The commercial antimicrobial triclosan induces high levels of antibiotic tolerance *in vitro* and reduces antibiotic efficacy up to 100-fold *in vivo*

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29 **Abstract**

30 The antimicrobial triclosan is used in a wide range of consumer products ranging from
31 toothpaste, cleansers, socks, and baby toys. A bacteriostatic inhibitor of fatty acid synthesis,
32 triclosan is extremely stable and accumulates in the environment. Approximately 75% of adults
33 in the US have detectable levels of the compound in their urine, with a sizeable fraction of
34 individuals (>10%) having urine concentrations equal to or greater than the minimal inhibitory
35 concentration for *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA).
36 Previous work has identified connections between defects in fatty acid synthesis and
37 accumulation of the alarmone guanosine tetraphosphate (ppGpp), which has been repeatedly
38 associated with antibiotic tolerance and persistence. Based on these data, we hypothesized that
39 triclosan exposure may inadvertently drive bacteria into a state in which they are able to tolerate
40 normally lethal concentrations of antibiotics. Here we report that clinically relevant
41 concentrations of triclosan increased *E. coli* and MRSA tolerance to bactericidal antibiotics as
42 much as 10,000 fold *in vitro* and reduced antibiotic efficacy up to 100-fold in a mouse urinary
43 tract infection model. Genetic analysis indicated that triclosan-mediated antibiotic tolerance
44 requires ppGpp synthesis, but is independent of growth. These data highlight an unexpected and
45 certainly unintended consequence of adding high concentrations of antimicrobials in consumer
46 products, supporting an urgent need to reevaluate the costs and benefits of the prophylactic use
47 of triclosan and other bacteriostatic compounds.

48

49 **Importance**

50 Added as a prophylactic to a wide range of consumer products, the fatty acid synthesis inhibitor
51 triclosan accumulates to high levels in humans and the environment. Based on links between
52 defects in fatty acid synthesis and accumulation of the alarmone ppGpp, we hypothesized that

53 triclosan would render cells tolerant to bactericidal compounds due to ppGpp-mediated
54 inhibition of biosynthetic capacity. Our data indicate that clinically relevant concentrations of
55 triclosan induces higher tolerance of *E. coli* and methicillin resistant *S. aureus* (MRSA) to a
56 panel of bactericidal antibiotics up to 10,000-fold. In a urinary tract infection model, mice
57 exposed to triclosan exhibited bacterial loads ~100-fold higher in the bladder than control
58 animals following ciprofloxacin challenge. These findings highlight an unexpected consequence
59 of antimicrobials in consumer products and support an urgent need to reevaluate the costs and
60 benefits of the prophylactic use of triclosan and other bacteriostatic compounds.
61

62 **Introduction**

63 The prophylactic use of antibiotics in consumer goods, ranging from animal feed to personal care
64 products, is widely believed to be a major contributor to the epidemic increase in antibiotic-
65 resistant pathogens (1-3). Prominent among these prophylactics are triclosan and triclocarban,
66 polychlorinated aromatic antimicrobials targeting fatty acid synthesis. Triclosan in particular is
67 found in a wide variety of consumer products, including: toothpaste, cleansers, socks, and baby
68 toys (1). Although the US Food and Drug Administration effectively banned the use of triclosan
69 in household soap in 2017, as of this writing Canada and Australia, among other countries, have
70 not elected to take similar action.
71

72 An inhibitor of enoyl-acyl carrier protein reductase, (4), at low concentrations (200 ng/ml)
73 triclosan is bacteriostatic, preventing cell growth but having little effect on viability over the
74 short term. At high concentrations (>10 μg/ml), triclosan is bactericidal, most likely killing cells
75 through disruption of plasma membrane integrity (4). Recent work from the Waters lab suggests
76 that at these higher concentrations triclosan can serve as an adjuvant, acting synergistically with

77 tobramycin and other drugs, increasing killing by ~100-fold in a *Pseudomonas aeruginosa*
78 biofilm model for CF lung infections (5). Triclosan is typically used as an antimicrobial additive
79 at these higher, bactericidal concentrations.
80
81 Because of its widespread use as a prophylactic, the high concentrations at which it is employed,
82 and its inherent stability, triclosan accumulates to high levels in the environment (6, 7).
83 Approximately 75% of adults in the US have detectable levels of the compound in their urine,
84 and >10% have urine concentrations greater than or equal to the minimal inhibitory
85 concentration for *Escherichia coli* (200 ng/mL) and methicillin-resistant *Staphylococcus aureus*
86 (MRSA) (100 ng/mL) (8, 9).
87
88 While the inverse relationship between antibiotic use and antibiotic efficacy is largely
89 attributable to the selection of heritable traits, non-heritable traits such as antibiotic tolerance and
90 persistence are also likely to be involved (10). In contrast to genetically-resistant bacteria, which
91 grow in the presence of an antibiotic, tolerant bacteria are able to survive antibiotic challenge for
92 longer periods of time than their more sensitive counterparts (10). Persister cells are the small
93 sub-set of an otherwise-sensitive population (~1 in 10^6) that exhibit levels of tolerance sufficient
94 to protect them from otherwise lethal concentrations of antimicrobial compounds (11). Increases
95 in antibiotic tolerance and persistence are confounding factors in the treatment of chronic *P.*
96 *aeruginosa* (12) and *S. aureus* (13) infections and are thought to contribute to the refractory
97 nature of medically relevant biofilms (14). Reduced growth rate and metabolic activity is
98 associated with increased antibiotic tolerance (10) and is a defining trait of persister cells.
99

100 Based on previous work identifying connections between defects in fatty acid synthesis and
101 accumulation of the alarmone guanosine tetraphosphate (ppGpp) (15), and the possible link
102 between ppGpp and antibiotic tolerance (16), we hypothesized that triclosan exposure may
103 inadvertently drive bacteria into a metabolically depressed state in which they are able to tolerate
104 normally lethal concentrations of antibiotics (17, 18). In particular, inhibiting fatty acid
105 synthesis stimulates interaction between acyl carrier protein and the hydrolase domain of the
106 bifunctional ppGpp synthase SpoT, resulting in accumulation of the alarmone and the
107 concomitant inhibition of biosynthetic capacity (19).
108
109 Here we report that clinically-relevant bacteriostatic concentrations of triclosan increased *E. coli*
110 and methicillin resistant *Staphylococcus aureus* (MRSA) tolerance to bactericidal antibiotics as
111 much as 10,000-fold *in vitro* and reduced antibiotic efficacy ~100-fold in a mouse urinary tract
112 infection model. Triclosan-mediated antibiotic tolerance is dependent on ppGpp synthesis:
113 although triclosan inhibited the growth of both wild-type and ppGpp mutant cells, only the latter
114 were highly susceptible to challenge with bactericidal compounds. In contrast, pretreatment with
115 another bacteriostatic drug, spectinomycin, a translation inhibitor that does not impact ppGpp
116 accumulation (20), induced high levels of antibiotic tolerance in both wild-type and ppGpp
117 mutant cells. Together, these data highlight an unexpected and certainly unintended
118 consequence of employing triclosan as a commercial antimicrobial, and support an urgent need
119 to reevaluate the costs and benefits of the addition of triclosan, and potentially other
120 bacteriostatic compounds, to consumer products.
121
122

