

EVOLUTION IN THE LAB: Biocide Resistance in *E. coli*

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The laboratory exercise described here accomplishes two goals:

- Demonstrating the observable occurrence of evolution, and
- Introducing students to the issues of misuse of antimicrobial compounds and the consequent spread of resistant bacteria.

Students culture *Escherichia coli*, a bacterium normally found in the gut of humans and other mammals; expose the bacteria to triclosan, a biocide used in many consumer products; and discover that the bacteria evolve resistance to this compound with surprising rapidity.

More than 40,000 cases of food-borne infection by the bacteria *Campylobacter*, *Staphylococcus*, *Salmonella*, and *E. coli*, resulting in about 500 deaths, occur every year in the United States (Cohen & Tauxe, 1986). These infections result from con-

tact with contaminated beef products, poultry, eggs, or water. In response to consumer concern about unsafe foods and other sources of infection, manufacturers have incorporated biocides, or antimicrobial compounds, into an astonishing variety of products, ranging from hand soaps to plastic toys to kitchen cutting boards (Henderson, 2000a; Henderson, 2000b; Stix, 1998; Travis, 2000). One of the most widely used compounds is triclosan (2,4,4'-Trichloro-2'-hydroxydiphenyl ether, or 5-Chloro-2-(2,4-dichlorophenoxy) phenol, also marketed as Irgasan DP300).

The possibility that pathogenic bacteria may evolve resistance to triclosan is a source of escalating concern among microbiologists and public health officials (Levy, 1998; Stix, 1998; Travis, 2000). If consumers think themselves protected by triclosan-containing products, they may become lax in their food-handling practices, and expose themselves to greater risk of food-borne disease (Levy, 1998). By killing susceptible, nonpathogenic bacteria, triclosan may also remove competitors that normally prevent pathogens from colonizing household surfaces, increasing the risk of dangerous infections by other routes.

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Triclosan has been classed as a biocide (a substance toxic to cells in general) or antimicrobial agent (a substance toxic to bacteria, fungi, and protists) because it kills or inhibits the growth of a wide spectrum of microbes. It is not considered an antibiotic (a microbial product that kills or inhibits the growth of susceptible microbes) because of its origin and its broad spectrum of toxicity.

Bacteria and fungi are well-known for evolving resistance to antibiotics (Levy, 1998), but are thought to be less likely to evolve resistance to biocides because these compounds often act by different mechanisms. However, recent research into the mechanism of action by triclosan (McMurray, Oethinger & Levy, 1998; Levy et al., 1999) indicates that it may act more like an antibiotic than a true biocide. Bacteria may, therefore, be more likely to evolve resistance to triclosan than previously thought.

This laboratory exercise can serve as a springboard for further investigation and discussion of biocide and antibiotic resistance and likely consequences; the relevance of evolution to medicine, public health, and students' lives; and the observability of evolutionary processes in a laboratory setting. The exercise also introduces students to techniques of bacterial culture that they can apply to other experiments.

Methods

Instructor Preparation

We acquired 99% pure triclosan, a white crystalline powder, from KIC Chemical Co. (451 Main St., Armonk, NY 10504-0437 USA).

Several days before the exercise, our laboratory preparator transferred *E. coli* (strain K-12) from stock cultures to tryptic soy agar plates, making a bacterial lawn (a more-or-less uniform sheet of bacteria covering the agar surface). She prepared one plate per lab section, with a few extras in case of contamination.

The laboratory preparator poured approximately 20 sterile petri dishes of tryptic soy agar and 20 tubes of sterile tryptic soy broth per team of three to five students. She also prepared a working solution of triclosan by dissolving the powder in a solution of 17.5% ethanol and 82.5% distilled water to a final triclosan concentration of 500 $\mu\text{g mL}^{-1}$. A stock of 500 mL each of triclosan and tryptic soy broth sufficed for the entire course (150 students).

