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The Effect of Natural Selection on the Resistance of *Escherichia coli* to Triclosan

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ABSTRACT

Triclosan is an antibacterial agent widely distributed as an ingredient in many consumer products used for skin care, oral care, deodorant formulations, surgical scrubs, and household cleansers. Studies have shown the mechanism of triclosan to be specific, making cellular resistance possible. Resistance to low levels of triclosan in *Escherichia coli (E. coli)* has been shown to be acquired through a missense mutation in the FabI gene. The purpose of this study was to determine if natural selection affects the resistance of *E. coli* to low levels of triclosan. An experimental group was exposed to a sub lethal concentration of triclosan that inhibited some bacterial growth but not all. Both the control groups and the experimental groups were acted upon by natural selection through serial transfers of the largest, fastest growing colonies. The final bacterial strains were tested for differences in zones of inhibition using the Kirby-Bauer method at two different triclosan concentrations. No evidence of resistance to a triclosan concentration of [0.097] g/ml was found in this experimental strain of *E. coli*. The possibility of resistance gained to a lower concentration of triclosan, [0.0316] g/ml, was found in the experimental strain of *E. coli*.

Keywords: *antibacterial, enoyl-acyl carrier protein reductase, Escherichia coli, triclosan, resistance.*

INTRODUCTION

Escherichia coli, more commonly referred to as *E. coli,* is a gram-negative bacteria that has been known to cause three main infections prone to humans: urinary tract infections, neonatal meningitis, and intestinal diseases. *E. coli* occur abundantly as normal flora in the lower part of the intestines of warm-blooded animals, including humans (Madigan *et al.*, 2000). This study was performed on *E. coli* because it has been used in related research projects dealing with triclosan resistance and is often used as a "model" organism for research.

Antibiotic resistance of any bacteria is acquired through the bacteria's ability to exchange genes and obtain new traits via mutation, plasmids, and genetic recombination. Bacteria multiply rapidly allowing mutations or genes beneficial for survival to spread quickly as nature selects for those traits more beneficial for survival (Raven and Johnson, 1999). Over time, surviving resistant bacteria can transmit their resistance gained by passing genetic material to both their progeny and neighboring bacteria. Today, as antibacterial agents are becoming more widely used, there are a greater number of bacteria that evolve in ways to avoid drugs' lethality, creating a critical health dilemma (Raloff, 2000).

Triclosan (2, 4, 4'-trichloro-2'-hydroxydiphenyl ether) is a chemical widely used and accepted internationally as an antibacterial cleansing agent. Originally developed in Switzerland in the 1960's, this chemical has been used for about thirty years and was first introduced as a surgical scrub in 1972 (Jones *et al.*, 2000). Triclosan's use has soared since its introduction; it is now an ingredient in many consumer products used for skin care, oral care, deodorant formulations, and household cleansers. Initially, triclosan was thought of as an antiseptic biocide, in which there are multiple mechanisms of cell toxicity. This makes cellular resistance unlikely (Heath *et al.*, 1999). Recent contrasting studies showed the mechanism of triclosan to be specific, classifying it instead as an antibiotic. McMurry *et al.* (1998) showed that triclosan acts by inhibiting the enzyme enoyl-ACP reductase, needed in bacterial lipid biosynthesis. Fatty acids are formed through four basic reactions of elongation as distinct genes encode each of the individual enzymes need for each step of elongation. The last reaction in each fatty-acid elongation cycle of *E. coli* is catalyzed by enoyl-ACP reductase (Heath *et al.*, 1999). FabI is the gene that encodes enoyl reductase. It has been shown in *E. coli* that triclosan inhibits lipid synthesis and that either over-expression of the FabI gene or missense mutations within the gene prevent the inhibition of lipid synthesis (McMurry *et al.*, 1998).

The ability of *E. coli* to acquire resistance to triclosan through mutations on the FabI gene suggests that the widespread use of triclosan could lead to resistant organisms. This would compromise the usefulness of triclosan and other antibacterials that involve the same cellular target (Heath *et al.*, 1999).