123 **Results**

124 **Triclosan pretreatment results in high levels of tolerance to bactericidal antibiotics *in vitro***

125 To assess if physiologically relevant levels of triclosan are sufficient to promote tolerance to
126 bactericidal antibiotics, we examined the relative sensitivity of *E. coli* (MG1655) and *S. aureus*
127 (FPR3757 an USA-300 MRSA strain) cultured in minimal inhibitory concentrations (MIC) of
128 triclosan to a panel of bactericidal antibiotics. Triclosan MICs for *E. coli* and MRSA were 200
129 ng/mL and 100 ng/mL, respectively under our growth conditions; similar to the triclosan
130 concentration found in the urine from individuals using triclosan-containing products (8, 9). In
131 all cases, triclosan was added 30 minutes prior to the addition of the specified bactericidal
132 antibiotic and both antibiotics were maintained in the culture for the remainder of the
133 experiment.

134

135 Triclosan had a dramatic protective effect on *E. coli* in an end point assay, increasing survival by
136 several orders of magnitude in the presence of three bactericidal antibiotics and providing nearly
137 complete protection against a fourth (Fig. 1). *E. coli* treated with triclosan exhibited a 1,000-fold
138 increase in survival in the presence of 50 μg/mL (~5x MIC) kanamycin, an inhibitor of peptide
139 bond formation. It also showed a 10,000-fold increase in survival in the presence of streptomycin
140 (50 μg /mL: ~2x MIC), an inhibitor of tRNA-ribosome interaction, and ciprofloxacin (100
141 ng/mL: ~3x MIC) a gyrase inhibitor (Fig. 1). Strikingly, triclosan rendered *E. coli* almost
142 completely refractory to treatment with the cell wall active antibiotic ampicillin (100 μg /mL;
143 ~10x MIC). Viable cell numbers were essentially identical in triclosan and triclosan-ampicillin
144 treated cultures at 2 hours, and 10% of cells in triclosan-ampicillin cultures were viable at 20
145 hours, suggesting triclosan increased persister frequency to all tested antibiotics.

146 Triclosan also protected MRSA cells from high concentrations of the glycopeptide antibiotic,
147 vancomycin, over the course of a 20-hour experiment (Fig. 2c). MRSA treated with 100 ng/ml of
148 triclosan were essentially refractory to 50 ng/ml vancomycin (10x the MIC) at 4 hours and
149 exhibited a viable cell count 200x that of untreated cells at 8 hours. Even at 20 hours the viable
150 cell count was several times higher in the presence of both triclosan and vancomycin than
151 vancomycin alone. This delayed reduction in viable cell count is consistent with induction of a
152 persistent state (10).
153
154 **Triclosan increases persister cell frequency**
155 To further assess the protective effect of triclosan, we next performed kinetic kill curves, in
156 which we measured colony-forming units (CFU) over a 20-hour time frame. If persister cells are
157 present, we expect to observe two slopes, one corresponding to the kill rate of the general
158 population and the second slope corresponding to the slower kill rate of persister cells (10). In
159 this manner, the kill curve is able to separate antibiotic tolerance at the population level from the
160 impact of triclosan treatment on persister levels.
161
162 For these experiments, we focused on ciprofloxacin, the broad-spectrum antibiotic used to treat
163 urinary tract infections (UTIs). Consistent with the results of the end point assay (Fig. 1),
164 triclosan substantially protected *E. coli* from ciprofloxacin-induced cell death throughout the
165 duration of the time course (Fig. 2a, b). Protection was particularly pronounced at the 2-hour
166 time point, where the slope of the kill curve for pre-treated cells diverged substantially from that
167 of untreated cells (Fig. 2a). A reduced kill rate suggests that the pre-treated population contains a
168 larger proportion of persister cells (21).

169 In agreement with previous work (16), persister population size was proportional to the
170 concentration of ciprofloxacin. 10% of triclosan treated MG1655 cells cultured in 100 ng/mL
171 ciprofloxacin remained viable after 2 hours (Fig. 2a), while only 0.1% cultured in the more
172 clinically-relevant 1,000 ng/mL ciprofloxacin were viable at the same time point (Fig. 2b). For
173 perspective, 0.1% is 1,000-fold higher than the expected frequency of persisters in an untreated
174 population (10). After 20 hours, 90,000 CFU/mL were viable in cultures treated with both
175 triclosan and 100 ng/mL ciprofloxacin (Fig. 2a), compared to 20 cells/mL in cultures treated with
176 ciprofloxacin alone. At 1,000 ng/mL ciprofloxacin, cultures treated with both triclosan and
177 ciprofloxacin contained 30 viable cells per mL (Fig. 2b). In contrast, no viable cells were
178 detected (<10 cells/mL) in cultures treated with 1,000 ng/mL ciprofloxacin alone. We observed
179 an increase in the abundance of persisters at drug concentrations above 1,000 ng/mL (Fig S1)
180 (16). Although this finding initially appeared counterintuitive, it is consistent with previous
181 reports suggesting that prophage induction in response to DNA damage is responsible for cell
182 death at lower concentrations of ciprofloxacin, while higher concentrations kill cells before
183 prophage are induced (16).
184
185 **Triclosan-mediated tolerance requires ppGpp**
186 Based on the well-established connection between defects in fatty acid synthesis and
187 accumulation of ppGpp, we speculated that triclosan-mediated tolerance was dependent on the
188 synthesis of the alarmone (15). To test this idea, we compared the relative viability of wild-type
189 *E. coli* and mutants unable to synthesize the alarmone (ppGpp0; *spoT*::*cat* Δ*relA*) 2 hours after
190 antibiotic challenge in the presence or absence of triclosan.
191

