

E. COLI ANTIBIOTIC RESISTANCE

Escherichia coli and Antibiotic Resistance to Tetracycline Antibiotics

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Acceptance of Senior Honors Thesis

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Abstract

Escherichia coli cells growing under ideal conditions are able to complete one reproduction cycle in as little as every twenty minutes. Since so many generations are able to be observed, one should theoretically be able to observe thousands of generations and determine evolution's effects over a short period of time. In this experiment, *E. coli* K12 cells were cultured under ideal growth conditions but in the presence of antibiotics as a selective environmental stress in order to select for resistance. This was accomplished by serially passing colonies that were in close contact with two different, but similar antibiotics over a period of more than 4,000 generations. The goal was to improve research in the antibiotic sensitivity properties of *E. coli* and to determine if the data are in agreement with the theory that bacteria or other species will accumulate new phenotypic traits via development of genotypic changes that will enhance the survival of the organism, especially under selective pressure (e.g. growth with the presence of an antibiotic). The diameter of *E. coli* colonies' zones of inhibition decreased over time in response to the antibiotics Doxycycline and Tetracycline indicating decreased sensitivity to these antibiotics. The cultures, however, appeared to have increased fitness cost as compared to the wild type.

Escherichia coli and Antibiotic Resistance to Tetracycline Antibiotics

Bacterial antibiotic resistance

The phenomenon of bacterial drug resistance was first documented in 1951 (1). Interest in bacterial antibiotic resistance continues to increase as some antibiotics are less effective against pathogens and as deaths from bacterial infections that do not respond to common antibiotic therapy are increasing. This problem is an area of interest in the scientific community for two reasons. First, those working in medical fields are concerned because human lives are at stake. Second, this issue is of importance to evolutionists because they believe that the mutations in bacteria responsible for some instances of drug resistance when faced with antibiotic therapy are favorable from the standpoint of the bacterial population, and thus offers real-time evidence for evolution. The argument is that the bacteria have adapted (so they can live to fight another day)—an example of an advantageous mutation (1). Evolutionist Colin Patterson commented that “the development of antibiotic-resistant strains of bacteria, and also of insects resistant to DDT and a host of other recently discovered insecticides are genuine evolutionary changes” (2). Bacterial antibiotic resistance is one of the most cited modern examples of evolution. It seems to fit the model so well that proponents of evolution use it as the primary example of beneficial mutations.

Mechanisms of Antibiotic Inhibition of Bacteria

Tetracycline (Tet) was first isolated in 1945 (3) and has been used extensively since the 1950's. Many bacterial pathogens have developed or acquired resistance to Tet (4). Tet inhibits the binding of aminoacyl-tRNA's to the A site of the 30S ribosomal

subunit, which inhibits protein synthesis (4, 5). There is a primary Tet binding site and up to 5 secondary Tet binding sites on the ribosome (6, 7).

Most cases of Tet resistance are due to resistance genes that either encode energy-dependent pumps to transport Tet outside of the bacteria, or make a ribosomal protection protein, which displaces Tet from the bacterial ribosome (4, 5). These resistance genes are passed as plasmids, integrons, and transposons, (4) and the passing of these genes among *E. coli* populations was discovered in 1960 (8). Clinical resistance to multiple antibiotics due to mutations alone, which is the focus of this study, has also been documented due to over-expression of existing chromosomal genes (9, 10). Resistance to Tet due to a mutation in the 16S rRNA of *Propionibacterium acnes* that interfered with the Tet binding site was documented in 1998 (11). Since then, 16S rRNA mutation sites that lead to Tet resistance in *Helicobacter pylori* have been well documented (12, 13, 14, and 15). Tet resistance in *Escherichia coli* genetically structured for point mutations at positions 965-967 and independently at 1058 in the rRNA (resulting in a single nucleotide change) have also been recently documented (16).

Doxycycline (Dox) is an analog of tetracycline and belongs to the same family of antibiotics. It was semisynthetically derived from Tet and exhibits more favorable pharmacokinetic properties than early tetracyclines (17).

E. coli and Antibiotic Resistance

E. coli were first discovered in 1885 by Theodore Escherich, a German pediatrician and bacteriologist. It is one of many species of bacteria living in the lower intestines of mammals, known as normal gut flora, and when located in the large intestine, *E. coli* assists with food absorption, waste processing, and vitamin K

production. However, there are pathogenic strains, and it is one of the most frequently isolated bacterial pathogens (18).

On average, *E. coli* cells living in a Petri dish with plenty of nutrients and incubated at the right temperature (thus keeping it in the log phase of growth) are able to duplicate their chromosomal DNA and split into two new cells every 20 minutes. One should be able to see the effects of evolution on an experimental time scale due to the rapid generation time if evolutionary change can occur over several thousand generations as in this study.

Scientists know more about *E. coli* than any other bacteria in the world. *E. coli* is useful as a research tool because so much is already known about its systems and biochemistry (19). Its entire genome has been sequenced (20), so finding any genetic aberrations and determining their effect in the phenotype is much easier than in other, unsequenced genotypes. However, it can still be difficult to determine the genetic causes for morphological or phenotypic changes (21). *E. coli* is a useful research tool, and microbiologists have extensively studied this species of bacteria in their attempts to understand antibiotic resistance.

E. coli and evolution

Speaking about *E. coli* in an evolutionary context, zoologist Pierre-Paul Grassé observed:

...bacteria, despite their great production of intraspecific varieties, exhibit a great fidelity to their species. The bacillus *Escherichia coli*, whose mutants have been studied very carefully, are the best example. The reader will agree that it is surprising, to say the least, to want to prove evolution

and to discover its mechanisms and then to choose as a material for this study a being which practically stabilized a billion years ago (1).

Although *E. coli* has allegedly undergone billions of year's worth of life history, it still has remained stable as an organism and essentially unchanged. The bacterial population has incurred some changes due to mutations and DNA transpositions, but these changes have occurred within narrow limits. Thus, the goal of this study is to add to the research in antibiotic sensitivity and resistance of *E. coli* and to determine whether or not the data agrees with the theory of evolution and the theory that antibiotic resistance will occur in the face of constant exposure to a low level of antibiotics, as it would be when subjected to antibiotic therapy in an infected patient.

Experimental Procedures

Preparation of E. coli Cultures

The experiment was designed to only test for antibiotic sensitivity due to random mutations. In order to do this, a defined culture was used and kept isolated from any other form of live or dead bacteria, from which it would be possible to gain antibiotic resistance through horizontal gene transfer (as in a plasmid).

