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Short communication

Mathematical modeling to predict the fitness cost associated with triclosan tolerance in *Salmonella enterica* serovars

Ulrike Rensch [a], Matthias Greiner [a, b], Günter Klein [a], Corinna Kehrenberg [a, *]

[a] Institute for Food Quality and Food Safety, University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, D-30173 Hannover, Germany
[b] Federal Institute for Risk Assessment (BfR), Department of Exposure, Max-Dohrn-Str 8-10, D-10589 Berlin, Germany

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ABSTRACT

Triclosan is a broad spectrum biocide that is incorporated in a wide variety of products for food industry, medical applications and domestic use to reduce microbial load and to improve hygiene. Despite the extended use of the biocide, bacterial tolerance to triclosan is still infrequent. Here, we support the hypothesis that the acquisition of a triclosan tolerance-mediating mutation in the target site for triclosan, the gene *fabI*, is associated with a fitness cost in *Salmonella enterica*. Growth competition experiments with mutants exhibiting the Gly_{93}-Val mutation in *fabI* and their wild-type strains revealed overall decreases in the relative proportions of mutants. For all four serovars investigated, the $\log_{10}$ difference values increased linearly over the experimental time of 96 h. Therefore, a linear regression model was used to characterize the changes in log ratios of wild-type strains and mutants over time. The calculated regression coefficients allowed an estimation of the fitness cost of triclosan tolerance in *Salmonella* mutants in comparison to their wild-type strains.

1. Introduction

Biocides play an important role in preventing bacterial disease. They are used for many practical applications such as cleaning or disinfecting to reduce microbial load and to improve hygiene (Sheridan, Lenahan, Duffy, Fanning, & Burgess, 2012). The broad-spectrum biocide triclosan is a chlorinated biphenyl ether, that has been used as active ingredient in antiseptics, preservatives and disinfectants for at least the past four decades (Condell et al., 2012). It is incorporated in a wide variety of products for food industry, medical applications and domestic use (Condell et al., 2012; Jones, Jampani, Newman, & Lee, 2000; Schweizer, 2001). Triclosan has a major target site of activity at subinhibitory concentrations, the bacterial enoyl acyl carrier protein reductase FabI (Heath et al., 1999; Lubarsky et al., 2012). At higher concentrations, triclosan may also impact cell membrane structure and functionality (Saleh, Haddadin, Baillie, & Collier, 2011). In *Salmonella enterica*, a phenotype tolerant to triclosan activity is reported to be the consequence of a single point mutation in *fabI* (leading to a Gly_{93}-Val amino acid exchange), although overexpression of FabI or enhanced efflux via multidrug transporters contribute to triclosan tolerance as well (Rensch, Klein, & Kehrenberg, 2013; Rensch, Nishino, Klein, & Kehrenberg, 2014; Webber, Randall, Cooles, Woodward, & Piddock, 2008). However, the de-repression of efflux systems such as AcrAB-TolC may provide cross-resistance to antibiotics and is therefore a matter of concern. In the laboratory setting, *S. enterica* mutants less susceptible to triclosan can be easily selected by incubating overnight cultures (Birošová & Mikulášová, 2009; Rensch et al., 2013) or by subsequently transferring in culture media with increasing triclosan concentrations (Braoudaki & Hilton, 2004; Karatzas et al., 2007). However, only few studies have reported the occurrence of *Salmonella* isolates from clinical, household or community settings exhibiting tolerance to elevated minimum inhibitory concentrations (MICs) of triclosan (Braoudaki & Hilton, 2005; Copitch, Whitehead, & Webber, 2010). This rare occurrence in the environment might be explained by reduced growth rates of triclosan tolerant *Salmonella* mutants in comparison to the wild-type strains, as has been shown by generation-time determinations in liquid media (Karatzas et al., 2007; Rensch et al., 2013). In addition, it has been demonstrated that triclosan selected *Salmonella* mutants are more susceptible to the aminoglycoside antibiotics kanamycin and gentamicin and to the biocide chlorhexidine (Rensch et al., 2013). Nevertheless, the assessment of the relative fitness of *S.* Typhimurium wild-type and mutant strains (inoculated in a 1:1 ratio) *in vivo* by using day-old chicks that were

* Corresponding author. Tel.: +49 511 856 7554; fax: +49 511 856 7694.
E-mail address: corinna.kehrenberg@tiho-hannover.de (C. Kehrenberg).

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inoculated with 10^4 CFU via oral gavage revealed that all triclosan tolerant mutant strains were able to colonize chicks and to persist in the avian gut during an observation time of 28 days (Webber et al., 2008). Although the results of this study indicated the successful establishment of a stable population of triclosan tolerant *Salmonella* Typhimurium in chickens, the relative numbers of mutants were lower compared to the wild-type strains at the end of the experiments (Webber et al., 2008). As the findings *in vitro* and *in vivo* could not explain the rare occurrence of triclosan tolerant field isolates, it was aimed at examining the fitness of triclosan-tolerant *Salmonella* mutants in direct growth competition experiments and to predict the fitness cost of triclosan tolerance (i.e. ability of susceptible bacteria to outcompete triclosan tolerant bacteria) for the first time by using a mathematical model.

2. Material and methods

Four *S. enterica* field isolates belonging to the serovars Enteritidis, Saintpaul, Livingstone, and Paratyphi B were used as wild-type strains and were included in the study together with their isogenic triclosan tolerant mutants. The origins of the strains and their characteristics have been described previously (Rensch et al., 2013). In brief, all mutants were selected during the determination of triclosan mutant prevention concentrations and exhibited high MIC values of triclosan (32 to ≥64 μg/ml) compared to their wild-type strains (MICs of 0.125–0.5 μg/ml). Sequence analysis detected a base pair exchange (GGT → GTT) in the *fabI* genes of all mutants that resulted in an amino acid exchange at codon 93 (Rensch et al., 2013). The level of expression of genes encoding efflux pump components or regulators indicated that neither the efflux pump AcrAB-TolC nor AcrEF were up-regulated in the mutant strains.