The lab preparator set up three workstations for each laboratory section of 24 students. Each workstation had the following items:

- A screw-top jar containing sterile cotton swabs

- A covered dish containing sterile 8 mm disks of filter paper (any diameter from 5 to 10 mm would work as well)
- Forceps labeled “Water,” “Ethanol,” and “Triclosan” sterilized by soaking in ethanol, and air-dried before use. (We simply laid these out on paper towels, but covering them may reduce contamination of petri dishes.)
- Waste disposal buckets for used swabs, petri dishes, and culture tubes (Filling these with enough disinfectant to cover the ends of the swabs may be a wise safety precaution.)
- A rack for culture tubes and a tray for petri dishes
- A box of Parafilm[®] and scissors for cutting Parafilm[®]
- Indelible pens for labeling culture tubes and petri dishes
- Labeled containers of the following:
 - Distilled water
 - Ethanol (17.5%)
 - Triclosan (500 $\mu\text{g mL}^{-1}$)
- A capped tube containing about 10 mL of sterile water, labeled “*E. coli*.”

Each lab room also contained an incubator cabinet set at 35 to 37° C.

Immediately before the first lab meeting, the lab preparator rubbed a sterile cotton swab across the surface of one of the prepared dishes with a lawn of *E. coli* and swirled the swab in the tube of distilled water (last item in the list above). This provided students with a dilute, spreadable suspension of *E. coli*.

Lab instructors divided students into teams of three to five, and assigned each team to start one petri dish for each treatment: water, ethanol, and triclosan. Alternatively, one could allow students to work alone, or assign each team a single treatment.

Student Activities

In outline, the experiment consisted of seven steps, repeated up to six times.

1. Transfer bacteria from a liquid culture to an agar plate.
2. Apply one of three paper disks soaked in water, ethanol, or triclosan.
3. Incubate 24 hours at 37° C.
4. Measure the width of the zone of inhibition (explained below).

5. Isolate from each plate the bacteria most resistant to the treatment applied (explained below).
6. Transfer these bacteria to liquid culture.
7. Incubate for 24 hours at 37° C.
8. Repeat.

To begin the experiment, students from each team transferred bacteria from the *E. coli* suspension provided by the instructor to petri dishes containing tryptic soy agar. They did this by swirling sterile cotton swabs in the bacterial suspension and spreading a lawn of bacteria on the agar surface of three petri dishes, using a separate swab for each dish. In subsequent rounds of the experiment, they transferred bacteria from their own liquid cultures, rather than from the initial suspension provided by the instructor.

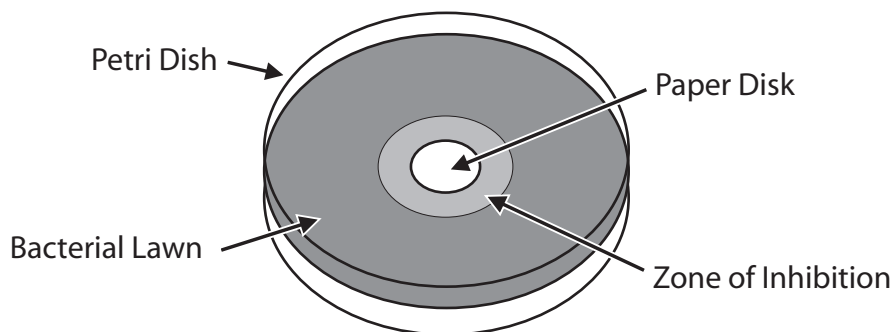
To apply the treatments to their plates, students dipped separate sterile disks of filter paper into distilled water, 17.5% ethanol, and 500 µg mL⁻¹ triclosan, and placed them, using the appropriate forceps, in the center of the agar surfaces of three petri dishes. After allowing a minute or so for the disks to adhere to the agar surfaces, students sealed the edges of the petri dishes with parafilm, labeled them with a team identifier and the treatment applied, and placed them in the incubator. If parafilm is unavailable, students could seal their dishes with transparent tape.

Cultures (both liquid and plate) were incubated at 35 to 37° C for 24 hours. To maintain uniformity of treatments, lab personnel moved students' cultures into a refrigerator (approx. 5° C) over weekends, either before or after the 24 hour incubation at 25 to 37° C.

After the incubation period, students measured the width of the zone of inhibition. This is the distance from the edge of the paper disk to the inner margin of the bacterial lawn (Figure 1). Because the concentration of the applied compound decreases with distance from the paper disk, the width of this zone is inversely correlated with the degree of resistance in the most resistant members of the bacterial population.

Students next isolated the most resistant bacteria in each dish by rubbing a sterile cotton swab across the inner margin of the zone of inhibition. If no zone of inhibition was apparent, they swabbed bacteria from

Figure 1.