An *E. coli* K12 culture was streaked for isolation on trypticase soy agar (TSA) (Becton, Dickson and Co.) for isolation. This step was to ensure the continuous purity of *E. coli*. Pure *E. coli* colonies were found in and passed from the fourth quadrant of the streak plate after one-day incubation at 37°C. A gram stain was performed to confirm the microbe's identity and its purity. The *E. coli* was then transferred into trypticase soy broth (TSB) (Becton, Dickson and Co.), and grown for 24 hours at 37°C. This *E. coli*

culture was used as the beginning stock culture for this experiment. All steps were performed using aseptic technique.

Testing for antibiotic sensitivity

The Kirby-Bauer (KB), or disk diffusion antibiotic sensitivity test, was the antibiotic sensitivity test used in this experiment (22). In this test, spread plates are prepared on Mueller Hinton II Agar plates (MHA II) (Becton, Dickson and Co.) using 0.1 mL of the broth culture, then wafers (filter paper discs) that are impregnated with the desired antibiotic concentration are placed on the spread plate. The antibiotic diffuses out from the disk into the agar in a gradient, so the agar closest to the disk has the highest concentration, and the concentration of the antibiotic decreases as you move further away from the disk.

The broth cultures of *E. coli* were grown until they had an optical density (OD) (or absorbance) of 0.8 – 1.0 at 600 nm wavelength. A KB test was performed in order to test for the best antibiotics to be used. The twelve different antibiotics tested were Tetracycline, Doxycycline, Thymol, Penicillin, Ampicillin, Bacitracin, Ciprofloxacin, Cephalosporin, Chloramphenicol, Vancomycin, Streptomycin, and Erythromycin. The organism was sensitive to Tetracycline, Doxycycline, Ciprofloxacin, Cephalosporin, Chloramphenicol, Streptomycin, and Erythromycin. Tetracycline and Doxycycline were chosen as the antibiotics to be used in this experiment because the organism was sensitive to both and both antibiotics have a similar mechanism of action.

Antibiotic Sensitivity Testing Successive E. coli Generations

Because the colonies nearest to the zones of inhibition of Tet and Dox (those exposed to the highest antibiotic concentration) may have the highest potential for

developing antibiotic resistance (high potential colonies), colonies from the margin of the zones of inhibition were inoculated in two different TSB's, one from the Tet zone and one from the Dox zone, for one day at 37°C. Note: The original cultures of Tet and Dox, which were derived from organisms growing at the margin of the zone of inhibition for the individual antibiotic, were propagated separately, but each was tested for sensitivity to both Tet and Dox at each serial pass. No antibiotics were administered to any of the broth cultures, which were used solely to generate enough bacteria to repeat the Kirby-Bauer test with organisms taken from the margin of the zones of inhibition.

The pair of inoculated broths of *E. coli* were grown to an optical density within the range of 0.62 to 1.0 at 600 nm (after the sixth passage, some cultures were unable to grow to an OD between 0.8 and 1.0, which was the initial OD target zone) which was considered acceptable. 0.1 ml from each *E. coli* broth was transferred and spread onto two separate MHA II plates in order to measure the new zones of inhibition for both antibiotics. On each plate the diameter of the zones of inhibition for each antibiotic were measured. For example, colonies for one culture of *E. coli* were only taken from the edge of the Tet zone of inhibition, but sensitivity to both antibiotics was always measured. The same procedure was used for the other culture as well. This process was repeated 12 times from inoculating colonies from the margin of the zones of inhibition to measuring the zones of inhibitions. All broths and MHA II plates were labeled including date, name of antibiotic, and the optical density at 600 nm wavelengths. All the steps were done using aseptic technique.

Results

Initial antibiotic sensitivity

The results for the initial antibiotic sensitivity test against twelve antibiotics are shown in figure 1. Tet and Dox were chosen for further studies based on the similarity in mechanism of action and comparable zones of inhibition.

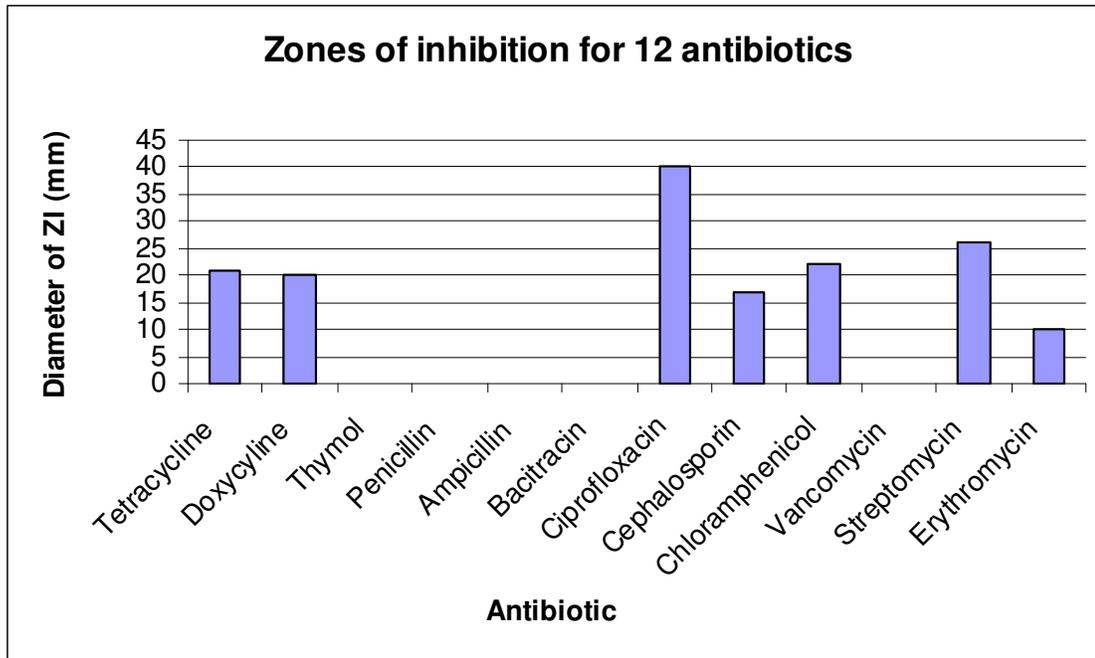


Figure 1 Zones of Inhibition for 12 Antibiotics

The initial zones of inhibition in mm of *E. coli* K12 for twelve antibiotics using the KB method of antibiotic sensitivity testing after 24 hour incubation at 37° C. All zone of inhibition measurements were performed on MHA II.