The purpose of this study is to determine whether or not low levels of triclosan are capable of producing resistant strains of *E. coli* through natural selection. This study will conduct serial transfers of the largest, fastest growing colonies of *E. coli* subject to a constant triclosan concentration, analogous to an everyday situation such as a cutting board becoming impregnated with a household cleanser, slowly releasing triclosan. This could expose nearby bacteria to sub lethal concentrations of triclosan, which is a

situation that could promote the development of resistance.

MATERIALS AND METHODS

The maximum concentration of triclosan that still allowed some bacterial growth was determined using 10-fold dilutions ([0.097], [0.0097], and [0.00097] g/ml) of the (97%) triclosan dissolved in methanol. The triclosan used throughout the experiment was purchased from Aldrich. Equal aliquots (0.25 ml) of each dilution were pipetted onto tryptic soy agar plates and the methanol was allowed to vaporize. The spread plate method was used on the plates by adding 0.3 ml of *E. coli* and then incubating the plates for 24 hours at 37 degrees centigrade and 53 RPM (continuous rotation.) Standard incubation of *E. coli* used throughout the experiment was 24 hours at 37 degrees centigrade, and 53 RPM unless otherwise specified. *E. coli* was purchased from Ward's Biology as a freeze-dried culture isolated from a urinary tract infection. The ten fold dilutions were followed by further 2.5-fold dilutions ([0.097], [0.0753], [0.0534], [0.0316], [0.0097], [0.0076], [0.0054], [0.0032], [0.00097] g/ml) following the same procedure outlined above. This determined the highest concentration of triclosan that still allowed some *E. coli* growth.

In order to ensure that countable colonies would be generated when plating, ten-fold dilutions of cultures from four colonies of *E. coli* were performed. These colonies had been incubated in tryptic soy broth at standard conditions. Using plates inoculated with the appropriate concentration of triclosan found previously ([0.0097] g/ml) that still allowed some bacterial growth, streak plates were made for each dilution (1 to 10^-3). The plates were incubated at standard conditions.

Ten serial transfers of *E. coli* grown on [0.0097] g/ml triclosan (three replicas) were carried out. At the same time, ten serial transfers of *E. coli* grown on a control plate (three replicas) containing methanol but lacking triclosan were carried out.

The same procedure was used for each serial transfer. Methanol was spread on both the control plates and the triclosan plates (0.25 ml) and allowed to vaporize. The difference between the control and the treatment plates was that the treatment plates had the appropriate concentration of triclosan dissolved in the methanol. On both types of plates, the streak plate method was carried out with *E. coli* from a broth culture to get isolated, countable colonies. For each transfer, both plates were incubated in an inverted position for the same amount of time (24-48 hours) at 37 degrees centigrade and 53 RPM. The four largest colonies from both types of plates (six total plates for each transfer) were transferred to appropriately labeled broth filled test tubes (six test tubes for each transfer). The broth filled test tubes were incubated at standard conditions. This represents one transfer and this procedure was repeated for ten transfers.

After ten transfers of both the triclosan plates and the control plates had been carried out, the Kirby-Bauer method using triclosan soaked disks was used to compare the zones of inhibition of both types of final plates (Alexander and Strete, 2001). Two concentrations of triclosan were used in this method, [0.097] and [0.0316] g/ml (the latter being a lower level of triclosan and the former concentration closer to that found in clinical use of triclosan). Statistical analysis using SigmaStat was performed comparing zones of inhibition of the experimental and control strains at both concentrations of triclosan to determine if the bacteria gained resistance.

RESULTS

The maximum concentration of triclosan that still allowed growth on 0.3 ml spread plates of *E. coli* was [0.0097] g/ml. Countable colonies resulted from no dilution and a 10^-1 dilution of the broth cultures from the four colonies of *E. coli* incubated at standard conditions.