Growth competition experiments were performed according to Guo, Abdelraouf, Ledesma, Nikolaou, and Tam (2012). For this, the strains were cultured overnight in Luria Bertani (LB) broth and diluted in a 10-fold dilution series to a concentration of 5×10^6 CFU/ml (wild-type and mutant strains, respectively). For the co-cultivation of strains starting both at approximately 5×10^4 CFU/ml, a volume of 200 μl of the diluted cultures were transferred in a flask containing 19.6 ml of fresh 37 °C pre-warmed LB-broth. The cultures were incubated at 37 °C with shaking at 130 rpm for 12 h to complete one competition cycle. At the end of every 12 h cycle, 100 μl of the mixed bacterial culture were diluted 1:100 with a 0.9% saline solution and 200 μl of the dilution were transferred into a new competition flask containing 19.8 ml pre-warmed LB broth. The CFU/ml of both the wild-type strain and the co-cultivated mutant were determined every 24 h by culture enumeration using Mueller-Hinton (MH) agar plates and, in parallel, MH agar plates supplemented with 2 μg/ml and 8 μg/ml triclosan, respectively. The agar plates were incubated 24 h at 37 °C. Differences between total counts of salmonellae determined on MH agar plates (growth of wild-type strains and mutants) and on MH agar plates supplemented with triclosan (only growth of mutant strains) were regarded as the amounts of triclosan susceptible wild-type strains.

In addition, *S.* Enteritidis wild-type and mutant strains were cultured in LB broth at pH 6 and pH 8, in brilliant green bile lactose broth and MH broth supplemented with 2% (v/v) lysed horse blood, and in LB broth using incubation temperatures of 25 °C and 41 °C to investigate how variations in pH, culture media components and incubation temperature affect the growth ability of mutants.

2.1. Data processing and modeling approach

The data used for statistical analysis comprise of the differences between $\log_{10}$ concentrations of the wild type ($\log_{10}C_W$) and mutant type ($\log_{10}C_M$), referred to as the $\log_{10}$ difference (*LD*), for incubation times of 0, 24, 48, 72 and 96 h, respectively. The regression models used three available observations of LD values for each incubation time (e.g., 15 observations for all serovars were available). The log difference is equivalent to the logarithm of the ratios of the respective count values ($LD = \log_{10}C_w - \log_{10}C_M = \log_{10}(C_w/C_M)$). Three independent growth competition experiments were conducted for 96 h under identical experimental conditions. The time-dependent competitive growth profiles were modeled using a simple linear regression model with intercept fixed as zero, fitted to the observed *LD* results for each serovar using time as covariate. The estimated regression parameter (denoted as K_C) can be interpreted as difference between the growth rate constant of the wild-type strain minus the growth rate constant of the isogenic mutant with an intercept term fixed at zero (Abdelraouf, Kabbara, Ledesma, Poole, & Tam, 2011; Guo et al., 2012). For each of the four serovars, a 95% uncertainty interval for the estimated regression parameter K_c was obtained by adding to the estimate (−2,2)-times its standard error (see Figs. 1–4). 95% uncertainty intervals for the predicted time-dependent mean *LD* (denoted as "confidence intervals") as well as for the predicted individual observations of *LD* for each serovar (denoted as "prediction intervals") were established (R command "predict" using the interval argument set to "confidence" or "prediction", respectively; R Core Team, 2013). The differentiation between the two types of uncertainty intervals allows to assess the biological variation of the mean LD due to the incubation time ("confidence interval") and the wider variation of individual LD values of co-cultured and mutant strains for replicate trials ("prediction interval"). Both types of uncertainty intervals were shown graphically (Figs. 1–4). The regression model results and graphics were obtained using the R package (R Core Team, 2013).

3. Results and discussion

Results from the *in vitro* growth competition experiments between the wild-type strains and their isogenic mutants revealed significant overall decreases in the relative proportions of mutant strains of all serovars. The differences in the concentrations were approximately 4.4×10^5–5.1×10^7 CFU/ml after 96 h, with the exception of *S.* Paratyphi B. For this serovar, a smaller difference of 2.7×10^3 CFU/ml between wild-type and mutant strain was observed. In a previous study in which growth kinetics of triclosan tolerant *Salmonella* mutants were investigated without competition, it could be shown that the growth rates of *S.* Paratyphi B were more similar than in the other serovars (Rensch et al., 2013). Nevertheless, an investigation including a larger number of *S.* Paratyphi B isolates would be necessary to examine whether this is a serovar-specific property. As shown for *S.* Enteritidis, variations in incubation temperature, pH or culture media components only had limited effect on the decrease in the proportion of the mutant when it was cultured in competition with the wild-type strain. Mutants were isolated in lower numbers under all conditions tested (Supplementary data, Figs. S1–S6). However, the results from all serovars suggested that the triclosan tolerance-mediating mutation Gly_{93} → Val in *fabI* is associated with a substantial fitness cost. According to Andersson and Hughes (2011), a reduction in the ability of a pathogen to reproduce and spread in the host population is regarded as a fitness cost. This is typically observed as a reduced bacterial growth rate and mutants with a high fitness cost are expected to be outcompeted and to disappear in a bacterial population (Andersson & Hughes, 2011). Hence, these findings support the hypothesis that the mechanism that confers triclosan tolerance limits the growth of tolerant strains in the absence of selection pressure. Similar observations have been made for