Optical Densities

The optical density of the broths was tracked to keep it as constant as possible throughout the experiment so that approximately the same number of bacteria (10^7 to 10^8) was plated each time a spread plate was performed. As shown in figure 2, the optical

density did have some variance. Note: As the experiment progressed, the time required for the broth cultures to grow to an optical density between 0.62 and 1.0 took an increasing amount of time as reported in figure 3. After the sixth passage (at day 22), some of the samples did not reach the initial OD target zone of 0.8 to 1.0 at 600 nm. The sample numbers in the figure 2 and 3 correspond to days as shown in table 1.

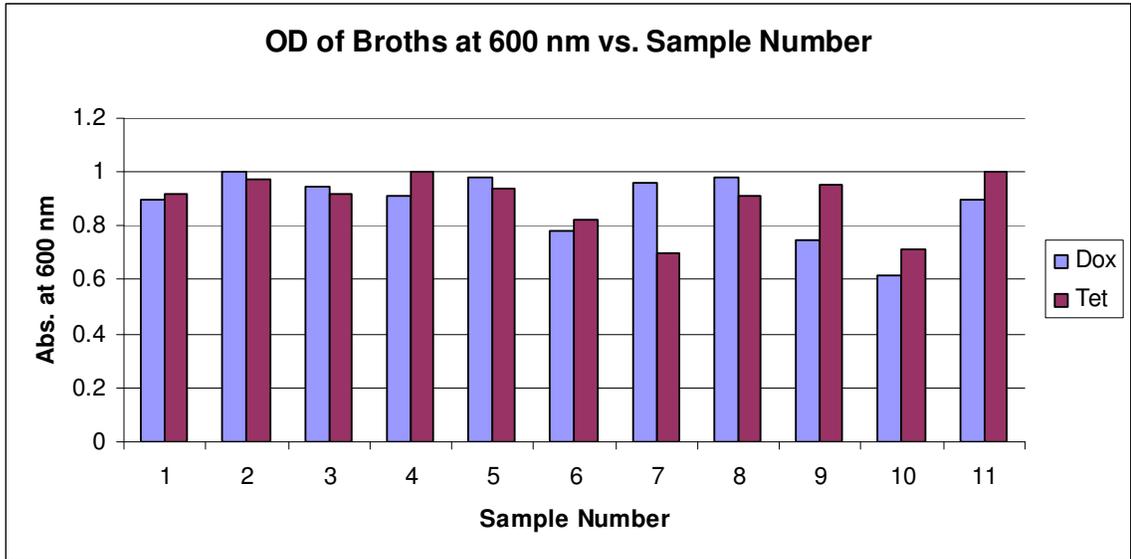


Figure 2. OD of Broths at 600 nm vs. Sample Number

The optical densities of both the Dox (blue) and Tet (pink) broth cultures over time.

Sample 6 (at day 22) was the first broth culture not able to reach an OD of 0.8 at 600 nm.

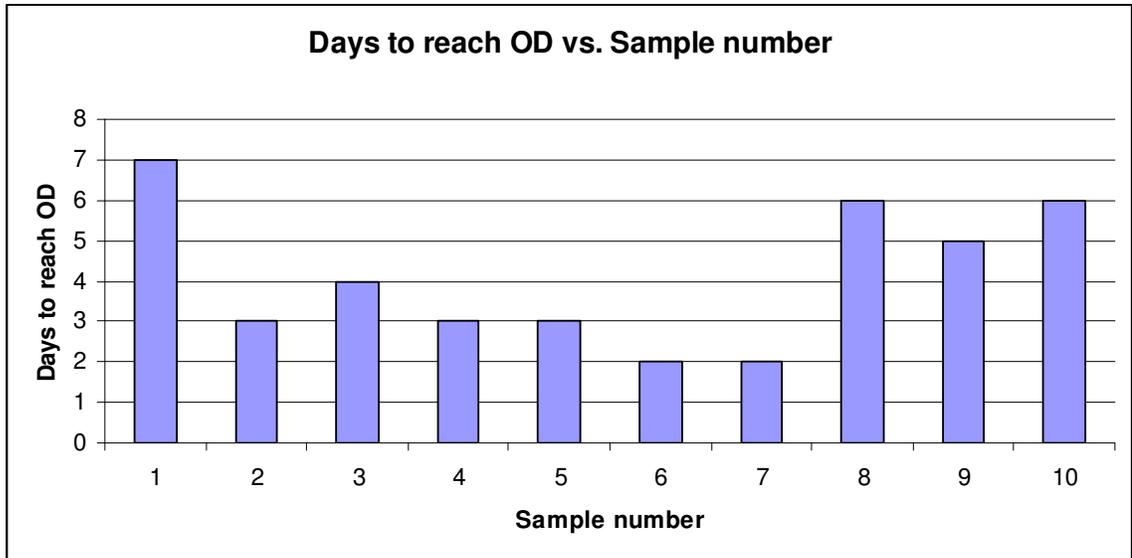


Figure 3. Days to Reach OD vs. Sample Number

The days allowed for the *E. coli* broth cultures to grow to an OD between 0.62 and 1.0 at 600 nm. Sample 6 was the first culture that did not grow to an OD of more than 0.8 at 600 nm.

Table 1.

Sample Number and Days to Reach OD

The correlation between the sample number and the number of days passed.

Sample Number	Days
1	7
2	10
3	14
4	17
5	20
6	22
7	24
8	30
9	35
10	41
11	59

Change in Antibiotic Sensitivity

The zones of inhibition for the doxycycline plates were tracked for the entire experiment. Figure 4 shows the antibiotic sensitivity against both tetracycline and doxycycline that developed over time for the doxycycline cultures. This reflects data from the bacteria passed from the margin of the zone of inhibition to doxycycline and then tested for sensitivity to both doxycycline and tetracycline. Table 2 shows the zones of inhibition for the antibiotic sensitivity tests to Dox and Tet in mm.