192 Although triclosan inhibited the growth of both wild-type and ppGpp0 cells, it was unable to
193 substantially protect ppGpp0 cells from any of the four bactericidal antibiotics we tested. These
194 include ampicillin and ciprofloxacin, as well as the translation inhibitors, kanamycin and
195 streptomycin (Fig. 3a, b). The ppGpp0 cells were more sensitive to kanamycin and streptomycin,
196 showing no viable cells after 60-minute treatment, thus measurements were performed at 30
197 minutes. Importantly, triclosan alone was not bactericidal to either wild-type or ppGpp0 cells
198 (Fig. S2).
199
200 In contrast to triclosan, pre-treatment with another bacteriostatic compound, spectinomycin,
201 increased tolerance to kanamycin, streptomycin, ampicillin, and ciprofloxacin in both wild-type
202 and ppGpp0 mutant cells (Fig. 3C). A translation inhibitor, spectinomycin does not impact
203 ppGpp levels in *E. coli* (20). While spectinomycin was still protective in the ppGpp0 cells, levels
204 of protection were slightly decreased compared to WT cells.
205
206 **Triclosan drives tolerance to ciprofloxacin in a murine model**
207 Due to its widespread use and inherent stability, triclosan is present in both human populations
208 and the environment (8). Thus, a key question is whether the tolerance we observed *in vitro* is
209 relevant *in vivo*. To determine the physiological relevance of triclosan-mediated tolerance, we
210 employed a mouse model of *E. coli* UTI. UTIs are one of the most prevalent bacterial infections,
211 impacting approximately 150 million people annually (22). Uropathogenic *E. coli* (UPEC) is the
212 main causative agent of both uncomplicated and complicated UTI (23). Pretreatment with
213 triclosan rendered the well-characterized *E. coli* cystitis isolate UTI89 ~10-fold more tolerant to

214 1,000 ng/mL ciprofloxacin than untreated cells at 2 hours, a level equivalent to the tolerance we
215 observed for *E. coli* MG1655 at the same time point (Fig. 1 and Fig. S3).
216
217 For *in vivo* experiments, we provided six-week old female wild-type C3H/HeN mice with
218 drinking water containing 1,000 ng/mL triclosan for 21 days. Control mice were given plain
219 water for the same duration. At 21 days, experimental and control mice were trans-urethrally
220 infected with ~5 x10^7 CFU of *E. coli* UTI89. At 24 hours post-infection, a subset of the mice was
221 treated with intraperitoneal ciprofloxacin (25 mg/kg). At 48 hours post-infection, all mice were
222 sacrificed and bacterial colonization assessed in urine and bladder.
223
224 After ciprofloxacin treatment, bacterial titers were >100-fold higher in the urine ($p<0.0001$) and
225 >10-fold higher ($p<0.0001$) in the bladders of triclosan-treated mice versus control animals (Fig.
226 4a, b), consistent with triclosan-induced tolerance occurring *in vivo*. Bacterial load at 24 hours
227 post-infection was nearly equivalent in triclosan-treated and control mice, indicating that
228 triclosan did not significantly impair UTI89 viability (Fig. 4a, b). Treated mice had triclosan
229 levels between 70-750 ng/mL in their urine, comparable to the MIC for *E. coli* (200 ng/mL) and
230 similar to reported triclosan levels in human urine (2.4 to 3,790 ng/mL) (8) (Fig. 4c). We also
231 detected two putative metabolized forms of triclosan, one with a mass consistent with the
232 previously reported sulfonated triclosan (24), and the other 96 daltons larger (Fig. S4). Whether
233 or not the modified forms of the drug are active against bacteria is unclear. Control mice had no
234 observable triclosan (below 1.6 ng/mL limit of detection).
235
236

237 **Discussion**

238 Our data indicate that environmentally relevant concentrations of triclosan reduce antibiotic
239 efficacy as much a 100-fold *in vivo* (Fig. 4) and highlight an unexpected and potentially
240 important role for triclosan as a contributor to antibiotic tolerance and bacterial persistence in
241 both community and healthcare settings. Triclosan-mediated tolerance is dependent on ppGpp
242 synthesis, most likely in response to inhibition of fatty acid synthesis (Fig. 3a) (15). This finding
243 is consistent with prior work implicating ppGpp in antibiotic tolerance and persister development
244 (16).

245

246 In contrast to previous studies of ppGpp-induced persistence that relied on either carbon
247 starvation or the addition of serine hydroxamate to induce accumulation of high concentrations
248 of ppGpp (100x above baseline) (25, 26), defects in fatty acid synthesis have at best a modest
249 impact on ppGpp levels (~5x over baseline) (15). This suggests that even relatively low levels of
250 ppGpp are sufficient to protect cells from a panel of antimicrobials. Specifically how modest
251 increases in ppGpp might confer tolerance to different antibiotics thus remains an open question.

252

253 We favor the hypothesis that ppGpp mediates changes in individual biosynthetic pathways that
254 render them tolerant of their cognate antimicrobial. For example, ppGpp-dependent down-
255 regulation of ribosomal RNA synthesis significantly curtails translation (27), potentially
256 conferring tolerance to the translational inhibitors kanamycin and streptomycin. Similarly,
257 increases in ppGpp are reported to curtail DNA replication (28)—both elongation and
258 initiation—providing a straightforward explanation for ppGpp-mediated ciprofloxacin resistance.

259

260 It is generally recognized as poor practice to prescribe a bacteriostatic compound prior to or
261 along with delivery of a bactericidal one (29), because of the potential that the former will
262 interfere with the activity of the latter. At the same time, their mechanisms of action, and
263 therefore the mechanisms by which these drugs drive tolerance, are likely to differ widely. While
264 the translation inhibitor spectinomycin provides protection against bactericidal compounds, it
265 does not induce accumulation of ppGpp (20), a fact supported by our finding that spectinomycin
266 induces tolerance to bactericidal compounds in both wild-type and ppGpp0 cells (Fig. 3c). At the
267 same time, ppGpp-dependent induction of antibiotic tolerance is likely to be a feature triclosan
268 shares with a related compound, triclocarban, which also inhibits an early step in fatty acid
269 synthesis, and is also a common additive in consumer products. Triclosan also stands out from
270 other bacteriostatic compounds by virtue of its widespread use and sheer abundance in the
271 environment. ~1 kg of Triclosan is produced for every 3 kg of other antimicrobials and estimates
272 indicate that ~100 metric tonnes are being deposited annually in the environment through waste
273 water treatment in the US alone (30).
274
275 Although triclosan has low toxicity (LD-50 4,350 mg/kg orally) (31), accumulating data link
276 long-term exposure with antibiotic resistance (32) and there are reports that triclosan may also
277 function as an endocrine disrupter (33, 34). Our analysis of the impact of triclosan on antibiotic
278 efficacy in a mouse UTI model (Fig. 4) highlights yet another deleterious "side effect" of this
279 ubiquitous antimicrobial. UTIs alone impact 150 million people worldwide (22) at a cost of $3.5
280 billion per year in the US alone (35). Complications associated with UTIs include pyelonephritis
281 with sepsis, renal damage, pre-term birth, *Clostridium difficile* colitis, sepsis, and death
282 particularly in the very old and the very young (23). Coupled with the well established

