A Comparison of Antibiotic Resistant *Escherichia Coli* to the Wild Type Strain

Matthew Thomas Schreiner

A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2011

Abstract

Antibiotic resistance is a growing problem that has rendered many antibiotics ineffective. Bacteria can gain resistance through spontaneous mutations and horizontal gene transfer. A better understanding of the overarching effects of antibiotic resistance on resistant strains is necessary when considering possible solutions to this issue. Tetracycline and doxycycline resistant strains of *E. coli* were compared to a wild type K-12 strain to determine if resistance engenders any fitness costs. The different strains were compared using antibiotic sensitivity tests, growth rate analysis and metabolic tests. The resistant strains actually grew at a slightly faster rate than the wild type strain. However, the metabolic test revealed that, unlike the wild type strain, the resistant strains were unable to ferment dulcitol. Thus, there may be a slight fitness cost associated with the resistant strains method of antibiotic resistance.

A Comparison of Antibiotic Resistant Escherichia Coli to the Wild Type Strain

Tetracycline

While working with a soil dwelling fungus in the late 1940s, Benjamin Duggar noted that the fungus secreted a golden yellow pigment in the hygrphorous substrate mycelium (1948). He gave the fungus the name *Streptomyces aureofaciens* because of the color and went on to discover that the pigment possessed remarkable antibacterial properties. When this new antibiotic was purified it also had a faint golden yellow, so Duggar called it aureomycin. Duggar's findings led to the discovery of an entire family of antibiotics known as the tetracyclines. The tetracycline family was the first major group of antimicrobial agents to which the term *broad-spectrum* was applied (Chopra, Hawkey & Hinton, 1992). The tetracyclines have been used since the 1940's against a wide range of Gram-positive and Gram-negative bacteria (Brodersen et al., 2000). The tetracycline family members that are clinically useful comprise a group of molecules that share the same 4-ring carbocyclic structure as a base skeleton (Chopra, Hawkey & Hinton, 1992). These include chlortetracycline (called aureomycin), oxytetracycline, tetracycline, demethylchlorotetracycline, methacycline, doxycycline and minocycline. The only chemical difference between them are the substituents found at carbons 5, 6 or 7. However, the various analogues do possess different pharmacokinetic properties.

There are three binding sites on the small, or 30S, ribosomal subunit for tRNA molecules (Brodersen et al., 2000). These sites are designated the A, (aminoacyl), P (peptidyl) and E (exit) sites. Tetracycline antibiotics work by binding to and obstructing access to these sites, thereby preventing protein synthesis. A study done in the late 1980s by Bernd Epe, Paul Woolley and Horst Hornig (1987) demonstrated that tetracyclines can

reduce tRNA's affinity for the 30S ribosomal subunit's P site by some 50%, and its affinity for the A site by 80%. Before the tetracycline molecules can interact with the ribosome, they must penetrate the cell's membrane. Tetracycline crosses the outer membrane through the use of porin proteins, especially the OmpF porin (Chopra, Hawkey & Hinton, 1992). Early observations indicated that tetracycline crosses the outer membrane as a cationic chelate of magnesium, which is supported by the fact that OmpF pores are cation selective. Once inside, tetracycline binds to the 30S and 50S subunits with up to 300 tetracycline molecules binding to each ribosome (White & Cantor, 1970). White and Cantor used fluorescent studies to provide strong evidence that tetracycline also uses chelation to magnesium ions to bind to ribosomal RNA. The magnesium ion was found to be present in the 30S subunit in the absence of tetracycline, which indicates it plays some role in the structure of the 30S subunit (Brodersen et al., 2000). Tetracycline can bind to multiple weak binding sites on both the 30S and 50S subunits, but it preferentially binds to a single strong binding site on the 30S subunit (Oehler, Polacek, Steiner & Barta, 1997). Single protein omission reconstitution experiments revealed the ribosomal proteins S3, S7, S8, S14 and S19 to be essential for the high-affinity binding site, with S7 and S14 being the most vital (Buck & Cooperman, 1990).

When tetracycline binds to the ribosome it causes changes in its 3-dimensional structure (Noah, Dolan, Babin & Wllenzien). Using an ultraviolet cross-linking procedure that discerns the pattern and frequency of 16S RNA cross-links, researchers were able to determine the effect of tetracycline on the ribosome. The antibiotic completely inhibits the C967-C1400 cross-link, while decreasing the G894×U244 cross-link by 50%. The fact that these structural changes occur at the same concentrations at which tetracycline

exhibits its inhibitory effect on the ribosome indicates the structural changes play a role in tetracycline's antibacterial function.

While tetracycline preferentially binds to the 30S subunit, it does not simply block the A site (Brodersen et al., 2000). The initial binding of a ternary complex of EF-Tu with tRNA to the A site still occurs in the presence of tetracycline, as ribosome-dependent guanosine triphosphate (GTP) hydrolysis by EF-Tu is unaffected. Using this information, Brodersen et al. generated a model to explain tetracycline's mode of action. They suggested that tetracycline does not affect the ternary complex, because the approach angle of tRNA, when bound to EF-Tu, differs enough from that of free A site tRNA that it avoids a steric clash with the bound tetracycline molecule. Additionally, tetracycline is positioned between H34 in the head of the 30S and the emodeled A site tRNA, but on the opposite side of the tRNA anticodon stem loop from the codon:anticodon interaction. This allows the decoding process to proceed without interference from tetracycline. Decoding results in GTP hydrolysis on EF-Tu, causing it to release from the complex. Immediately following EF-Tu's release, tRNA tries to rotate into the A site, which leads to a steric clash with tetracycline. This results in tRNA's ejection from the ribosome. The available structural and biochemical data support this model and explain tetracycline's effectiveness as an antibiotic. Tetracycline acts catalytically, as it not only prevents protein synthesis, it also leads to the unproductive hydrolysis of GTP, which is a very expensive waste of energy for the cell.

Chloramphenicol

Another antibiotic that functions by inhibiting the ribosome is chloramphenicol. Chloramphenicol is also considered a broad-spectrum antibiotic, and works well against Gram-positive and Gram-negative bacteria (Moreira, Oliveira, Teixeira & Moraes, 2005). It has been available for clinical use since 1948. In contrast to tetracycline, chloramphenicol binds to the 50S subunit of *E. coli* ribosomes (Pestka, 1969). Once bound, chloramphenicol inhibits peptidyl transfer function. Studies have shown that at inhibitory concentrations, chloramphenicol also inhibits the binding of phenylalanyl-oligonucleotide to the ribosome. This supports the hypothesis that the antibiotic works by interfering with the aminoacyl-end of charged tRNA, preventing it from binding to the ribosome. Research by Oleg Jardetzky (1962) suggests chloramphenicol's mode of action critically depends on the steric configuration and conformation of the molecule, especially its propanol moeity. Any alteration to the propanol moiety leads to a complete loss of antibacterial function. The overall effect of chloramphenicol is the suppression of protein synthesis (Rosenkranz, 1988). Because of this, chloramphenicol is classified as a bacteriostatic agent as it does not so much kill exposed bacteria, but arrests their growth. This allows the host's defenses a chance to eliminate the invading bacteria.