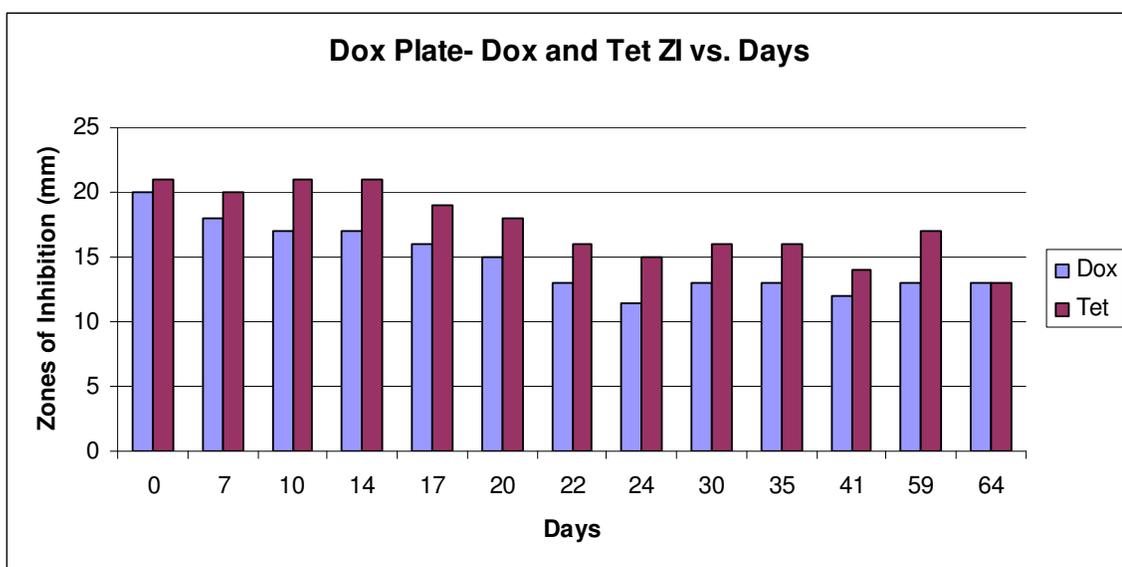


Figure 4. Dox Plate-Dox and Tet Plate ZI vs. Days

The diameters of the zones of inhibition in mm for cultures tested for Tet (Dox plate-Tet) in pink and Dox (Dox plate-Dox) in blue sensitivities on the plates propagated from organisms from the margin of the zone of inhibition of Dox. This graphically represents the sensitivity to Tet and Dox for the bacteria selected from the margins of the zone of inhibition to Dox.

Table 2.

Dox Culture Days with corresponding Dox and Tet ZI's

The diameter of the zones of inhibition in mm for both Dox and Tet from the culture propagated from colonies near the margin of the Dox antibiotic disk.

Days	Dox Culture	
	Dox ZI	Tet ZI
0	20	21
7	18	20
10	17	21
14	17	21
17	16	19
20	15	18
22	13	16
24	11.5	15
30	13	16
35	13	16
41	12	14
59	13	17
64	13	13

The zones of inhibition for the tetracycline plate were also tracked for the entire experiment. Figure 5 shows the sensitivity to both Tet and Dox that changed over time on the Tet plates. Table 3 shows the diameters of the zones of inhibition for the antibiotic sensitivity tests to Tet in mm.

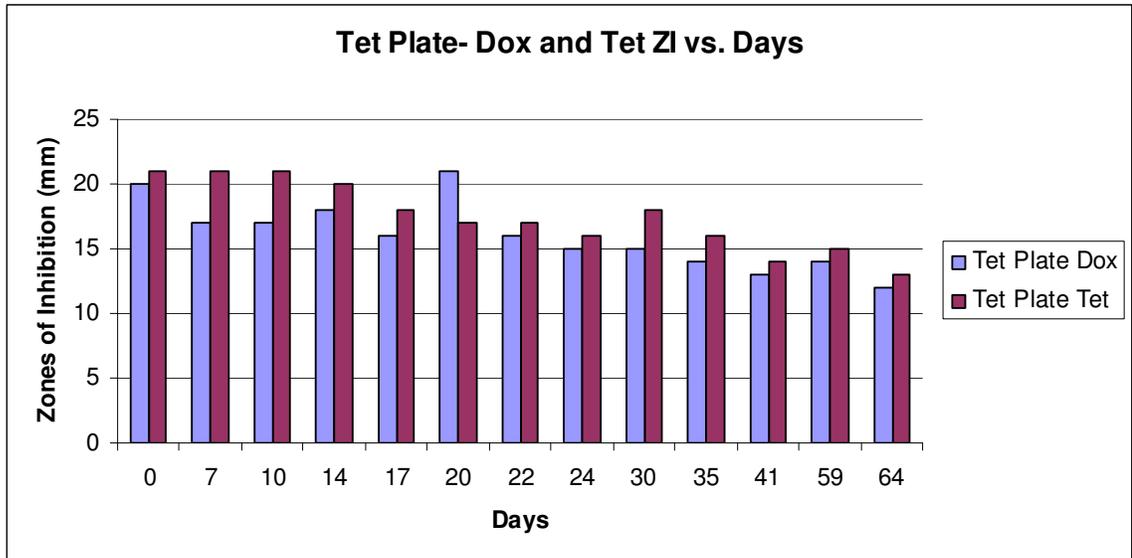


Figure 5. Tet Plate-Dox and Tet ZI vs. Days

The diameters of the zones of inhibition for cultures tested for Tet sensitivity (Tet plate-Tet) in pink, and Dox sensitivity (Tet plate-Dox) in blue, on the plates derived from high potential colonies for decreased Tet sensitivity over time. This graphically represents the sensitivity to Tet and Dox for the bacteria selected from the margins of the zone of inhibition to Tet.

Table 3.

Tet Culture Days with corresponding Dox and Tet ZI's

The diameters of the zones of inhibition for both Dox and Tet from the culture propagated from colonies near the margin of the Tet antibiotic disk.

Days	Tet Culture	
	Dox ZI	Tet ZI
0	20	21
7	17	21
10	17	21
14	18	20
17	16	18
20	21	17
22	16	17
24	15	16
30	15	18
35	14	16
41	13	14
59	14	15
64	12	13

Figure 6 compares the diameters of the zones of inhibition to Dox for the culture that was propagated in the presence of Dox (Dox plate-Dox) with the diameters of the zones of inhibition for Tet for the culture that was propagated in the presence of Tet (Tet plate-Tet).