283 connection between antibiotic tolerance and recurrent/chronic infections (12, 13), our findings
284 reinforce the need for substantial caution—as well as consideration of unintended
285 consequences—in evaluating the costs and benefits of antimicrobial additives in consumer
286 products.
287
288 **Methods**
289 **Materials and Strains**
290 Triclosan, ampicillin, kanamycin, streptomycin, ciprofloxacin, and vancomycin were purchased
291 from Sigma-Aldrich. Stock solutions were made in water for ampicillin (100 mg/mL),
292 kanamycin (50 mg/mL), streptomycin (100 mg/mL) and ciprofloxacin (10 mg/mL). Triclosan
293 was dissolved in ethanol (10 mg/mL), and vancomycin was dissolved in DMSO (100 mg/mL). *E.*
294 *coli* MG1655 and *S. aureus* FPR3575 were both lab strains and *E. coli* UTI89 was isolated from
295 a patient with a urinary tract infection (36). *E. coli* were grown in Luria-Bertani broth (LB) and
296 *S. aureus* was grown in tryptic soy broth (TSB). Growth temperature was 37 °C for all
297 experiments.
298
299 **Determination of Minimum Inhibitory Concentration (MIC)**
300 To determine the MIC for the panel of antibiotics utilized in this study, *E. coli* and *S. aureus*
301 were grown to OD-600 = 0.1 in LB or TSB, respectively. Cells were then back-diluted 1,000-
302 fold and transferred to a 96-well plate containing 2-fold dilutions of respective antibiotics and
303 cultured at 37 °C for 16 additional hours with vigorous shaking in a BioTek Eon plate reader.
304 MIC was calculated as the lowest antibiotic concentration, preventing development of detectable
305 turbidity at OD-600.

306 **Assays for antibiotic tolerance and persistence**

307 To assay tolerance and persistence, *E. coli* and *S. aureus* were grown to an OD-600 = 0.2 in LB
308 or TSB, respectively. Cells were then back-diluted to an OD-600 = 0.1 in media containing
309 triclosan at indicated concentrations and cultured for an additional 30 minutes, before being
310 challenged with bactericidal antibiotics. For dot plating, 10 µL of a 10-fold dilution series was
311 plated on antibiotic-free LB agar or TSB agar as appropriate. For determination of colony
312 forming units (CFU), 100 µL of a 10-fold dilution series was spread on antibiotic-free LB agar or
313 TSB agar plates. Cells were incubated for ~12 hours at 37 °C prior to quantification. CFUs were
314 normalized to CFUs at the initial time point to correct for the ~2-fold increase in cell number in
315 untreated cultures during the 30-minute pre-treatment period. Relative persistence is defined as
316 the CFU's of the triclosan treated sample divided by the CFU's of the non-treated sample.

317

318 **UTI mouse work**

319 Six-week old female wild-type C3H/HeN mice were obtained from Envigo. Mice were treated
320 with or without 1,000 ng/mL (100 ppm) triclosan in the drinking water for 21 days. At 21 days,
321 mice were anesthetized by inhalation of 4% isoflurane and mouse bladders were trans-urethrally
322 infected with approximately 5 x10^7 CFU of *E. coli* UTI89 in 50µl PBS(37). Briefly, a single
323 UTI89 colony was inoculated in 20 ml of Luria Broth (LB) and incubated at 37 °C under static
324 conditions for 24 hours. Bacteria were then diluted (1:1,000) into fresh LB and incubated at 37
325 °C under static conditions for 18 to 24 hours. Bacteria were subsequently washed three times
326 with PBS and then concentrated to approximately 5 x10^7 CFU per 50 µL. At 24 hours post-
327 infection, mice received 25mg/kg of ciprofloxacin intraperitoneal. 48 hours post-infection, mice
328 were euthanized, and bladders were harvested and urine was collected. Bladders were

329 homogenized in PBS and bacterial load present in bladders and urines was determined by plating
330 serial dilutions on LB agar supplemented with antibiotics when appropriate. Statistical analyses
331 were performed using the Mann–Whitney U test with GraphPad Prism software (version 6.0 for
332 Mac). All animal studies were performed in accordance with the guidelines of the Committee for
333 Animal Studies at Washington University School of Medicine.
334
335 **Measurement of triclosan and metabolites in mouse urine**
336 Since triclosan has been observed to adsorb to plastic surfaces, sample handling was performed
337 in glass vessels whenever possible (24). A stock solution of 1 mg/mL triclosan (Sigma) was
338 prepared in methanol and a 100 μg/mL $^{13}C_{12}$-triclosan (99%) internal standard in MTBE was
339 purchased from Cambridge Isotope Laboratories (Andover, MA). A dilution series of 1,000, 200,
340 40, 8, 1.6, and 0.32 ng/mL triclosan was prepared in pooled, untreated mouse urine and spiked
341 with 100 ng/mL $^{13}C_{12}$-triclosan internal standard. Samples were diluted 1:1 in methanol, spun
342 down at 20,000×*g* for 10 minutes, and filtered through 0.45 μm 13 mm diameter PVDF syringe
343 filters (Millipore). Finally, cleaned samples were diluted 1:1 in HPLC-grade water (Sigma).
344
345 Using a Shimadzu UFLC (Kyoto, Japan), 10 μL of each sample was injected onto a fused core
346 phenyl-hexyl column (100 mm × 2 mm × 2.7 μm) with a 0.4 mL/min flow rate (Ascentis
347 Express, Supelco). Triclosan was eluted from the column as follows: Solvent A (0.1% formic
348 acid) and Solvent B (90% acetonitrile with 0.1% formic acid) were held constant at 80% and
349 20%, respectively, for 0.1 minutes. Solvent B was increased to 98% by 5 minutes, held at 98%
350 for 1 minutes, and then reduced again to 20% in 0.1 minutes. The column was equilibrated in
351 20% Solvent B for 3 minutes between runs.