Experimental setup, showing placement of filter paper disk in the center of a petri dish of tryptic soy agar. Students measured the width of the zone of inhibition from the edge of the paper disk to the inner margin to the bacterial lawn.

the area adjacent to the paper disk. If isolated bacterial colonies were visible within the zone of inhibition, they swabbed those.

Next, students transferred the bacteria collected from their dishes to liquid culture by swirling each cotton swab in a separate tube of tryptic soy broth. They labeled the tube according to the treatment applied to the dish from which the bacteria were taken, and incubated the capped tubes at 35 to 37° C for 24 hours. This step allowed the putatively resistant bacteria to multiply in preparation for the next round of selection.

Students repeated the above process of liquid culture, transfer to petri dish, treatment, measurement of zone of inhibition, isolation of the most resistant bacteria, and transfer to liquid culture for up to six rounds. They discarded used petri dishes, liquid cultures, and cotton swabs after each round.

Thus, each team of students cultured three separate lineages of bacterial populations subjected to repeated episodes of the same treatment: triclosan, ethanol, or water. After each round of exposure to treatment, students recorded the width of the zone of inhibition in each dish in a common database, which instructors later distributed to all students.

Data Analysis

Instructors provided the compiled raw data from all student teams in a spreadsheet data file via the campus computer network. Students computed the mean (average) width of zone on inhibition for each treatment on each measurement date and graphed means against time. Students were told to determine whether zones of inhibition were wider initially on triclosan plates and

whether widths of zones of inhibition in any treatment changed during the course of the experiment.

Tips & Explanations

By applying the treatment in the center of the agar surface, students created a concentration gradient as the compound in the paper disk diffused out into the agar. This is, the concentration of water, ethanol, or triclosan was highest adjacent to the disk and progressively lower farther from it.

We found that using paper disks to apply the treatments was preferable to applying the liquids directly onto the agar. Liquids do not soak into agar immediately, but run irregularly across the surface, especially if the dish is tilted or jostled. This creates a very irregular distribution of concentrations, and students cannot see where the treatment was applied. The paper disk holds the liquid in place and allows students to apply a larger volume. It also provides a visual landmark to show where the center of the concentration gradient is located.

Using cotton swabs instead of inoculating loops allowed us to avoid flame sterilization. We considered this an important safety benefit because it allowed students to transfer bacteria without faculty supervision. Swabs also make spreading bacteria onto agar easier and less likely to puncture the agar surface.

Students may need help to recognize *E. coli* and to distinguish it from contaminants. *E. coli* forms light gray-brown, glistening, smooth-surfaced colonies and lawns. Other bacteria usually form colonies of different colors (often yellow) or textures (rough-surfaced). Fungi grow in filaments (hyphae) and often resemble white, gray, green, or black fuzzy masses of cottony threads.

To reduce contamination, instruct students in sterile technique:

- Do not allow cotton swabs to touch any surfaces or solutions other than the bacterial cultures as directed. Do not touch agar or culture broth with fingers or anything other than cotton swabs.
- Keep petri dishes closed at all times except during the actual transfer of bacteria, and then open them as briefly and as narrowly as possible.
- When opening petri dishes for transfer, hold the lid over the agar surface and as close to it as possible.
- Make a uniform bacterial lawn on an agar surface by swirling a sterile cotton swab in a liquid *E. coli* culture and gently rubbing the swab back and forth over the entire surface of the agar. Rub gently to avoid puncturing the agar surface.

- Students should place used swabs, liquid cultures, and petri dishes in designated waste containers. These materials should be sterilized before disposal, and treated and labeled as biological waste.
- Students should wash their hands with soap (preferably triclosan-free) and water after handling bacterial cultures.

Results

Students began with 73 culture lines (25 exposed to triclosan, and 24 each exposed to ethanol and water). Of these, 71 culture lines completed four rounds of treatment exposure, 48 completed five rounds, and 22 completed six rounds. Other culture lines were lost due to miscommunication among lab instructors regarding the intended duration of the experiment. Much to our surprise, neither students nor lab instructors noted any contamination in the cultures.