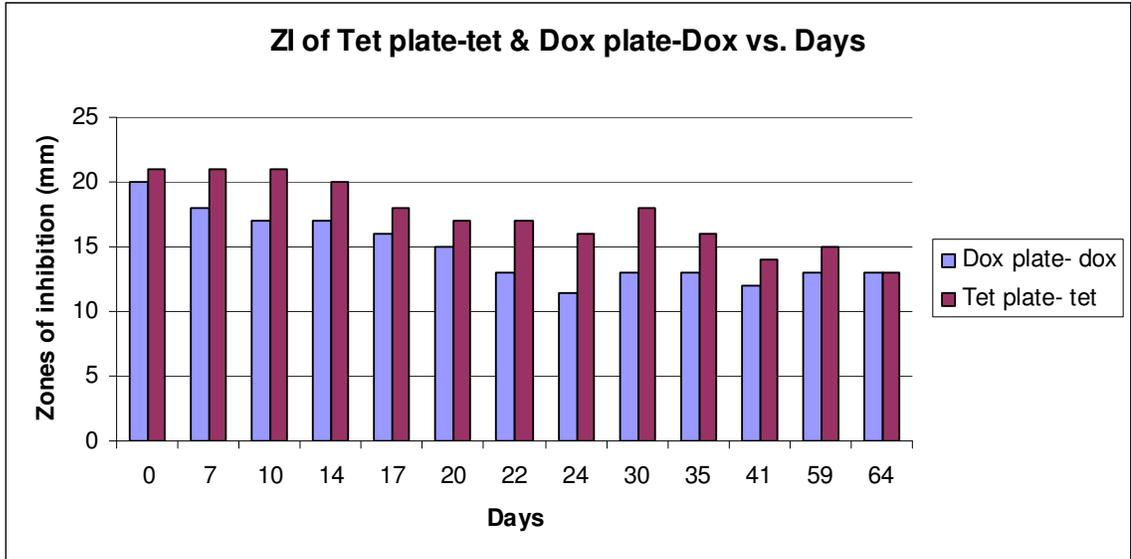


Figure 6. ZI of Tet Plate-Tet and Dox Plate-Dox vs. Days

The diameters of the zones of inhibition in mm for the cultures tested for sensitivity to Tet and Dox for the plates derived from high potential colonies for decreased Tet (Tet plate-Tet) and Dox (Dox plate-Dox) sensitivity over time. This graphically represents the sensitivity to Dox for the bacteria propagated in the presence of Dox and the sensitivity to Tet for the bacteria propagated in the presence of Tet.

Figure 7 compares the diameters of the zones of inhibition to Dox for the culture that was propagated in the presence of Tet (Tet plate-Dox) with the diameters of the zones of inhibition for Tet for the culture that was propagated in the presence of Dox (Dox plate-Tet).

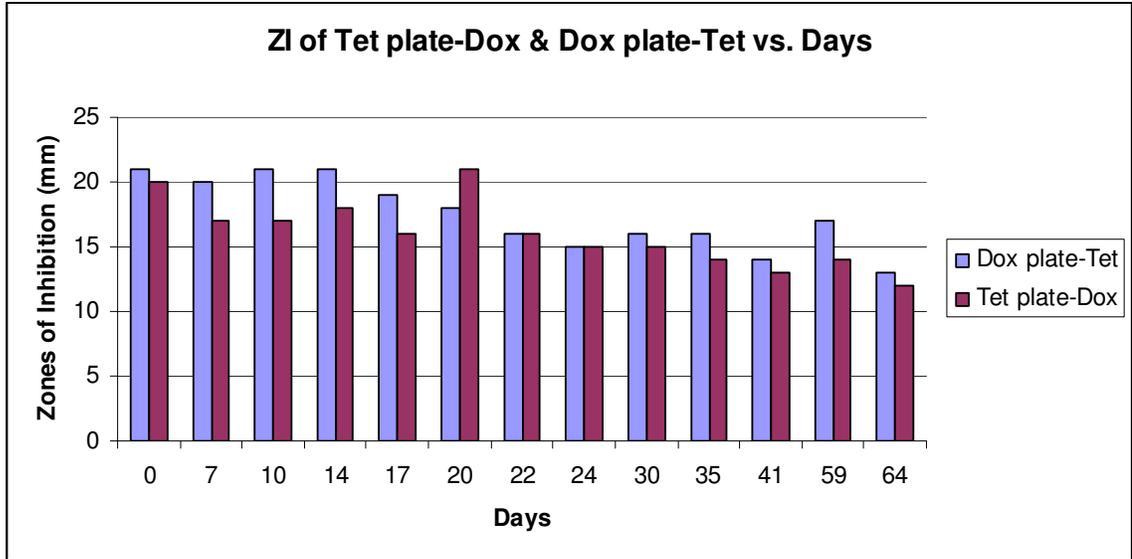


Figure 7. ZI of Tet Plate-Dox and Dox Plate-Tet vs. Days

The diameters of the zones of inhibition in mm for the cultures tested for sensitivity to Tet and Dox for the plates derived from high potential colonies for decreased Tet (Tet plate-Dox) and Dox (Dox plate-Tet) sensitivity over time. This graphically represents the sensitivity to Dox for the bacteria propagated in the presence of Tet and the sensitivity to Tet for the bacteria propagated in the presence of Dox.

Figure 8 compares the diameters of the zones of inhibition to Dox for the culture that was propagated in the presence of Tet (Tet plate-Dox) with the diameters of the zones of inhibition for Dox for the culture that was propagated in the presence of Dox (Dox plate-Dox) as well as the average of the two zones of inhibition. This shows the antibiotic sensitivity a single antibiotic that arose by two separate means (one by culturing with Dox, and the other with Tet).