352 Triclosan was detected using an AB Sciex API 4000 QTrap mass spectrometer (AB Sciex, Foster
353 City, CA) running in negative ion electrospray ionization mode (ESI) using a Turbo V ESI ion
354 source. Triclosan was detected using the instrument settings listed in Supplementary Data Table
355 1. A precursor ion scan was performed for the 35 m/z product ion to determine the mass
356 spectrum of triclosan, $^{13}C_{12}$-triclosan, and any potential metabolites (Fig. S4a). Because triclosan
357 contains three chlorine atoms, its mass spectrum includes prominent isotope peaks (M+2, M+4)
358 corresponding to the natural abundance of ^{37}Cl (Fig. S4b). To improve sensitivity, product ions
359 from the two most abundant isotopologues were detected and added together prior to peak
360 integration. Peaks for triclosan and internal standard were integrated with Analyst software (AB
361 Sciex) and normalized. Normalized peak areas varied linearly with triclosan concentration above
362 1.6 ng/mL.
363
364 Pooled urines from 3 to 4 mice were spiked with 100 ng/mL internal standard and cleaned as
365 described above. Samples were analyzed by LC-MS/MS and triclosan was quantified using the
366 standard curve (Fig. S4e).
367
368 **Statistical analysis**
369 Values for the *in vitro* data are expressed as the mean ± standard error of the mean from n=3
370 replicates. *In vitro* data was analyzed using a two-tailed Student's t-test with statistical
371 significance determined when $p<.05$. For the mouse data, the Mann-Whitney U test was used to
372 test for statistical significance. Values represent means ± SEM derived from at least 3
373 independent experiments. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$; ****, $P<0.00005$; ns,
374 difference not significant.

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382

383 **Author Contributions**

384 CSW, PAL, ALFM, SJH, JIR and JPH designed the research studies. CSW performed the *in*
385 *vitro* assays. ALFM and AJLL performed the animal experiments and acquired data. JIR and
386 JPH performed triclosan detection and quantification experiments and acquired data. CSW
387 ALFM, JIR, and PAL analyzed data. CSW, ALFM, and JIR prepared the figures. CSW, PAL,
388 and ALFM wrote the manuscript. CSW, PAL, ALFM, SJH, JIR and JPH reviewed and edited the
389 manuscript.

390
391

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498 **Figures**

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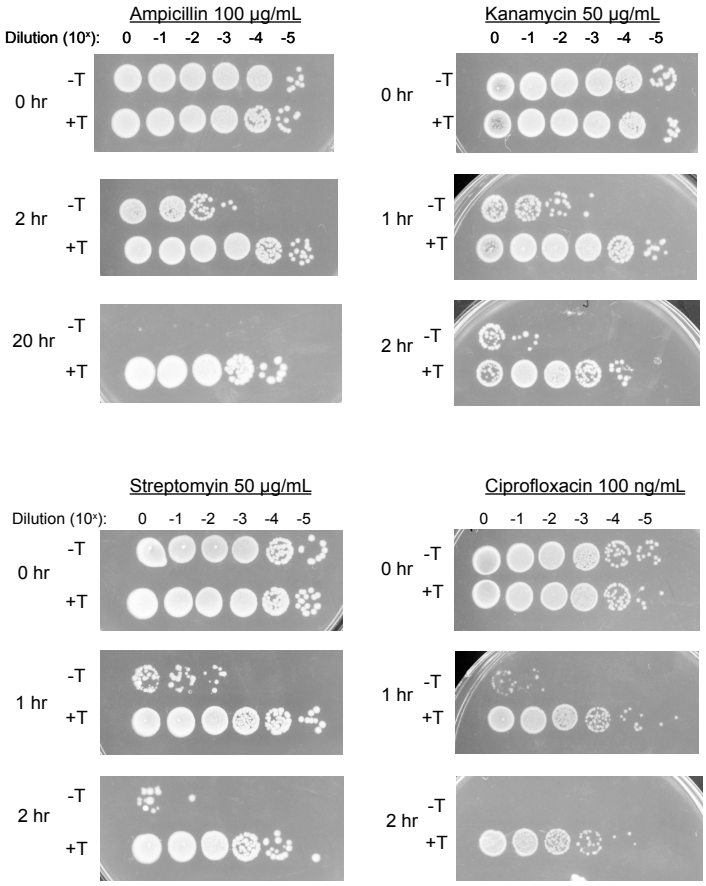

500

501 **Figure 1: Triclosan induces tolerance to multiple antibiotics.** *E. coli* (MG1655) were cultured

502 to OD600 = 0.2, split and cultured for an additional 30 minutes with (+T) or without 200 ng/ml

503 triclosan (-T). Indicated bactericidal antibiotics were then added and cells cultured for an

504 additional 2 to 20 hours prior to dilution plating. Each experiment was replicated three

505 independent times with only representative data shown.