Escherichia Coli

The microbe known as *Escherichia coli* was first identified in 1885 when Theodor Escherich, a German pediatrician, cultured what he called "Bacterium coli" from the feces of healthy individuals (Lederberg, 2004, p. 116). This bacterium, which is found almost universally in the colon, hence *coli*, was renamed *Escherichia coli* in honor of its discoverer in revision of bacteriological nomenclature. The most famous strain of *E. coli*

is the K-12 strain, which was isolated at Stanford University in 1922 from human feces and used as a stock strain in their bacteriological department. In 1946, Joshua Lederberg's pioneering work with the K-12 strain led to his discovery of sexual recombination, for which he received the Nobel Prize in 1958. Since then, *E. coli* has been used in thousands of genetic studies and is possibly one of the most studied organisms in science. *E. coli*, a member of the Enterobacteriaceae family, is a small Gram-negative rod that is approximately 3 μm long and 1 μm in diameter (Dougan et al., 2001; Reshes, Vanounou, Fishov & Feingold, 2008). One reason *E. coli* is such a popular choice in laboratories is its extremely fast life cycle, as it can reproduce or duplicate itself every 20 minutes on rich media. Recent studies have shown that the *E. coli* genome consists of a conserved core of genes that provide the information necessary for survival (Dobrindt, 2005). For instance, in the case of the K-12 strain, as much as 18% of the genome is made up of foreign DNA that was acquired through horizontal transfer (Lawrence & Ochman, 1998). The flexibility of the *E. coli* genome has led to a wide variety of enterobacterial species that are typically subdivided into three categories, commensal or non-pathogenic, intestinal pathogenic and extraintestinal pathogenic strains (Johnson & Russo, 2005). This ability to adapt is due to the ease in which *E. coli* can mix and match various genes. For instance, gene islands can be transferred between cells using vectors such as bacteriophages (Dougan et al., 2001). Furthermore, *E. coli* can circulate genes that confer specific phenotypes, such as antibiotic resistance, on extrachromosomal elements in the form of plasmids. The flexibility of *E. coli* allows them to colonize efficiently a wide range of environments and has led to many pathogenic and antibiotic resistant strains.

Antibiotic Resistance

Nobel laureate Alexander Flemming fired the first shot against the microscopic world in 1928, when he discovered the antibiotic properties of a substance he named penicillin. Since then, man has been involved in a new, escalating war with bacteria. Certain strains of bacteria have slowly gained a resistance to the antibiotics that once killed them. To counter this, new antibiotics have been produced, but as time passed their effectiveness also decreased. An article by *Time Magazine* stated that, "Modern medicine has engaged disease-causing microbes in an escalating arms race, so that as soon as drug developers launch a new weapon--an antibiotic, for example--their microbial foes respond by shoring up their own defenses" (Nash, 2001, p. 2). Studies have revealed a direct relationship between antibiotic use and antibiotic resistance. On a global scale, it can be shown that countries that use antibiotics more frequently have a larger incidence of antibiotic resistant bacteria (Cizman, 2003). A more recent study revealed a significant correlation between the quantity of tetracycline used in a population and the levels of tetracycline resistant *Escherichia coli* found in that population's waste (Alali et al., 2009). In another such study a direct correlation between antibiotic exposure and resistance was established for multiple strains of bacteria (Alonso-Hernando, Capita, Prieto, & Alonso-Calleja, 2009).

Bacteria typically possess antibiotic resistance via one or a combination of three general mechanisms. They can alter the target molecule of the drug, destroy the drug or pump the drug out (Wright, 2005). These mechanisms can be reached via random mutations and horizontal gene transfer. Horizontal gene transfer involves sharing genetic information between members of the same, or other species of bacteria (Davison,

Woolhouse & Low, 2000). Genes that impart resistance to specific antibiotics can be encoded in integrons, transposons or plasmids, which are passed back and forth (Alekshun & Levy, 2007). In the presence of antibiotics these resistance genes will be highly selected for and will permeate the population. This is helped along by the fact that antibiotics can modify bacterial physiology to make it more receptive to foreign DNA. This phenomenon was demonstrated in a study that examined the effects of stress on intergeneric mating in bacteria, and is likely due to denaturation of enzymes responsible for degrading foreign DNA (Shafer, Kalinowski & Puhler, 1994).

Additionally, random mutations can impart antibiotic resistance to a wide variety of drugs. Gram-positive bacteria can gain resistance to fluoroquinolones in this way. Fluoroquinolones interact with the enzymes DNA gyrase and topoisomerase IV, which are required for DNA replication (Hooper, 2002). By binding with complexes on each of these enzymes, fluoroquinolones can block DNA replication, which ultimately ends in bacterial cell death. Spontaneous mutations can alter the amino acid sequence of the two enzymes inhibiting the drug from binding. While a mutation in one of the target enzymes will reduce a strain's susceptibility to fluoroquinolones, mutations in both enzymes result in fully resistant bacteria.

Spontaneous mutations can also enhance the removal of antibiotics from the interior of the bacterial cell. All bacteria are equipped with efflux pumps that function in the removal of toxins, but the normal quantity of these pumps is not enough to translate into any sort of significant antibiotic resistance. Certain mutations can alter the expression of these pumps to increase their potential. *E. coli* develop low level multi-antibiotic resistance through a mutation that cripples the regulatory protein marR, leading

to an over expression of the efflux pump AcrAB (Grkovic, Melissa & Skurray, 2001). Similarly, a mutation in the *N. gonorrhea* repressor mtrR leads to elevated expression of the mtrCDE operon. This operon encodes a multidrug efflux complex that confers increased levels of resistance to antimicrobial hydrophobic agents. Lastly, *P. aeruginosa* gain a resistance in much the same way. A mutational inactivation of the MexR regulatory protein generates overproduction of the MexAB-OprM pump. Consequently, this reduces *P. aeruginosa's* susceptibility to a wide range of drugs.

E. Coli Antibiotic Resistance

In most cases, tetracycline resistance results from bacteria acquiring resistance genes via horizontal transfer and not from mutations (Baum & Marre, 2005). When it comes to tetracycline resistance in *E. coli,* there are two principle mechanisms: drug efflux and ribosomal protection. Efflux is the more common of the two. In fact, 23 of the 33 distinct tetracycline resistance genes, known as *tet* genes, code for efflux pumps that transport tetracycline actively out of the cell. The other 10 resistance genes code for proteins that protect the ribosome from tetracycline. This second set of proteins has genetic sequences that are very similar to bacterial elongation factors (EF-G and EF-Tu) (Alekshun & Levy, 2007). Quite a few of the ribosomal protection protein (RPP) determinants are located on mobile genetic elements, which allows them to spread easily between bacterial populations via horizontal transfer events (Connell, Tracz, Nierhaus & Taylor, 2003). Included in this class are the RPPs Tet(O), Tet(M), Tet(S), Tet(T), Tet(Q), TetB(P), Tet(W), and OtrA. Tet(O) and Tet(M); the best studied of these determinants, are soluble cytoplasmic proteins with molecular weights of ~72kDa. Researchers have determined that these two RPPs confer antibiotic resistance by forcing tetracycline to

release from the ribosome, thereby allowing protein synthesis to continue. When tetracycline binds to the ribosome it renders it functionally paralyzed with an open A site, which provides a kinetic window for Tet(O) to act. This allows Tet(O) to bind selectively to tetracycline-blocked ribosomes. Once bound, Tet(O) causes a slight conformational change in h34 that results in the ejection of the tetracycline molecule.