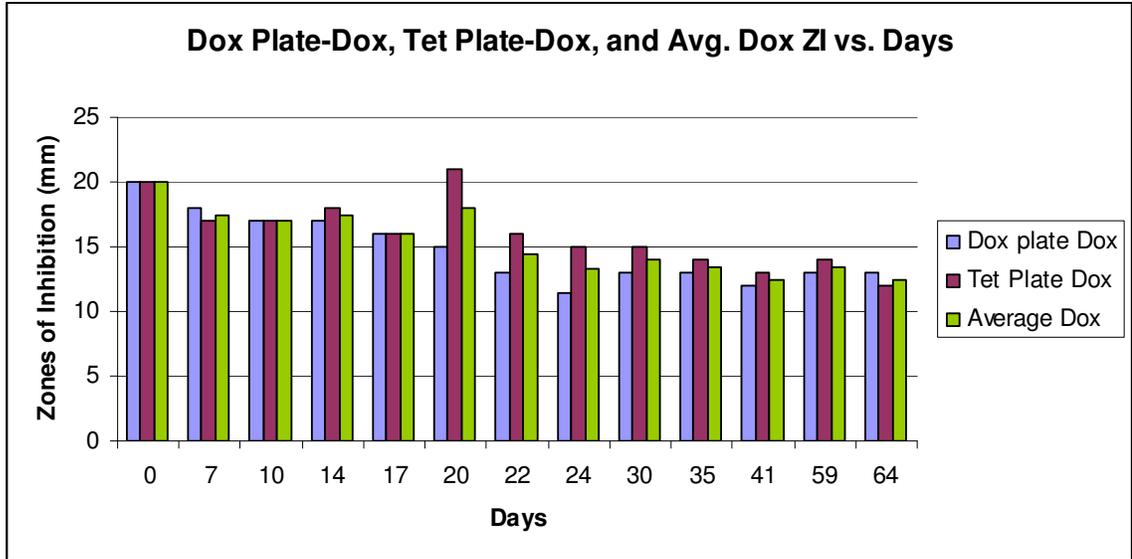


Figure 8. Dox Plate-Dox, Tet Plate-Dox, and Average Dox ZI vs. Days

The diameters of the zones of inhibition in mm for the cultures tested for sensitivity to Dox for the plates derived from high potential colonies for decreased Dox (Dox plate-Dox) and Tet (Tet plate-Dox) sensitivity over time as well as the average between the two. This graphically represents the sensitivity to Dox for the bacteria propagated in the presence of Dox and Tet as well as the average of the two. This shows the change in antibiotic sensitivity to a single antibiotic (Dox) derived by two separate means (one by culturing with Dox, and the other with Tet).

Figure 9 compares the diameters of the zones of inhibition to Tet for the culture that was propagated in the presence of Tet (Tet plate-Tet) with the diameters of the zones of inhibition for Tet for the culture that was propagated in the presence of Dox (Dox plate-Tet) as well as the average of the two zones of inhibition. This shows the antibiotic sensitivity a single antibiotic that arose by two separate means (one by culturing with Dox, and the other with Tet).

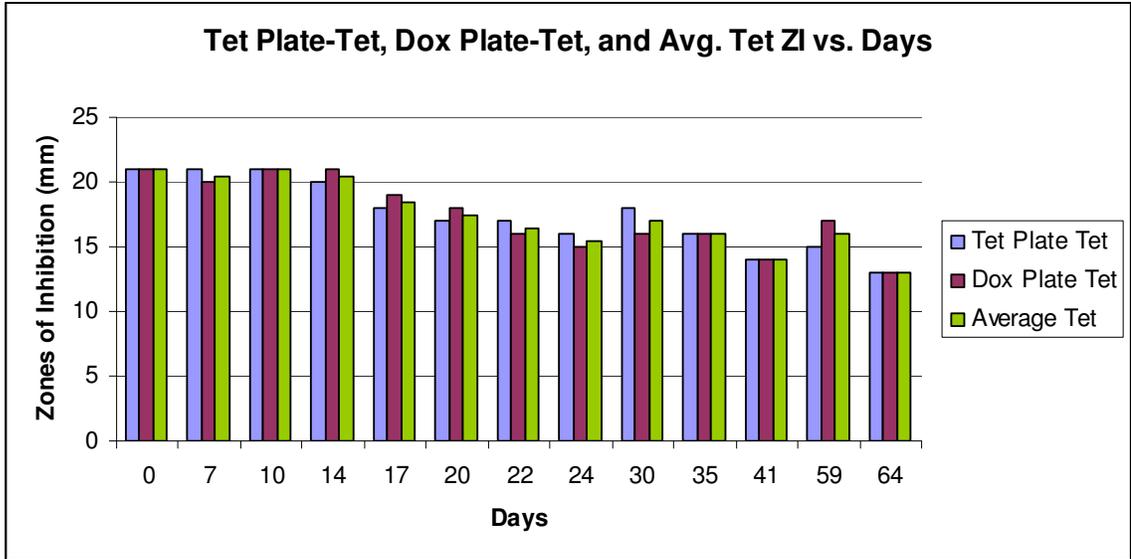


Figure 9. Tet Plate-Tet, Dox Plate-Tet, and Average Tet ZI vs. Days

The diameters of the zones of inhibition in mm for the cultures tested for sensitivity to Tet for the plates derived from high potential colonies for decreased Tet (Tet-Plate Tet) and Dox (Dox Plate-Tet) sensitivity over time as well as the average between the two. This graphically represents the sensitivity to Tet for the bacteria propagated in the presence of Dox and Tet as well as the average of the two. This shows the change in antibiotic sensitivity to a single antibiotic (Tet) derived by two separate means (one by culturing with Dox, and the other with Tet).

Optical Density Related to Zones of Inhibition

As a control, the zones of inhibition for the plates prepared from the Dox broths were compared to their corresponding optical densities which produced figure 10. Figure 10 compares the data points and linear trends for the Dox broths vs. the diameters of the zones of inhibition for both Tet and Dox.