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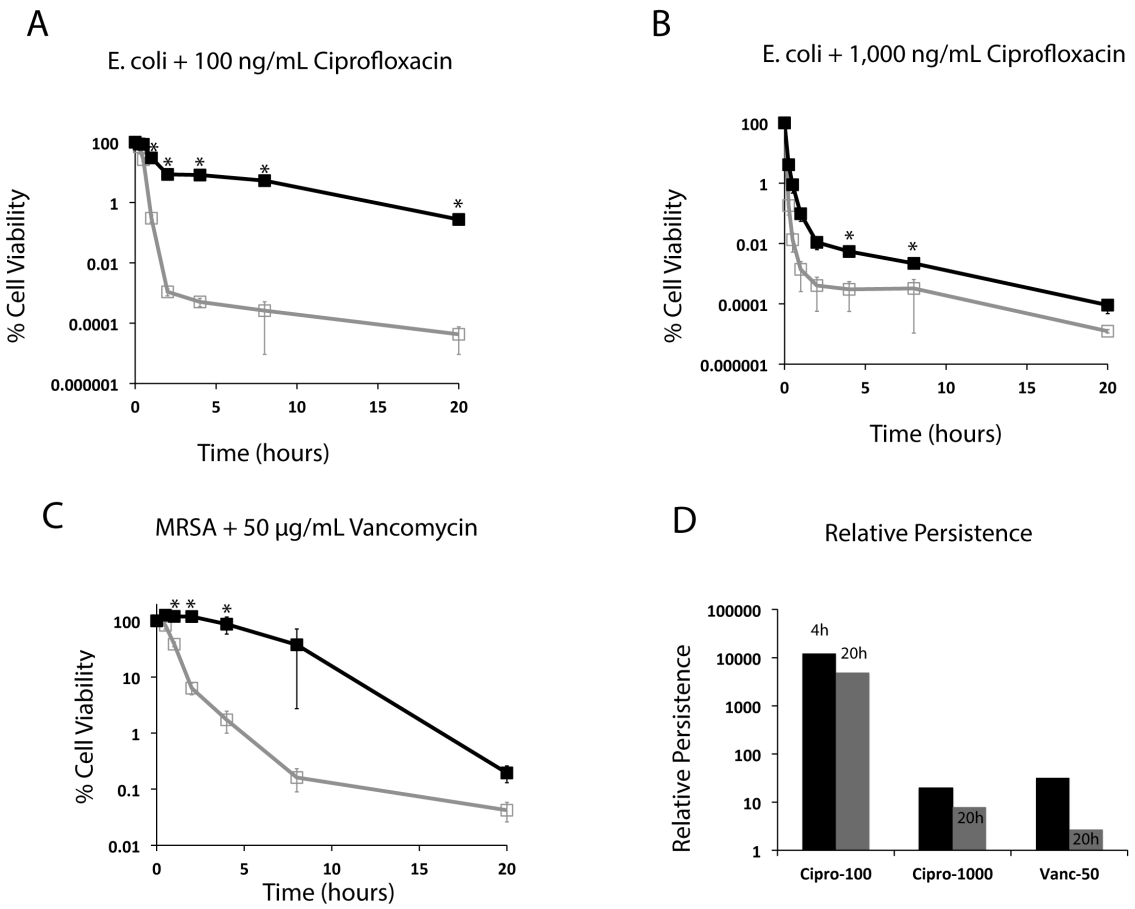

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510 **Figure 2: Kinetic analysis of triclosan-induced persistence.** *E. coli* (MG1655) and MRSA

511 (FPR3757) cells were cultured to OD-600 = 0.2, split and cultured for an additional 30 minutes

512 with (black line, closed squares) or without triclosan (grey line, open squares). At t=0, 100

513 ng/mL (A) or 1,000 ng/mL ciprofloxacin (B) was added to *E. coli* cultures and 50 ng/ml

514 vancomycin was added to MRSA cultures (C). Relative persistence in the presence of triclosan

515 (CFU+T/CFU-T) was calculated from the 4- and 20-hour time points (D). Values are the mean of

516 three independent biological replicates with error bars representing the standard error of the

517 mean. Asterisks represent significant difference between the triclosan treated and non-treated

518 using a Student's two-tailed t-test with * = $p < 0.05$.

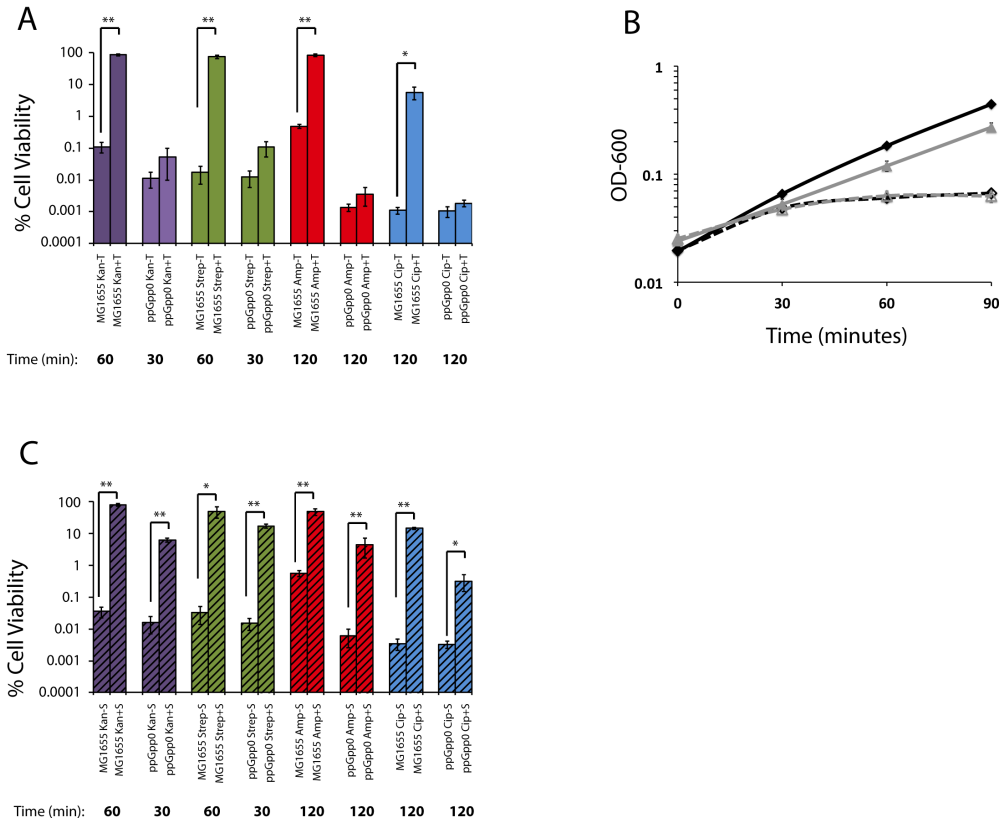

Figure 3. ppGpp is needed for triclosan induced tolerance. Cell viability of MG1655 and ppGpp0 *E. coli* with (+T) or without (-T) pretreatment with triclosan after challenge with antibiotic (A). Growth curves of MG1655 (black curve) or ppGpp^0 (gray curve) in LB with (dashed lines) or without (solid lines) triclosan (B). Cell viability of MG1655 and ppGpp0 *E. coli* with (+T) or without (-T) pretreatment with spectinomycin after challenge with antibiotic (C). Values are the mean of three independent biological replicates with error bars representing the

526 standard error of the mean. Asterisks represent significant difference between the triclosan

527 treated and non-treated using a Student's two-tailed t-test with * = $p < 0.05$ and ** = $p < .001$.