Drug efflux pumps are non-specific resistance mechanisms that allow the cell to remove a broad range of structurally unrelated compounds (Kumar & Schweizer, 2005). Efflux pumps are classified into one of five families. These include the major facilitator superfamily (MFS), the adenosine triphosphate binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family and the resistance-nodulation-cell division (RND) superfamily. The *E. coli* genome has been shown to possess seven RND transporters, and five of these are confirmed to participate in drug efflux. Researchers determined that the driving force of these tetracycline efflux systems involves a proton gradient. One study performed by Midory Kaneko, Akihito Yamaguchi and Tetsuo Sawai (1985) used different pH solutions to show that tetracycline transport was carried out by an electrically neutral antiport system that swapped protons and a cationic form of tetracycline. In fact, all RND transporters discovered to date have been shown to function via a substrate/H^+ antiport mechanism (Kumar & Schweizer, 2005).

Multi-component efflux pumps found in gram-negative organisms are formed from the combination of a periplasmic membrane fusion protein component and an outer membrane protein. The outer membrane proteins (OMP) of *E. coli* play a critical part in this method of resistance to tetracycline antibiotics. Researchers examining this crucial role performed by outer membrane complexes discovered an upgregulation of the

proteins FimD, Tsx OmpW, OmpC and TolC and a downregulation of LamB in response to tetracycline (Zhang, Jian, Xiang, & Wang, 2008). Using genetically modified *E. coli* strains, they were able to ascertain that of these six proteins, upregulation of TolC and OmpC and downregulation of LamB was required for tetracycline resistance. The protein TolC works with AcrAB to form an efflux pump that is responsible for the removal of toxins such as antibacterial drugs, detergents, organic solvents and haemolysin directly from the cytosol. OmpC is a small, classical porin that, along with the large porin OmpF, is transcriptionally regulated by the EnvZ/OmpR two-component signal transduction regulatory system. In normal conditions the large OmpF porin is highly expressed, but in hostile circumstances, which are defined as a medium containing antibiotics or detergents, the cell upregulates OmpC in order to decrease membrane permeability. Thus, TolC and OmpC work synergistically to export antibiotics out of the cell while simultaneously inhibiting their entry into the cell. Lastly, LamB is a trimeric outer membrane porin protein that acts as a channel for maltodextrins and maltose. Downregulation of LamB decreases permeability of *E. coli's* membrane, which blocks tetracycline from entering the cell.

Cost of Resistance

Most mutations that confer resistance actually decrease the overall fitness of the bacteria in a normal environment (Andersson & Levin, 1999). One study revealed multi-antibiotic resistant *E. coli* are more susceptible to outside influences and are significantly more sensitive to heat than normal strains (Duffy, Walsh, Blair & McDowell, 2006). Alteration to an enzyme may impart antibiotic resistance, but it leaves the enzyme less stable and inhibits its original function (Wang, Minasov & Shoichet, 2002). Researchers

have discovered that even resistance genes obtained through horizontal transfer result in an overall decrease in the fitness of the bacteria (Gillespie, 2001). For instance, the TcR gene of plasmid pBR327par', which grants tetracycline resistance, was shown to have deleterious effects to cell growth (Lee & Edlin, 1985). In some cases, the resistant strain will spontaneously revert to the healthier, sensitive strain in a drug free environment (Massey, Buckling & Peacock, 2001). These findings coincide with observational studies that demonstrated that decreasing the use of antibiotics typically lowers levels of resistant bacteria (Baum & Marre, 2005). The fitness cost of these mutations is precisely why regulating the use of antibiotics is an effective method for combating resistance (Ntagiopoulos, Paramythiotou, Antoniadou, Giamarellou & Karabiniset, 2007).

While the majority of studies performed on antibiotic resistance reveal an associated fitness cost, this is not always the case (Andersson & Levin, 1999). Researchers have also discovered antibiotic resistant mutants with no measurable costs. In one instance, a mutation with no observable cost conferred resistance to high concentrations of streptomycin to *S. typhimurium* and other enteric bacteria. A factor that makes conclusive results difficult to procure is the observed difference between bacteria grown in vitro and in vivo. In an unpublished study cited by Andersson and Levin (1999), certain mutant bacteria that possess no fitness cost on laboratory medium (in vitro) struggle to grow in laboratory mice (in vivo). Conversely, mutants can show no cost in laboratory mice, but have large costs in vitro.

Methods and Materials

E. coli Cultures Used

The purpose of this experiment was to compare antibiotic resistant *E. Coli* strains to a wild type K-12 control strain. The antibiotic resistant strains used in this experiment were the result of a study performed by Cac Thanh Bui. In her experiment, Bui developed *E. coli* strains that were resistant to the antibiotics tetracycline and doxycycline from several strains of *E. coli* K-12, which were provided by Dr. Randall Hubbard and the Liberty University Biology Department. She did this by passing many generations of the cultures in the presence of the two antibiotics. Bui finished her work after passing each strain 64 times, which roughly equates to 10,000 generations. The strain passed in the presence of tetracycline strain was labeled Tet 64, and the strain passed in the presence of doxycycline was labeled Dox 64. The K-12 control strain was provided by Dr. Randall Hubbard. These three strains of *E. coli* were used in all of the tests performed in this study.

Preparation of *E. coli* Cultures

Before any tests were performed on Bui's *E. coli* strains, fresh cultures were prepared. The Dox 64, Tet 64 and K-12 strains had been stored in BBL™ trypticase soy broth (TSB; Becton, Dickson, & Co.) tubes in a refrigerator at 23°C. These strains were streaked for isolation on BBL™ trypticase soy agar (TSA; Becton, Dickson, & Co.) and incubated at 37°C. Representative colonies were re-incubated in TSB at 37°C and Gram stained to make sure of identity and purity. After the purity of the cultures was established they were used for the remaining tests.