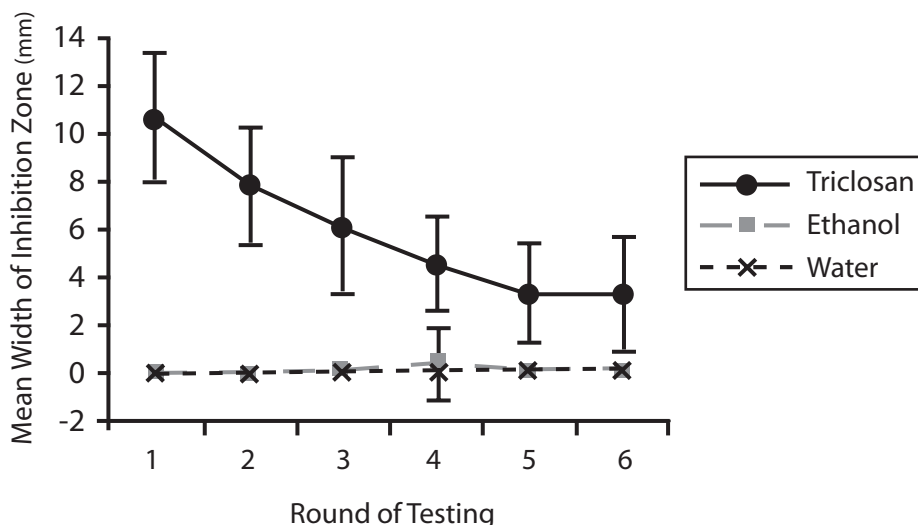
Triclosan cultures had wider zones of inhibition initially than other treatments (triclosan: 2.7 – 15.0 mm, \bar{x} = 10.6, ethanol: 0.0 – 1.0 mm, water; all 0.0 mm), and the difference was statistically significant ($F = 372$, d.f. = 2, $p < 0.0001$). Mean width of zones of inhibition in triclosan treatments shrank progressively throughout the experiment, reaching a mean of 3.1 mm after six rounds of treatment (Figure 2). The difference between initial mean zone width and mean zone width after six rounds of treatment was statistically significant (paired $t = 6.3$, two-tailed $p < 0.002$). Differences between initial mean zone width and mean width after four and five rounds, although smaller, were also statistically significant. Zones of inhibition in other treatments showed no trend.

Although we show 95% confidence intervals (Figure 2) and report statistical tests, we did not ask students to carry out these statistical analyses. However, these analyses are relatively easy to do using spreadsheets or statistical programs, and more advanced students should be capable of carrying them out.

Discussion

As shown by the initial widths of zones of inhibition in the three treatments, triclosan killed *E. coli* or inhibited their reproduction, whereas water and 17.5% ethanol did not. We were somewhat surprised that the ethanol treatment did not inhibit bacterial growth or reproduction, but it may be the case that the ethanol evaporated from the paper disk rather than diffusing into the agar, obviating any concentration gradient, and perhaps even diffusing (in the gaseous state) out of the petri dish entirely.

Figure 2.



Mean widths of zones of inhibition. Treatments were water, 17.5% ethanol, and triclosan ($500\mu\text{gL}^{-1}$). Round of testing refers to the number of exposures to treatment. Error bars encompass 95% confidence intervals on each mean.

The bacteria apparently evolved resistance to triclosan rapidly, as shown by the rapid reduction in the width of the zone of inhibition (Figure 2). The continuing reduction in the width of the zone of inhibition may indicate that the bacteria accumulated several resistance mutations during the course of the experiment. McMurray, Oethinger & Levy (1998) found five point mutations in different lineages of triclosan-resistant *E. coli*, and it is intriguing to speculate that our bacteria may have accumulated several of these.

The results reinforce several key concepts of evolutionary biology and can help combat some common misconceptions. Contrary to the claims of some, microevolution can be observed in the laboratory, under controlled conditions. Also, notable evolutionary change can occur rapidly, especially in organisms with short life-spans and rapid reproduction, such as bacteria. Finally, note that some triclosan dishes had very narrow zones of inhibition from the start (2.7 mm in one case). This indicates that triclosan resistance mutations were present in the bacterial population before exposure to triclosan. In other words, triclosan resistance arises by normal random mutation, not in a directed response to the presence of triclosan in the environment.

Despite the apparent conclusiveness of these results, some alternative explanations are possible. Because students used the same triclosan solutions

throughout the experiment, if the concentration of triclosan decreased over time, zones of inhibition would have shrunk as observed. This reduction in concentration may have occurred by breakdown of triclosan in solution (unlikely), or by precipitation of triclosan out of solution. However, lab instructors recognized the latter possibility partway through the experiment and began stirring the triclosan solutions before each lab.