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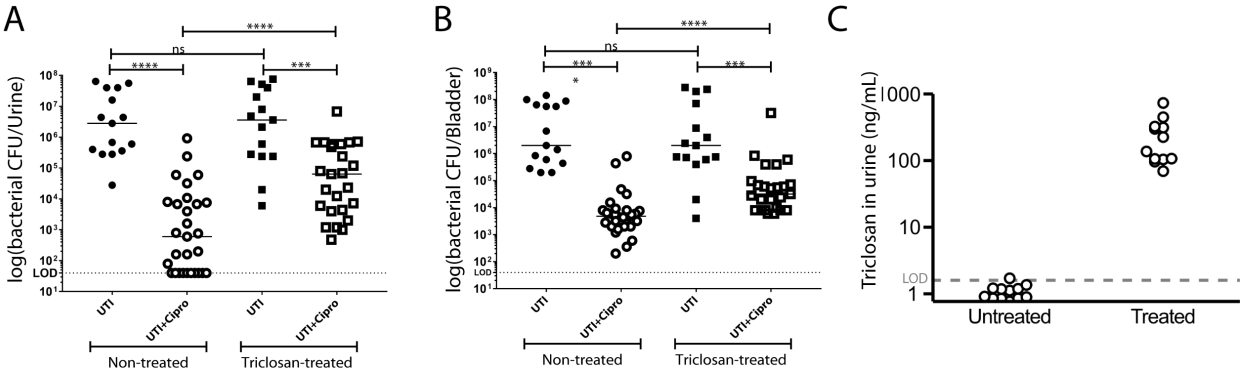
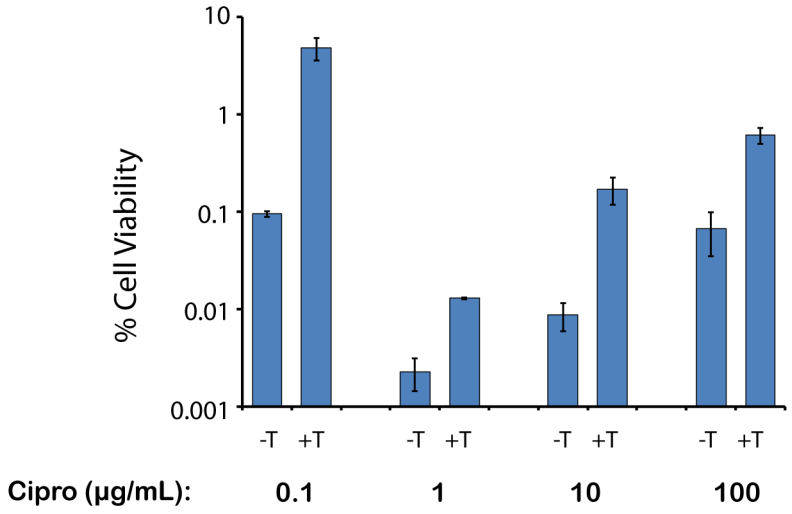


Figure 4: Triclosan reduces ciprofloxacin efficacy up to 100-fold in a mouse UTI model. For each round of the experiment, 15 mice were given water containing triclosan (1,000 ng/mL) for 21 days, and 15 control mice received plain water. At 21 days, all mice were infected with *E. coli* UTI89 (~5 x10^7 CFU). 24 hours post-infection, 10 mice from each group were given 25 mg/kg of ciprofloxacin intraperitoneally. 48 hours post infection, urine (A) was taken and mice were sacrificed and bladders (B) were harvested. To compare the mice groups, Mann-Whitney U test was used, $p<0.05$ was considered statistically significant: *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$; ns, values were not statistically different. The horizontal bar represents the median value. The horizontal broken line represents the limit of detection (LOD) of viable bacteria. Data are from 3 independent experiments. (C) Free triclosan levels measured by LC-MS/MS of triclosan in untreated and treated mouse urine. (45 mice were untreated and 45 mice were treated, these mice were divided in three independent experiments. Urine from 3-4 mice was pooled for triclosan analysis). The horizontal broken line represents the limit of detection (LOD) of triclosan.

565 **Supplementary Data**



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568 **Supplementary Data Figure 1. Triclosan protects at high concentrations of ciprofloxacin.**

569 MG1655 cells were grown to OD-600 = 0.1 before triclosan was added for a final concentration

570 of 200 ng/mL for 30 minutes. Ciprofloxacin was added at the labeled concentration.

571 Ciprofloxacin was washed off, and cell viability was determined. Values are shown as averages

572 of three replicates with error bars showing the standard error of the mean.

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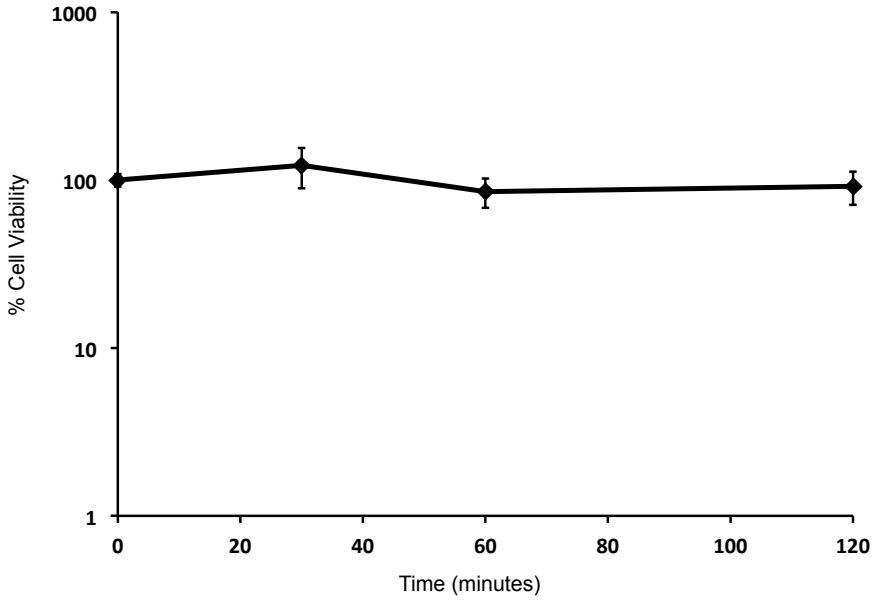

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582 **Supplementary Data Figure 2. Triclosan is not bactericidal to the ppGpp0 cells.** ppGpp0
583 cells were grown to OD-600 = 0.2 before triclosan was added for a final concentration of 200
584 ng/mL. Cells were plated at each time pointed and colony-forming units were quantified. Each
585 point represents the average of three biological replicates with the error bars representing the
586 standard error of the mean.