Measuring Antibiotic Resistance

In order to determine the degree of antibiotic resistance, the Tet 64 and Dox 64 strains were subjected to Kirby-Bauer (KB) disc diffusion tests. The results of these tests were then compared to the K-12 strain, which was used as a control. First, each strain was incubated in a TSB tube at 37°C. Once the bacteria had been allowed to grow for at least 48 hours, the tubes were diluted with TSB to an optical density (OD) of 0.5 at 600nm. A pipette was used to transfer 0.2mL from each tube to a separate TSA plate, which was spread out using a sterile spreader. Doxycycline and tetracycline antibiotic discs (Becton, Dickson, & Co.) were placed on the Dox 64 and Tet 64 plates respectively. The K-12 (wt) plate received both discs. The paper discs contain 30μg of the antibiotic, which diffuses into the agar creating a concentration gradient. This creates a zone of inhibition (ZI) in which sensitive bacteria are unable to grow. The diameter of the ZI corresponds to the level of sensitivity of the particular strain being tested. The *E. coli* colonies growing closest to the disc were re-incubated and the KB test was repeated several times to remove any non-resistant colonies from the Dox 64 and Tet 64 cultures. After this precaution, the diameter of the ZI from the Dox 64, Tet 64 and K-12 strains were measured and recorded. The percent difference of the ZI were calculated using the equation:

$$(\text{Diameter}_{WT} - \text{Diameter}_{Res}) / [(\text{Diameter}_{WT} + \text{Diameter}_{Res}) / 2] \times 100\%.$$

This same technique was used to determine the Tet 64 and Dox 64 strains' sensitivity to a broad range of antibiotics. K-12 was used as a control. The antibiotics tested using KB disc diffusion tests were chloramphenicol, ciprofloxin, erthromycin, kanamycin, SXT, streptomycin, ampicillin, oxacillin, bacitracin, penicillin, and

vancomycin. The resulting inhibition zones were measured for each antibiotic and recorded.

Growth Rate Determination

This test was used to compare the growth rates of the resistant strains with the wild type strains. The six cultures used, Dox 61, Dox 63, Tet 64, Tet 65 and the wild type ancestor strains Tet 1 and Dox 1, were grown in TSB at 37°C to an OD of 0.5 at a wavelength of 600nm. A pipette was used to transfer 0.2mL from each of these tubes to fresh tubes containing 10mL of TSB. These tubes were incubated at 37°C and every two hours the tubes were gently agitated to homogenize the contents and the OD was measured. These measurements continued until there was no increase in OD for over 6 hours. A tube containing pure TSB was used to form a baseline OD for comparison. The rate of change in OD for each strain corresponds to their relative growth rates.

Gram Stains

In order to compare the morphological features of the various strains, gram stains of each strain was prepared. The strains used were Dox 64, Tet 64 and K-12. The Dox 64, Tet 64 and K-12 strains were plated on TSA with doxycycline and tetracycline discs respectively. Samples for gram staining were taken from the edge of the ZI as well as from an area of the plate distant from the antibiotics. The K-12 sample served as a control. A Leica DME microscope equipped with a Moticam 2000 and Motic DS Capture software was used to take pictures of each culture at 1000X. A picture of a Reichert micrometer was also taken in order to provide a rough scale.

Metabolism Tests

The Tet 64, Dox 64 and K-12 strain were used in a series of tests to determine their metabolic capabilities. Bergey's Manual of Determinative Bacteriology was used to determine what wild type *E. coli* are normally capable of (Holt, Krieg, Sneath, Staley & Williams, 1994). Specific reagents were incubated with each strain. These reagents include indole, methyl red, ornithine decarboxylase, D-glucose (to test for acid production/gas production), L-arabinose, dulcitol, glycerol, lactose, maltose, D-mannose, raffinose, salicin, D-sorbital, sucrose, trehalose, D-xylose, and a nitrate reduction test.

Results

Antibiotic Resistance Tests

For the first round of the KB tests, Dox 64, and Tet 64 were tested for their susceptibility to doxycyline and tetracycline respectively. K-12 was used as a control. Each plate received three antibiotic discs and the diameters recorded are the average of the three ZI. The ZI are measured in mm and are recorded in Table 1.

Table 1. Dox and Tet ZI

E. coli Strains	Tet ZI diameter (mm)	Dox ZI diameter (mm)
Dox 64	14.0±0.75	12.5±0.75
Tet 64	14.0±0.75	13.0±0.75
K-12	21.0±0.45	19.0±0.53

The *E. coli* strains Dox 64, Tet 64 and the wt strain K-12 were plated on TSA with antibiotic diffusion discs. The resulting ZI diameters were measured and averaged. Standard deviation (±) was calculated with n=11.

The results of the first Kirby-Bauer test show that the Dox 64 and Tet-64 strains are considerably less sensitive to tetracycline and doxycycline than the wt strain K-12. This phenomenon is the result of raising 10,000 generations of *E. coli* in the presence of these antibiotics. Interestingly, Dox 64 and Tet 64 have almost the same sensitivity profile. In the case of tetracycline, the ZI was reduced by 7mm for both Dox 64 and Tet 64. Doxycycline's ZI reduction was very close for both strains, as Dox 64 reduced the ZI by 6.5mm and Tet 64 reduced the ZI by 6mm. This similarity between strains would suggest that their methods of antibiotic resistance are not specific to either doxycycline or tetracycline.

The percent differences between the wt K-12 stain's ZI and the two resistant strain's zones Dox 64 and Tet 64 were calculated and are shown in Table 2.

Table 2. Percent difference of ZI diameters

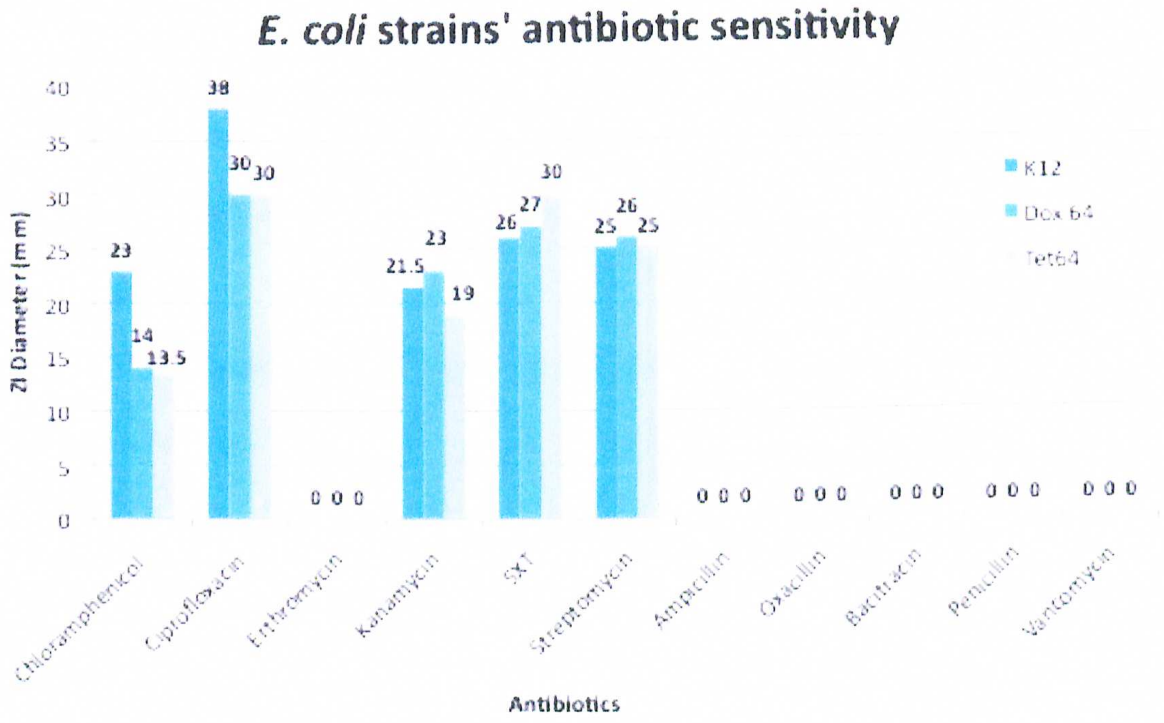
E. coli Strains	% Diff. Tet	% Diff. Dox
Dox 64 vs K-12	50.0%	41.3%
Tet 64 vs K-12	50.0%	37.5%