We plan to address these concerns in the future by altering the experimental design in two ways. First, we will instruct students and lab preparators to shake or stir triclosan solutions thoroughly before each use. Second, we will test the

original stock culture of *E. coli* for resistance to the triclosan solution at the end of the experiment. If the solution retains the original concentration, we should see the same degree of resistance (or lack thereof) at the end of the experiment as at the beginning.

We also plan to control the amount of water, ethanol, and triclosan applied more carefully. In future labs, we will instruct the students to pipette uniform volumes of liquid onto the filter paper disks.

Although triclosan is not considered an antibiotic, this exercise is nonetheless relevant to evolution of antibiotic resistance. The evolution of resistance to antibiotic drugs by pathogenic bacteria is nothing new (Levy, 1998). Some bacteria that cause life-threatening disease are already resistant to all of the more than 100 antibiotic drugs in use today. These multi-drug resistant strains are untreatable. Other bacteria, such as some strains of *Staphylococcus aureus* (which causes blood poisoning, wound infections, and pneumonia), have recently shown resistance to vancomycin, the antibiotic of last resort, and are considered likely to evolve multi-drug resistance in the near future. Some strains of *S. aureus* are already resistant to triclosan (Sasatusu, 1993).

Triclosan has been classed as a biocide or an antimicrobial compound, rather than an antibiotic, because it

was presumed to inhibit bacterial growth and reproduction by a variety of mechanisms. Antibiotics, in contrast, usually interfere with a specific metabolic pathway. However, the mechanisms of triclosan's action were unknown until recent research (McMurray, Oethinger & Levy, 1998; Levy et al., 1999) showed that it blocks a single step in the synthesis of fatty acids. Triclosan-resistant *E. coli* strains were shown to differ from susceptible strains by single base-pair substitutions in the DNA (McMurray, Oethinger, and Levy 1998). Thus, triclosan appears to act more like an antibiotic than previously thought, and resistance to triclosan, like antibiotic resistance, can arise by point mutations. These findings imply that the evolution of triclosan resistance may be more likely than previously thought, and more likely than implied by manufacturers of triclosan-containing products (Larkin, 1999).

The enzyme blocked by triclosan is also the target of an antibiotic used to treat tuberculosis (Travis, 2000). Because bacteria frequently exchange drug-resistance genes via small loops of DNA called plasmids (Davies, 1994), some microbiologists have become concerned that triclosan-resistant bacteria could transfer a plasmid to *Mycobacterium tuberculosis* that would make it resistant to the antibiotic. This scenario is made more likely by the use of triclosan-containing gloves and soaps in hospitals treating tuberculosis patients.

The applicability of our results to real-world evolution is a matter for fruitful discussion with students. Resistance genes typically carry costs as well as benefits. Energy invested in circumventing the action of an antimicrobial compound is not available for reproduction, and therefore resistant bacteria usually reproduce at a slightly slower pace than susceptible strains. In an environment devoid of the antimicrobial, susceptible bacteria will out-reproduce and replace resistant ones. Only in an environment where the antimicrobial is prevalent will the benefit of resistance outweigh its cost.

Procter & Gamble, makers of several household products containing triclosan, criticized similar studies for this very reason. "These were not real world conditions. In the real world [germs] have to compete with a host of other organisms. We're really not worried about [triclosan use] leading to superbugs or resistant organisms," said a Procter & Gamble spokesperson (quoted in Hellinghausen, 1998).

The increasing prevalence of triclosan in household products calls this optimistic position into question. A recent survey of national and regional supermarkets in the U.S. found triclosan (or another antimicrobial, triclocarban) in 76% of liquid soaps and 29% of bar soaps (Henderson, 2000b). You might assign your students to visit local supermarkets and grocery stores to compile their own triclosan statistics, and ask themselves whether triclosan has become a normal part of the environment.

Note: We have now repeated this experiment with a new class of students, with an additional control. At each round of plate culture, students exposed previously unexposed and unselected *E. coli* from the original stock culture to the triclosan solution, using the same methods as for the selection treatment. The zone of inhibition in this control remained about 10 mm wide throughout the experiment, and the results from the other treatments were nearly identical to those of the original experiment, indicating that the decreased width of the zone of inhibition in the selection treatment was not due to chemical breakdown or precipitation in the triclosan solution.

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