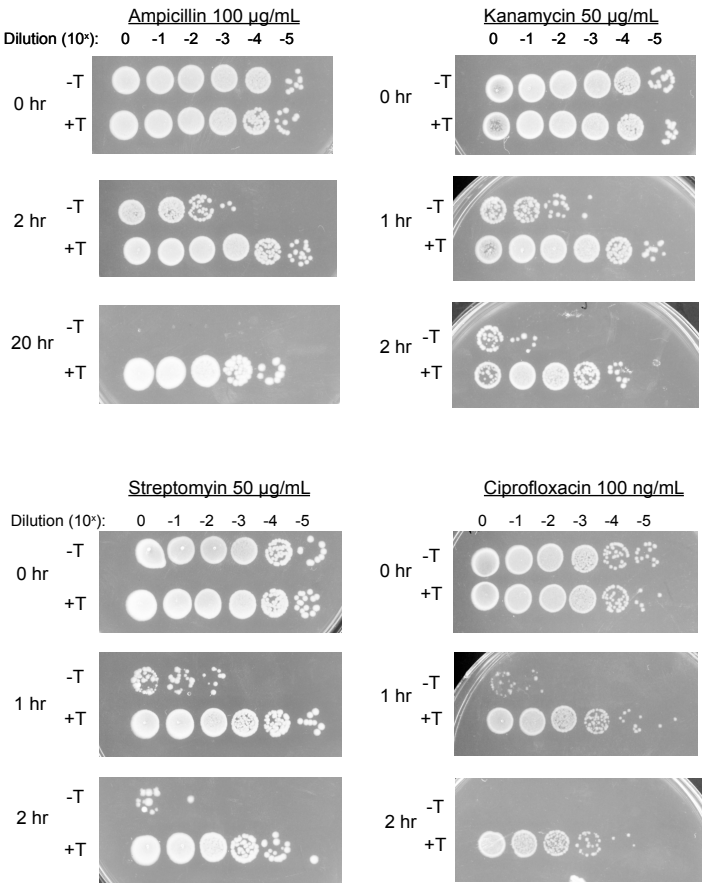

Figure 1: Triclosan induces tolerance to multiple antibiotics. *E. coli* (MG1655) were cultured to OD600 = 0.2, split and cultured for an additional 30 minutes with (+T) or without 200ng/ml triclosan (-T). Indicated bactericidal antibiotics were then added and cells cultured for an additional 2 to 20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

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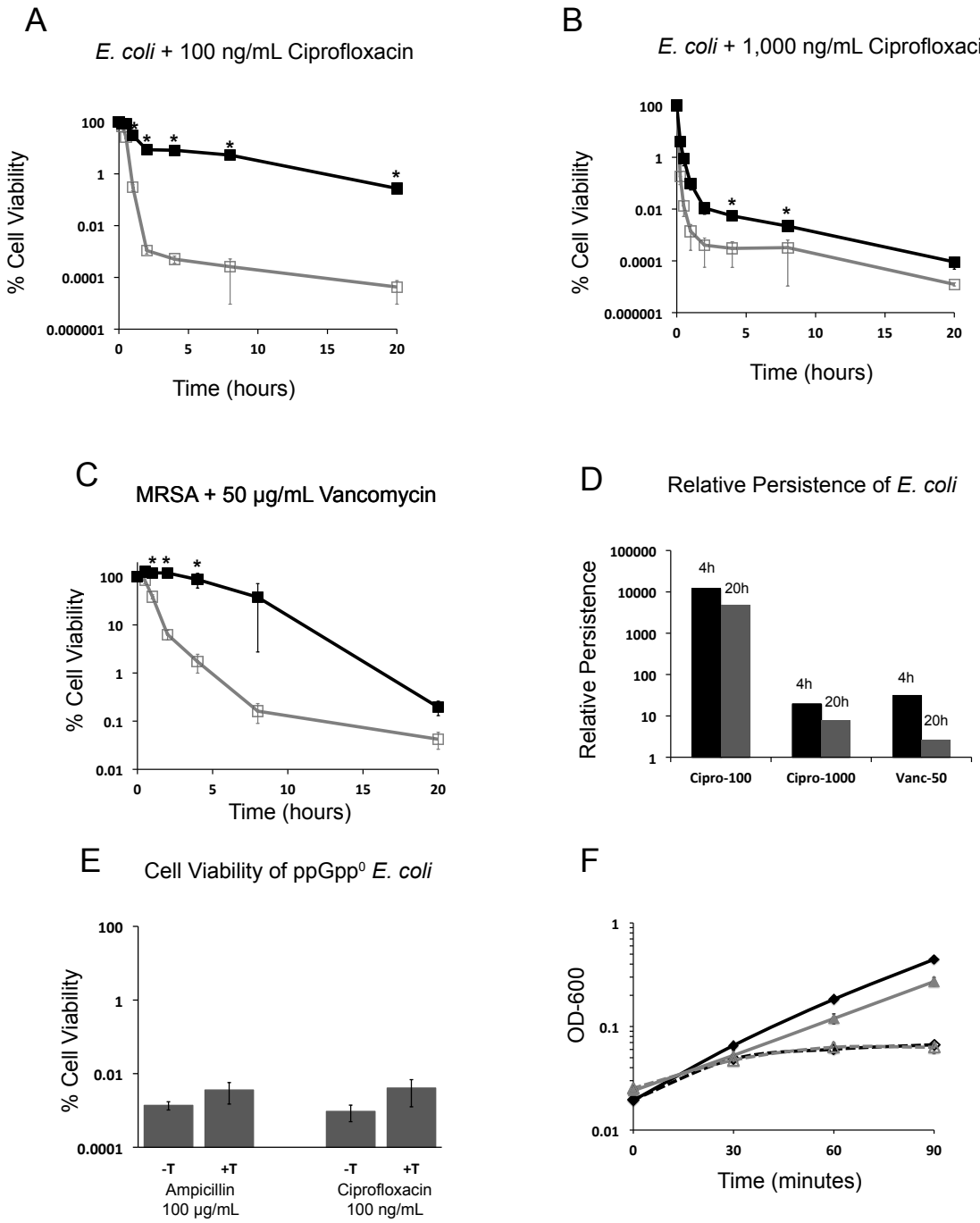

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258 **Figure 2: Kinetic analysis of triclosan-induced persistence.** *E. coli* (MG1655) and MRSA

259 (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). Cell viability of $ppGpp^0$ *E. coli* with (+T) or without (-T) pretreatment with triclosan after 2 hour challenge with ampicillin or vancomycin (E). Growth curves of MG1655 (black curve) or $ppGpp^0$ (gray curve) in LB with (dashed lines) or without (solid lines) triclosan (F). 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 $p < 0.05$.