The *E. coli* strains Dox 64, Tet 64 and the wt strain K-12 were plated on TSA with antibiotic diffusion discs. The diameters of the resulting ZI were compared using the percent difference formula.

Table 2 indicates that tetracycline was 50% less effective against Dox 64 and Tet 64 as compared to the wt strain. Doxycycline was 41.3% and 37.5% less effective against Dox 64 and Tet 64 respectively.

The second set of KB tests used a wide variety of antibiotics to ascertain the limits of Dox 64 and Tet 64's antibiotic resistance. The antibiotics tested using KB disc diffusion tests were, chloramphenicol, ciprofloxacin, erthromycin, kanamycin, SXT, streptomycin, ampicillin, oxacillin, bacitracin, penicillin, and vancomycin. K-12 was used as a control. The relative effectiveness of each antibiotic is shown in Figure 1.

Figure 1.



The *E. coli* strains Dox 64, Tet 64 and K-12 were plated with a variety of antibiotic diffusion discs. The diameters of the resulting ZI were measured and compared. (For chloramphenicol n=3)

As Figure 1 shows, the sensitivity profile of the three strains is very similar for most of the antibiotics. The only major difference between the two resistant strains and the wt K-12 strain is with the antibiotics chloramphenicol and ciprofloxacin. Table 3 compares the effectiveness of those antibiotics using percent difference.

Table 3. Percent difference of ZI diameters for Chloramphenicol and Ciprofloxacin

E. coli Strains	% Diff. Chloramphenicol	% Diff. Ciprofloxacin
Dox 64 vs K-12	48.6%	23.5%
Tet 64 vs K-12	52.1%	23.5%

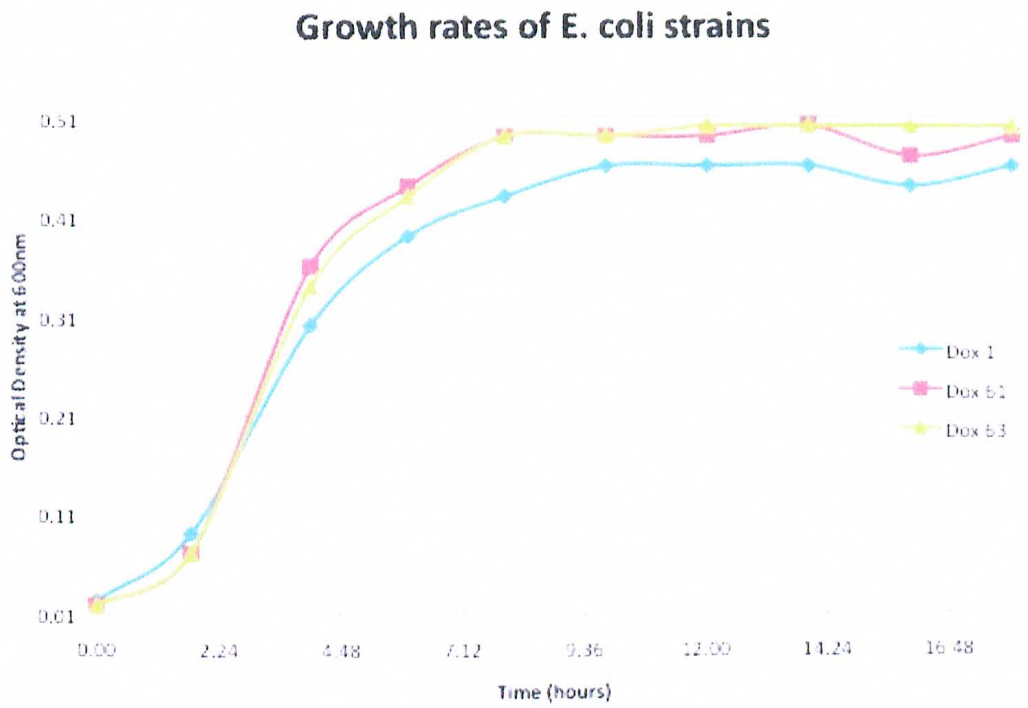
This table compares the effectiveness of chloramphenicol and ciprofloxacin on Dox 64 and Tet 64 as compared to K-12.

Table 3 shows that chloramphenicol is 48.6% and 52.1% less effective on Dox 64 and Tet 64 respectively, when compared to the wt strain. This is very close to the level of increased resistance Dox 64 and Tet 64 experience in the presence of doxycycline and tetracycline. This suggests that the mechanism of resistance is non-specific.

Growth Rate

The changing optical densities of the different *E. coli* strains were recorded as they grew to gauge their relative growth rates. The three Dox strains tested were the ancestor wt strain, Dox 1, which was used as a control and two resistant strains Dox 61 and Dox 63. The OD of the three growing strains was recorded every two hours over a period of 18 hours and the results are shown in Figure 2.

Figure 2.