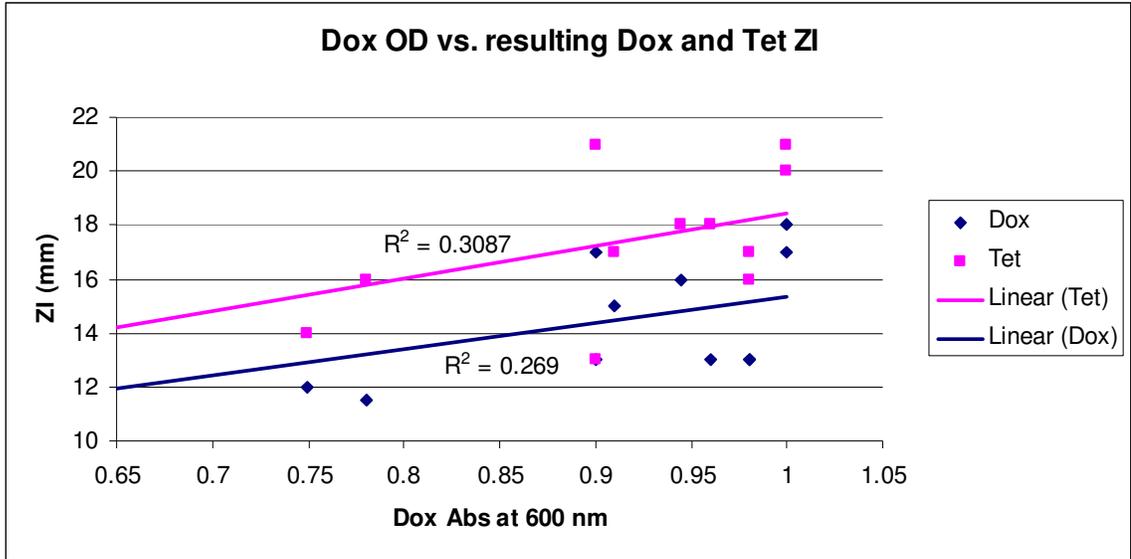


Figure 10. Dox OD vs. resulting Dox and Tet ZI

The linear trends of the optical densities for the culture incubated in the presence of Dox vs. the diameters of the zones of inhibition around both the Tet (Dox plate-Tet) and Dox (Dox plate-Dox) antibiotic discs.

The zones of inhibition for the plates prepared from the Tet broths were also compared to the corresponding optical densities which produced figure 11. Figure 11 depicts the data points for the Tet broth optical densities vs. the zones of inhibition for both Tet and Dox.

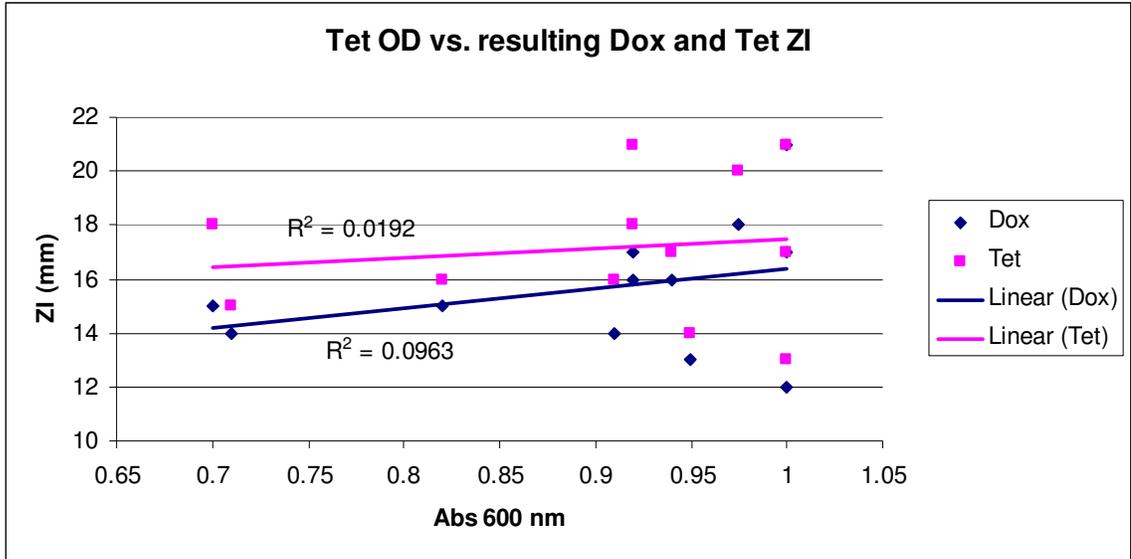


Figure 11. Tet OD vs. resulting Dox and Tet ZI

The linear trends of the optical densities for the culture incubated in the presence of tetracycline and the resulting diameters of the zones of inhibition around both the Tet (Tet plate-Tet) and Dox (Tet plate-Dox) antibiotic discs.

Over time, the growth of the *E. coli* in the broths considerably slowed. After the sixth passage, some of the broths were not even able to grow above an absorbance of 0.8 at 600nm.

Discussion

Zones of Inhibition

E. coli cultures were incubated for a total of 64 days. Our objective was to keep colonies in the log phase of growth, which would result in 72 reproduction cycles per day, or 4,608 total divisions. In this amount of time, our *E. coli* showed a decrease in the diameter of the zone of inhibition and therefore a decrease in the sensitivity to these antibiotics.

Our results show that *E. coli* has the capacity to have a decrease in antibiotic sensitivity over time, but whether or not it can become completely resistant to either antibiotic due to random mutations alone is inconclusive from our data (figures 4-9).

Optical Densities

As noted above, the bacteria in the broths after the sixth passage appeared to be less fit than they were at the beginning of the experiment and were unable to reach optical density of more than 0.8 at 600nm. The author believes that while these cells are less sensitive to the antibiotics (as observed by the diameters of the zones of inhibition) when in the presence of Dox or Tet (as on the MHA II plates), they are probably less competent than wild type *E. coli* (i.e. less metabolically able to support rapid growth), which could reach optical densities of 8.0 – 1.0 in one to two days.

Optical Density Related to Zones of Inhibition

There appears to be a strong correlation between the OD that the broths were able to reach and the zones of inhibition that were produced from them (Figures 10 and 11). The lower OD's correspond to smaller zones of inhibition. The data seem to suggest that the less sensitive cultures are not able to grow as quickly as the wild type cultures, or at least not able to grow to higher OD's (above 0.8 at 600 nm).

Fitness Cost

Ribosomes are the translational centers of the cell. The tetracycline group of antibiotics inhibits the ribosomes of bacteria by binding the 30S rRNA and blocking the binding of aminoacyl-tRNA's to the A site and therefore slow or stop the production of proteins. It logically follows that if the *E. coli* have acquired mutations in their ribosomes that decrease the efficacy of the antibiotics (i.e. change the ribosome in some way), that it

could be possible to see a decrease in their growth rate, due to less efficient translation and production of needed proteins.

For example, the streptomycin resistance mutation of *E. coli* (*rpsL*) is due to a mutation in the ribosome which makes the bacterium resistant to streptomycin, but with overall translational fitness costs to the bacteria (23, 24). In the presence of streptomycin, these *E. coli* are more fit (they survive streptomycin treatment), but if removed from the streptomycin environment, they are less fit than wild type cells (do not reproduce as well).