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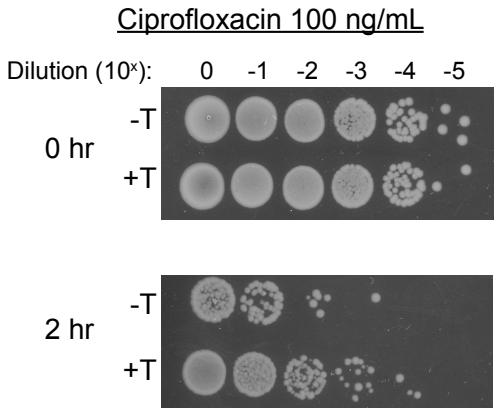

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596 **Supplementary Data Figure 3. Triclosan induces ciprofloxacin tolerance in the**

597 **uropathogentic *E. coli* UTI89.** UTI89 cells were grown up to OD-600 = 0.2, split and cultured

598 for an additional 30 minutes with (+T) or without 200ng/ml triclosan (-T). Ciprofloxacin was

599 added to obtain a final concentration of 100 ng/mL. Cells were dot-plated at the 0- and 2-hour

600 time points. The plating efficiency was repeated three independent times with a representative

601 image shown.

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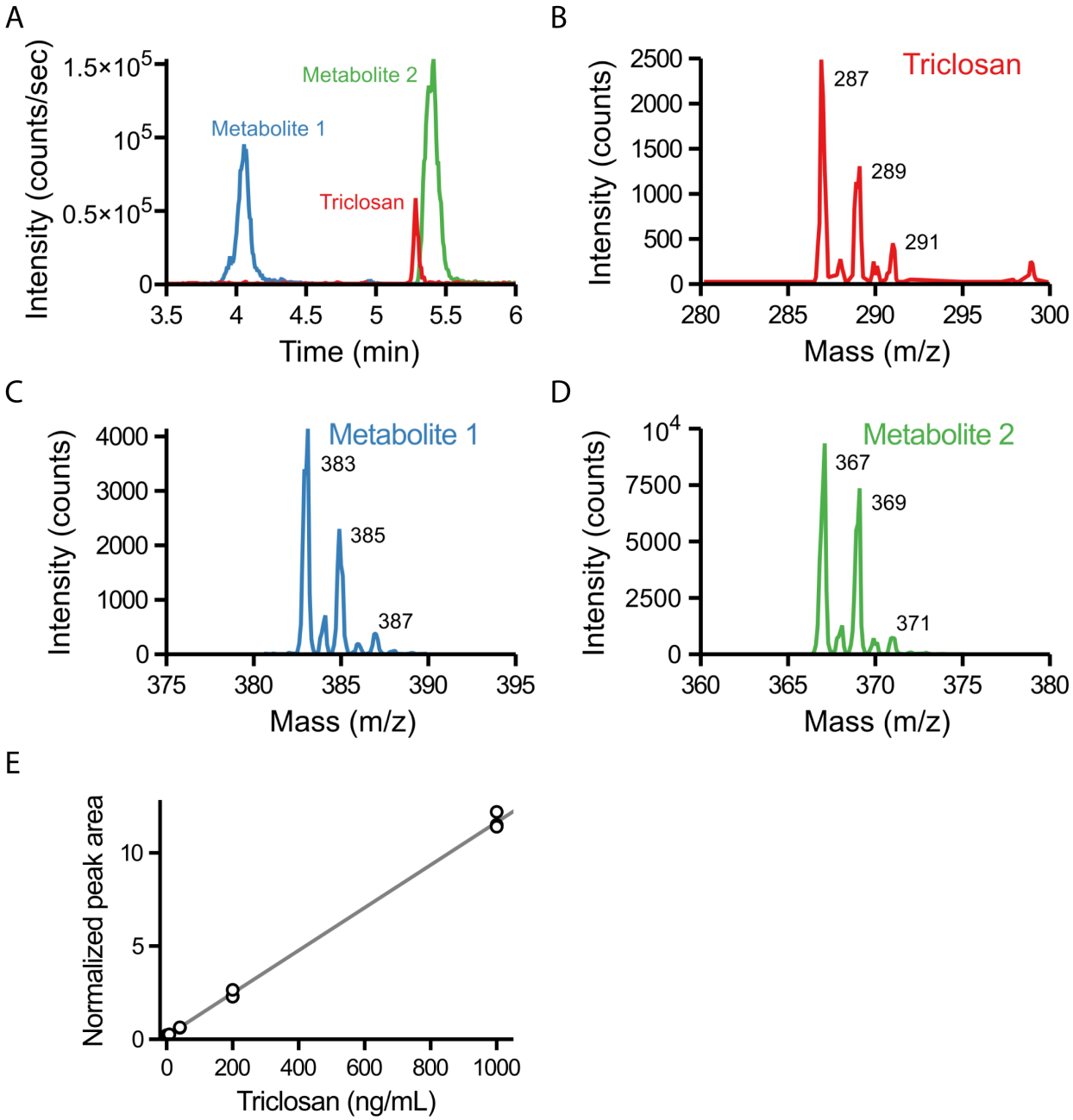

Supplementary Data Figure 4. Measurement of free and metabolized triclosan in triclosan-treated mouse urine. Chromatogram of a representative LC-MS/MS experiment showing the elution profile of metabolite 1 (blue), triclosan (red) and metabolite 2 (green) (A). Mass spectra of triclosan, metabolite 1, and metabolite 2, respectively, measured using precursor ion scans for

616 the chloride product ion (35 m/z) (B, C, and D). Triclosan peak area detected by LC-MS/MS
617 varies linearly with concentration down to 1.6 ng/mL (E).
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Instrument Settings		
Ion Spray Voltage	-4.5 kV	
Heater Temperature	500 °C	
Nebulizer Gas	40	
Auxiliary Gas	40	
Declustering Potential	-10 V	
Collision Energy	-40 V	
Ions Detected (m/z)		
Triclosan	286.8 → 35	288.7 → 35
$^{13}C_{12}$-Triclosan	298.84 → 35	300.74 → 35
Metabolite 1	382.8 → 35	384.8 → 35
Metabolite 2	366.7 → 35	368.8 → 35

Supplementary Data Table 1. MS/MS settings for triclosan detection