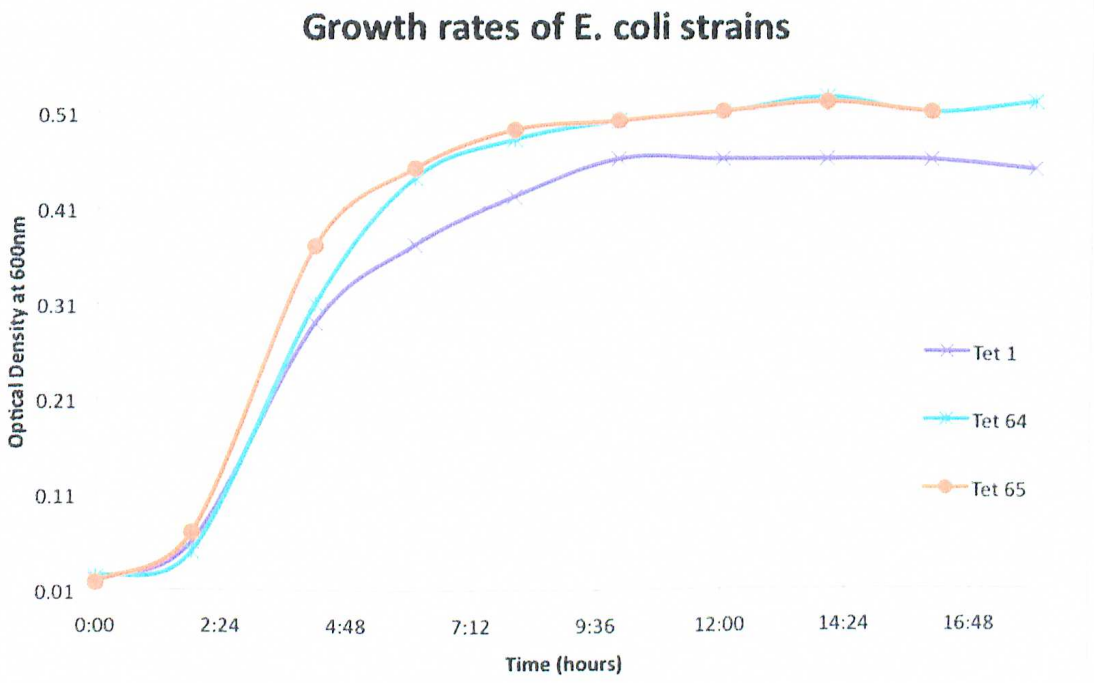


A line graph of the changing optical densities of the *E. coli* strains Dox 1, Dox 61 and Dox 63 growing in TSB.

The graph indicates that all three strains grow at almost the same rate. While, the two resistant strains, Dox 61 and Dox 63, do have a slight advantage after the first 2 hours, the difference is almost negligible.

Likewise, three different Tet strains were tested. These included the ancestor wt strain Tet 1, as well as the resistant strains Tet 64 and Tet 65.

Figure 3.



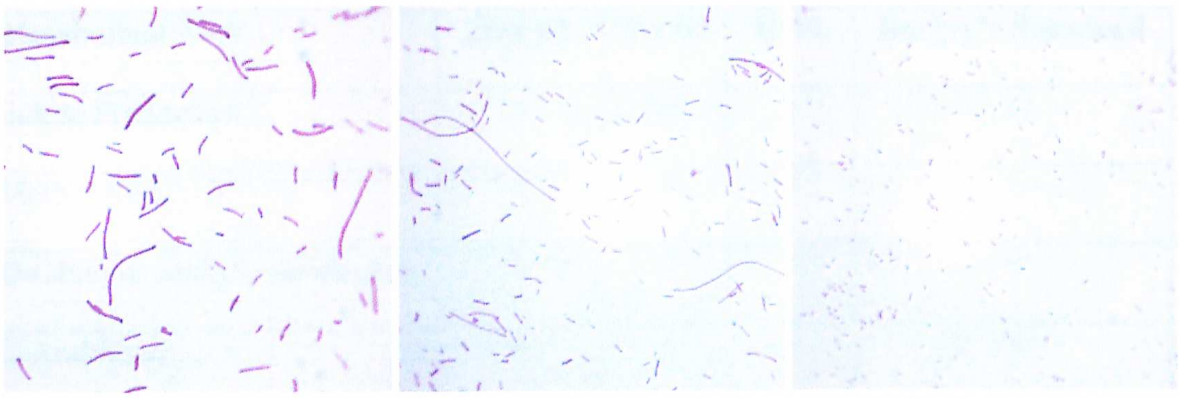
A line graph of the changing optical densities of the *E. coli* strains Tet 1, Tet 64 and Tet 65 growing in TSB.

The graph depicting the growth rates of the three Tet strains shows similar growth characteristics to the Dox strains shown in Figure 2. All three strains grow at nearly the same rate. Again, the two resistant strains, Tet 64 and Tet 65, seem to have a slight advantage, but the difference is small. Interestingly, in both cases the wt control strain's growth leveled out and stabilized at a lower final OD than any of the resistant strains.

Gram Stain

The gram stains show the *E. coli* strains experience a morphological change in the presence of the antibiotic. Figure 4 shows representative pictures taken of the Dox 64, Tet-64 and K-12 strains.

Figure 4. Pictures of *E. coli* strains at 1000X



(Left Tet 64: from edge of ZI, Middle: Dox 64 from edge of ZI, Right: K-12) These pictures show the general shape and size of each *E. coli* strain. As shown, cells exposed to doxycycline and tetracycline grew much longer than the wild type K-12 strain.

The gram stains reveal that tetracycline and doxycycline cause morphological changes in the shape and size of the *E. coli* cells. Exposure causes them to grow many times longer than is normal. Samples from all three stains obtained at the edge of the antibiotics' ZI all showed abnormally long growth (K-12 sample from edge of ZI not shown). This is almost certainly a result of antibiotic exposure since a sample of the same culture taken away from the antibiotic disc revealed normal cell shape (not shown). Additionally, these morphological changes are not unique to the resistant strains as the control (K-12) behaved similarly. A micrometer was used to provide a rough measurement for the abnormal growth. A representative cell was estimated to be ~40μm long, which is 13 times longer than a normal *E. coli* cell.

Metabolic Tests

The results of the metabolic tests are recorded in Table 4. These tests show the capabilities of the Tet 64, Dox 64 and K-12 strains and compare them to the standard as described by Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Table 4. Metabolism Tests

Metabolism Test	**Dox 64**	**Tet 64**	**K-12**	**Bergey's Standard**
Indole Production	+	+	+	+
Methyl Red	+	+	+	+
D-Glucose acid/gas production	+	+	+	+
L-Arabinose	+	+	+	+
Dulcitol	-	-	+	d
Glycerol	+	+	+	d
Lactose	+	+	+	+
Maltose	+	+	+	+
D-mannose	+	+	+	+
Raffinose	-	-	-	d
Salicin	-	-	-	d
D-Sorbitol	+	+	+	+
Sucrose	-	-	-	d
Trehalose	-	+	+	+
D-Xylose	+	+	+	+
Nitrate Reduction	+	+	+	+

Three *E. coli* strains, Dox 64, Tet 64 and K-12, were tested for their ability to metabolize a series of reagents. These results are compared against the standard metabolic profile of *E. coli* as seen in Bergey's Manual of determinative bacteriology. (A + symbol indicates a positive result, a d indicates a positive result for most strains, and a – symbol indicates a negative result)

The only discrepancies that occur between the resistant strains Dox 64 and Tet 64, and the control strain K-12 are with the trehalose and dulcitol tests. In the case of trehalose metabolism, Dox 64 failed to ferment the sugar. Moreover, Dox 64 and Tet 64 were unable to ferment dulcitol, while K-12 could. These results seem to indicate that the two resistant strains have lost an ability that the wild type strain possesses.