The zone of inhibition was measured using the Kirby-Bauer method at two different concentrations of triclosan to see if there was a significant difference between the treatment and the control groups. Three disks were placed on each plate for a total of 18 measurements at each concentration.

The first triclosan concentration tested was [0.097] g/ml. The data passed both the Normality Test (P = 0.1838) and the Equal Variance Test (P = 0.6527). In Figure 1, it is shown that the mean of the treatment group was found to be 25.1 mm with a standard error of 0.332 mm. The mean of the control group was found to be 25.1 mm with a standard error of 0.329 mm. The t-test showed that there was not a statistically significant difference between the measurements of the zones of inhibition of the treatment and control groups (P = 0.9068).

The second triclosan concentration tested was [0.0316] g/ml. The data passed both the Normality Test (P = 0.4389) and the Equal Variance Test (P=0.3434).

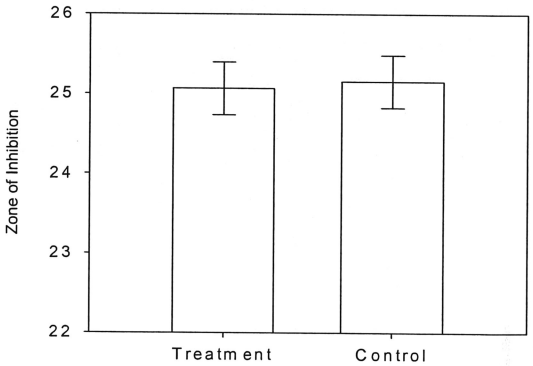

Figure 1. Collective average of the zones of inhibition of each group at a triclosan concentration of [0.097] g/ml. Mean (± 1 s.e.)

In Figure 2, it is shown that the mean of the treatment group was found to be 23.3 mm with a standard error of 0.232 mm. The mean of the control group was found to be 24.1mm with a standard error of 0.291 mm. A t-test showed that the difference in the mean values of the treatment and control groups is greater than would be expected by chance; there is a statistically significant difference between the measurements of the zones of inhibition of the two groups (P = 0.0313). The 95 percent confidence interval for the difference of the two means was -1.67 to -0.0892.

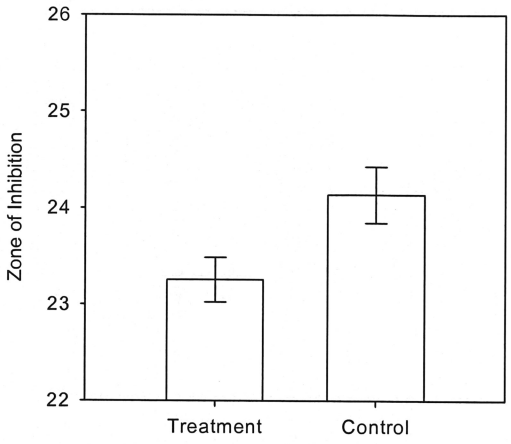

Figure 2. Collective average of the zones of inhibition of each group at a triclosan concentration of [0.0316] g/ml. Mean (± 1 s.e.)

DISCUSSION

The difference in the mean values of the two groups tested at a triclosan concentration of [0.0316] g/ml was greater than what is expected by chance, which could mean that some resistance was gained. The higher triclosan concentration [0.097] g/ml showed no evidence of resistance, which shows that if the *E. coli* gained any resistance, it was to a low level of triclosan only. The experimental findings could have been affected by sources of error in measuring zones of inhibition, as recording to the nearest tenth of a millimeter cannot be very exact with the human eye. This study could be a basis for more conclusive studies in the future. The bacteria in this experiment were left to mutate spontaneously at the FabI gene, which could take place more readily if factors favoring evolution would have been more prominent. More than ten transfers could be carried out or mutations could be induced by way of ultraviolet light or mutagenic chemicals. If mutations were induced, there would be higher chances of mutation on the FabI gene for resistance to occur and for natural selection to take place. Also, mixing the triclosan in with the agar would give a more even distribution of the chemical, resulting in less error.

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