Increased fitness costs have been documented for pathogenic strains of *E. coli* that gain resistance to quinolones (25, 26) as determined by the decrease in the number of virulence factors the resistant *E. coli* strains possess. Although some studies have shown that it is possible for wild type *E. coli* cultures to acquire spontaneous antibiotic resistance to fluoroquinolones without the loss of any virulence factors (27), these antibiotics have a different mechanism of action than tetracycline antibiotics. They disrupt DNA gyrase and topoisomerases, instead of ribosomes.

In 2007, the first fitness evaluation was performed on Tet resistant strains of *E. coli* by Erjavec *et al.* They performed fitness tests on 110 Tet resistant *E. coli* isolates and determined that there was a statistically significant decrease in the virulence potential of the Tet resistant strains of *E. coli* (28).

The zones of inhibition of our *E. coli* seem to have leveled off around 13 mm for both the Dox and Tet cultures (figures 4-9). This could be coincidence, but the author believes that further mutations are too detrimental for the cells in order to be viable.

Studies have shown that fitness costs for mutations in the ribosome that lead to antibiotic resistance tend to be high (23, 24).

Evolution of E. coli

In the case of sexual organisms (such as humans), a species is a group of organisms who can breed amongst themselves, but not with another species. In the case of bacteria (which are asexual), species are defined based on their phenotypic similarity, genetic makeup, and biochemical traits (36). In *E. coli*, the cultures have to be morphologically similar and over 70% of their DNA has to reassociate under moderately restrictive condition (36) in order to be considered of the same species. Our *E. coli* organisms did not evolve into a new species over a period of 4,600 generations. Under intense artificial selection adaptation was observed (as evidenced by the decrease in the diameter of the zones of inhibition), however, no new traits were observed, so no evidence of macroevolution was observed.

Evolution of E. coli vs. evolution of man

One could argue that macroevolution is just the sum total of all the adaptation and microevolution that occurs. While this may be true, it would be beneficial to ask why we are not able to observe this effect even among organisms whose generational periods extend even beyond ours. In a study done by Tim Cooper, Daniel Rosen, and Richard Lenski, 12 lines of *E. coli* cells were propagated for 20,000 generations in glucose-limited media to determine what mutations they would develop separately from each other but under the same selective stress (21). In this study, the bacteria did have some changes, but they were still *E. coli* after 20,000 generations. If one was to translate 20,000 generations to a human evolutionary time scale, given a 15-20 year generational

time, this translates to 300,000 to 400,000 years. One would expect an organism under intense selective pressure to be able to evolve into a more fit organism in this amount of time if evolutionary change is possible in several thousand generations.

According to human evolutionary theory, *Homo sapiens* evolved approximately 250,000 years ago and the next most recent species lived as little as 12,000 years ago (*H. floresiensis*). Since the evolution of *H. sapiens* about 250,000 years ago, there have been at least 3 other contemporaneous species (*H. heidelbergensis*, *H. rhessiensis*, and *H. neanderthalensis*) that have come and gone (37). The number of years ago that an organism lived is much less relevant in the study of evolution than the number of generations. This is because evolution is supposed to take place through random mutations that are passed on to the next generation. *Homo sapiens* are estimated to have evolved about 250,000 years ago from *Homo erectus*. This change is said to have started to occur when the cranial capacity began increasing somewhere around 400,000 years ago (37). This would mean that the evolution from *H. erectus* to *H. sapiens* could take place in about 150,000 years. If the average generation time was 15-20 years, this gives only 7,500-10,000 generations for the new species to evolve. Over the course of 4,600 generations, our *E. coli* cells have only managed to minimally decrease their zones of inhibition and definitely have not evolved into a new species.

Macroevolution vs. Microevolution

The term microevolution was first coined by Russian entomologist Iuri'i Filipchenko in 1927 while trying to reconcile evolution with Mendelian genetics (31). Microevolution describes evolution that occurs below the species level, and can result in subspecies. Macroevolution describes evolutionary change at or above the species level.

These terms should not be confused. Microevolution has been scientifically proven, and is not under scrutiny. This phenomenon is due to a number of factors, including “natural selection, gene flow, random genetic drift, and mutation.” (32). This is made possible in part by sexual organisms with heterozygous genotypes in the population. When organisms with heterozygous genotypes mate, some of the offspring display the recessive phenotypes while the rest display the dominant phenotypes. When environmental pressures change, some of these recessive phenotypes may be more fit than other phenotypes to survive and reproduce. The net result in the population can be a shift in the phenotype to recessive traits. A well-known example of this includes Darwin’s finches. They were all still finches, but they had several differences, such as beak characteristics, behaviors, and song melodies (34). Through microevolution, subspecies can form (as in dogs) but they have not been shown to evolve into an entirely new species.

The problem with calling bacterial antibiotic resistance a “genuine example of evolutionary change,” (2) is that it doesn’t qualify as macroevolution. Usually, the genes for antibiotic resistance are already in the population of bacteria whose genomes are at least as diverse as eukaryotic organisms (29). As plasmids get passed from one bacterium to another, the genes for antibiotic resistance become more prevalent within populations (30). *In vitro* mating of Tet sensitive strains of *E. coli* to Tet resistant strains, and *in vivo* tests in mice and chickens, have shown that Tet resistance genes are transferrable in both circumstances (30). This can look similar to macroevolution, but before antibiotics were even discovered, these genes were present in the population. For example, only four years

after penicillin began to be mass produced, resistant strains *Staphylococcus aureus* were isolated (35).

In the *E. coli* cells used in this study, the population was controlled, which eliminated the possibility of acquiring antibiotic resistance genes from other organisms and allowed the organisms to be tested for the hypothesis that mutations can result in favorable genetic changes. This left random mutations to do the work of decreasing sensitivity to the antibiotics. As can be seen from our experiment, some mutations will render the bacteria more fit in this environment, but their ability to reproduce as quickly as wild type cells was hampered (figures 2 and 3).

Further Experimentation

Further experimentation is being performed to determine whether or not the *E. coli* cultures will continue to show decreased antibiotic sensitivity in successive generations or whether they will ever gain complete resistance to these antibiotics, and how long it takes if they do. Fitness tests should be performed in future experiments to evaluate their overall fitness as compared to normal K12 cells. Further tests should be done to determine whether the changes seen in the population of *E. coli* were due to genetic mutations or were a result of adaptation, also known as phenotypic plasticity, which is considered both non-genetic and reversible (33). The DNA from the adapted strain could be compared to the DNA from the original *E. coli* K12 culture to see if any genetic change, although unspecified as to effect, had occurred during the course of these experiments.

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