Discussion

The antibiotic sensitivity tests confirmed that Dox 64 and Tet 64 had become less sensitive to tetracyclines after being raised by serial passage in the presence of doxycycline and tetracycline respectively. Tetracycline was 50% less effective against Tet 64 and Dox 64 than the wt strain K-12. Similarly, doxycycline was 41.3% less effective against Dox 64 and 37.5% less effective against Tet 64 when compared to K-12. Additional tests also showed that Dox 64 and Tet 64 had become less sensitive to drugs with which they had not previously come in contact. Chloramphenicol was 48.6% less effective against Dox 64 and 52.1% less effective against Tet 64 when compared to K-12. Ciprofloxacin was 23.5% less effective against Dox 64 and Tet 64 when compared to K-12. The drugs tetracycline, doxycycline and chloramphenicol all function by inhibiting prokaryotic ribosomes (Brodersen et al., 2000; Rosenkranz, 1988). Thus, while they inhibit the ribosome through different mechanisms, it is not merely a coincidence that the Dox 64 and Tet 64 strains have decreased the relative effectiveness of the three antibiotics by almost the exact same amount. Since neither the Dox 64 nor Tet 64 strain had any previous exposure to chloramphenicol, they could not have generated a specific mechanism of resistance to the drug. Therefore, it is safe to assume that Dox 64 and Tet 64 possess a non-specific multidrug resistance mechanism. Dox 64 and Tet 64 also share

the same levels of resistance to tetracycline and doxycycline, which would indicate the two strains possess the same mechanism of resistance. This should come as no surprise since tetracycline and doxycycline are virtually identical in structure (Chopra, Hawkey & Hinton, 1992). Of the two most common methods of tetracycline resistance among *E. coli*, efflux pumps and ribosome protection, the most likely is efflux pumps (Baum & Marre, 2005). Ribosomal protection is unlikely since the tetracyclines and chloramphenicol bind to different ribosomal subunits (Brodersen et al., 2000; Pestka, 1969). Furthermore, ciprofloxacin's mode of action is to inhibit DNA gyrase, so ribosomal protection would not account for Dox 64 and Tet 64's decreased sensitivity to it (Nawaz, Rauf, Akhtar & Khalid, 2006). Therefore, Dox 64 and Tet 64 most likely possess low-level multidrug resistance due to efflux pumps.

Next, a growth rate test was performed on the Dox strains (Dox 63, Dox 61 and Dox 1) and the Tet strains (Tet 65, Tet 64 and Tet 1) to determine if their newly acquired antibiotic resistance included a fitness cost. In the first set, Dox 1, the wt control strain, was compared to Dox 61 and Dox 63. The growth rates of the three strains were very similar. Dox 1 initially had a faster growth rate, but was quickly caught and slightly passed by the resistant strains. The three Tet strains were even closer initially. However, Tet 1 peaked sooner then the resistant strains Tet 64 and Tet 65. The difference between the resistant strains and the wt strains was not large enough to warrant any conclusions on the relative fitness of the different strains.

The Gram stains of the different strains showed they all experienced a morphological change in the presence of tetracycline or doxycycline. These antibiotics caused the *E. coli* cells to grow many times longer than normal. However, there was no

observable difference in the shape or size of the different strains. All three (Tet 64, Dox 64, K-12) reacted identically in the presence of the antibiotics, and all three grew normally in the absence of antibiotics.

The metabolic tests performed on the different strains revealed a couple of interesting abnormalities. In almost every case, Dox 64, Tet 64 and K-12 had identical results. However, K-12 was able to ferment dulcitol, while the resistant strains could not. Furthermore, Dox 64 was unable to ferment trehalose, while Tet 64 and K-12 could. This could simply be an anomaly since Tet 64 and Dox 64 are probably identical strains. Nonetheless, the dulcitol test does seem to indicate that Tet 64 and Dox 64 lost a metabolic ability when they gained antibiotic resistance. Since the growth rate test was conducted in TSB tubes (a nutrient rich medium), this small deficiency would not have impeded them in that test (PML Microbiologicals, 2009). This would explain why potentially handicapped strains grew at the same rate as the wt K-12 strain.

More research must be conducted to determine whether Dox 64 and Tet 64's antibiotic resistance is linked to an inability to ferment dulcitol or if that is simply a coincidence. Additionally, any fitness costs that might be detected in vitro must be viewed cautiously. Previous studies have shown that in vitro fitness is not always a good indicator of in vivo fitness (Andersson & Levin, 1999).

Equally important is determining how Dox 64 and Tet 64 gained low-level multidrug resistance to doxycycline, tetracycline, chloramphenicol and ciprofloxacin. One possible conclusion is that repeated exposure to the antibiotics caused Dox 64 and Tet 64 to up-regulate efflux proteins, which allow them to survive at higher concentrations of doxycycline and tetracycline. This would also explain why the

resistance is non-specific, as well as only being partial resistance. A test that isolated known *E. coli* drug efflux proteins and compared concentrations between strains could be used to confirm this hypothesis.

In conclusion, the antibiotic resistant *E. coli* strains, Dox 64 and Tet 64 were shown to possess low level multidrug resistance. This is likely due either to decreased membrane permeability, increased drug efflux, or a combination of the two. In addition to decreased sensitivity to doxycycline and tetracycline, both strains also possessed decreased sensitivity to chloramphenicol and ciprofloxacin. The resistant strains (Dox 64, Tet 64) did not exhibit any associated fitness costs when compared to the K-12 (wt) strain using a growth rate test. However, unlike the K-12 strain, the resistant strains were not able to ferment dulcitol as shown in the metabolism tests.

Acknowledgements

I would like to thank Dr. Hubbard for providing all the materials and cultures I used in this experiment. His advice and knowledge were invaluable. I would also like to thank Matthew Henry for assisting me in the lab on many occasions. Lastly, I would like to express my gratitude to my thesis chair, Dr. Hubbard, and my readers Dr. Sattler and Dr. Adu-Gyamfi for helping me edit and revise my thesis.

References

Alali, W. Q., Scott, H. M., Christian, K. L., Fajt, V. R., Harvey, R. B., & Lawhorn, D. B. (2009). Relationship between level of antibiotic use and resistance among *Escherichia coli* isolates from integrated multi-site cohorts of humans and swine. *Preventative Veterinary Medicine, 90*(3-4), 160-167.

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell 128*(6), 1037-1050.

Alonso-Hernando, A., Capita, R., Prieto, M., & Alonso-Calleja, C. (2009). Comparison of antibiotic resistance patterns in *Listeria monocytogenes* and *Salmonella enterica* strains pre-exposed and exposed to poultry decontaminants. *Food Control*, 20(12), 1108-1111.

Andersson, D., & Levin, B. (1999). The biological cost of antibiotic resistance. *Current opinion in Microbiology 2*(5), 489-493.

Baum, H. V., & Marre, R. (2005). Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *International Journal of Medical Microbiology, 295*(6-7), 503-511.

Brodersen, D. E., Clemons, W. M., Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T., & Ramakrishnan, V. (2000). The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30s ribosomal subunit. *Cell, 103*(7), 1143-1154.

Buck, M. A., & Cooperman, B. S. (1990). Single protein omission reconstitution studies of tetracycline binding to the 30S subunit of *Escherichia coli* ribosomes. *Biochemistry, 29*(22), 5374-5379.

Chopra, I., Hawkey, P. M., & Hinton, M. (1992.) Tetracyclines, molecular and clinical aspects. *Journal of Antimicrobial Chemotherapy, 29*(3), 245-277.

Cizman, M. (2003). The use and resistance to antibiotics in the community. *The international Journal of Antimicrobial Agents 21* (4), 297-307.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., & Taylor, D. E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrobial Agents and Chemotherapy, 47*(12), 3675-3681.

Davison, H. C., Woolhouse, M., & Low. J. (2000). What is antibiotic resistance and how can we measure it? *Trends in Microbiology, 8*(12), 554-559.

Dobrindt, U. (2005). (Patho-)genomics of *Escherichia coli. International Journal of Medical Microbiology, 295*(6-7), 357-371.

Dougan, G., Haque, A., Pickard, D., Frankel, G., O'Goara, P., & Wain, J. (2001). The *Escherichia coli* gene pool. *Current Opinion in Microbiology, 4*(1), 90-94.

Duffy, G., Walsh, C., Blair, I. S., & McDowell, D. A. (2006). Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in food matrices. *International Journal of Food Microbiology 109*(3), 179-186.

Duggar, B.M. (1948). Aureomycin: A product of the continuing search for new antibiotics. *Annals of the New York Academy of Science, 51*, 177-181.

Epe, B., Woolley, P., & Hornig, H. (1987). Competition between tetracycline and tRNA at both P and A sites of the ribosome of *Escherichia coli. FEBS Letters 213*(2), 443-447.

Gillespie, S. H. (2001). Antibiotic resistance in the absence of selective pressure. *International Journal of Antimicrobial Agents 17*(3), 171-176.

Grkovic, S., Melissa, H. B., & Skurray, R. A. (2001). Transcriptional regulation of multidrug efflux pumps in bacteria. *Seminars In Cell & Developmental Biology 12*(3), 225-237.

Holt, J. H., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams S. T. (1994). *Bergey's Manual of Determinative Bacteriology* (9th ed.). Baltimore, MA: Williams and Wilkins.

Hooper, C. D. (2002). Fluoroquinolone resistance among Gram-positive cocci. *The Lancet Infectious Diseases 2*(9). 530-538.

Jardetzky, O. (1962). Studies on the mechanism of action of chloramphenicol. *The Journal of Biological Chemistry, 238*(7), 2498-2508.

Johnson, J. R., & Russo, T. A. (2005). Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli. International Journal of Medical Microbiology, 295*(6-7), 383-404.

Kaneko, M., Yamaguchi, A., & Sawai, T. (1985). Energetics of tetracycline efflux system encoded by Tn10 in *Escherichia coli. FEBS Letters, 193*(2), 194-198.

Kumar, A., & Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. *Advanced Drug Delivery Reviews, 57*(10), 1486-1513.

Lawrence, J. G., & Ochman, H. (1998). Molecular archaeology of the *Escherichia coli* genome. *PNAS, 95*(16), 9413-9417.

Lederberg, J. (2004). *Escherichia coli* K-12. *Microbiology Today, 31*, 116.

Lee, S. W., & Edlin, G. (1985). Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli. Gene 39*(2-3), 173-180.

Massey, R., Buckling, A., & Peacock, S. (2001). Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. *Current Biology 11*(22), 1810-1814.

Moreira, M. A. S., Oliveira, J. A., Teixeira, L. M., & Moraes, C. A. (2005). Detection of a chloramphenicol efflux system in *Escherichia coli* isolated from poultry carcass. *Veterinary Microbiology, 109*(1-2), 75-81.

Nash, J. M. (2001). The antibiotic crisis. Time.com. Retrieved from http://www.time.com/time/magazine/article/0,9171,998967-2,00.html

Nawaz, H., Rauf, S., Akhtar, K., & Khalid, A. M. (2006). Electrochemical DNA biosensor for the study of ciprofloxacin-DNA interaction. *Analytical Biochemistry 354*(1), 28-34.

Noah, J. W., Dolan, M. A., Babin, P., & Wollenzien, P. (1999). Effects of tetracycline and spectinomycin on the tertiary structure of ribosomal RNA in the *Escherichia coli* 30 s ribosomal subunit. *The Journal of Biological Chemistry, 274*, 16576-16581.

Ntagiopoulos, P. G., Paramythiotou, E., Antoniadou, A., Giamarellou, H., & Karabinis, A. (2007). Impact of an antibiotic restriction policy on the antibiotic resistance patterns of Gram-negative microorganisms in an intensive care unit in Greece. *International Journal of Antimicrobial Agents 30*(4), 360-365.

Oehler, R., Polacek, N., Steiner, G., & Barta, A. (1997). Interaction of tetracycline with RNA: Photoincorporation into ribosomal RNA of *Escherichia coli*. *Nucleic Acids Research, 25,* 1219-1224.

Pestka, S. (1969). Studies on the formation of transfer ribonucleic acid-ribosome complexes X. phenylalanyl-oligonucleotide binding to ribosomes and the mechanism of chloramphenicol action. *Biochemical and Biophysical Research Communications, 36*(4), 589-595.

PML Microbiologicals, (2009). *Tryptic soy broth* (PDF). Retrieved from http://www.pmlmicro.com/assets/TDS/1012.pdf

Reshes, G., Vanounou, S., Fishov, I., & Feingold, M. (2008). Cell shape dynamics in *Escherichia coli. Biophysical Journal, 94*(1), 251-264.

Rosenkranz, H. S. (1988). Chloramphenicol: Magic bullet or double-edge sword? *Mutation Research/Reviews in Genetic Toxicology, 196*(1), 1-16.

Shafer, A., Kalinowski, J., & Puhler, A. (1994). Increased fertility of *Corynebacterium glutamicum* recipients in intergeneric matings with *Escherichia coli* after stress exposure. *Applied and Environmental Microbiology, 60*(2), 756-759.

Wang, X., Minasov, G., & Shoichet, B. (2002). Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *Journal of Molecular Biology, 320*(1), 85-95.

White, J. P., & Cantor, C. R. (1971). Role of magnesium in the binding of tetracycline to *Escherichia coli* ribosomes. *Journal of Molecular Biology, 58*(1), 397-400.

Wright, G. D. (2005). Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Advanced Drug Delivery Reviews, 57*(10), 1451-1470.

Zhang D., Jian, B., Xiang, Z., & Wang, S. (2008). Functional characterisation of altered outer membrane proteins for tetracycline resistance in *Escherichia coli. International Journal of Antimicrobial Agents, 32*(4), 315-319.