Antibiotic resistance against rifampicin in E. Coli

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Introduction

Every fifteen minutes, one person in the United States dies because of an infection that antibiotics can no longer treat effectively.[1] This striking estimate comes from a major report released recently on the imminent – and very current – threat posed by antibiotic resistance. Not only is antibiotic resistance a problem, but it is a crisis. More than 2.8 million antibiotic resistant infections occur in the US alone each year, causing over 35,000 deaths[2]. Over 700,000 people worldwide die each year from antibiotic resistant infections, and if radical change isn't immediately implemented, these numbers will continue to rise exponentially.[3] The British government commissioned a study predicting a worst-case scenario regarding the progression of antibiotic resistance, where more people will die by the year 2050 from antibiotic resistant infections than from cancer.[4] Our antibiotic resistance crisis has largely arisen from our irresponsibility regarding the usage and distribution of these precious drugs. Every time we administer antibiotics, we are applying a selective pressure to a bacterial population, thus giving rise to natural selection and the possibility of bacterial mutations that confer resistance. The CDC estimates that approximately 30% of all prescribed antibiotics are unnecessary; this accounts for 47 million excess prescriptions.[5] And it may seem that these superfluous prescriptions are driving our resistance epidemic, however, 70% of all antibiotics produced in the U.S. are used in agriculture.[6] These multi-billion dollar meat corporations want to maximize revenue (i.e. production at minimal cost) and so employ antibiotics sub-therapeutically (to promote weight gain and increase feed efficiency), as well as for prophylaxis and metaphylaxis. This overuse of antibiotics gives bacteria ample opportunity for evolution and adaptation to the selective pressure. Antibiotic resistance occurs due to the advent of mutations that allow bacteria to defend against these drugs; this causes the antibiotic to no longer be effective in eliminating infection. These mutations that confer antibiotic resistance provide the bacteria with a selective advantage, and they will survive and reproduce in the presence of antibiotics. The resulting bacterial strain can then pass resistance genes to other bacteria via horizontal gene transfer. Additionally, bacteria can develop resistance to a number of antibiotics, resulting in strains called superbugs.

Antibiotic resistance can arise via a number of mechanisms, including gene expression, the activation of membrane pumps, and permeability changes. In this paper, we will be focusing on a mechanism of rifampicin resistance in E. Coli. More specifically, a discrete mutation in the rpoB gene.

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[1] CNN Health, https://www.cnn.com/2019/11/13/health/superbug-amr-drug-resistant-cdc-2019-report/index.html.

[2] CDC, https://www.cdc.gov/drugresistance.

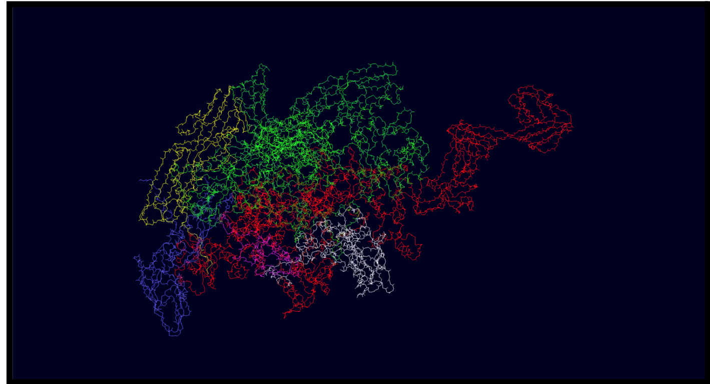
[3] "We're Losing the War against Bacteria, Here's Why | NYT," video, https://www.youtube.com/watch?v=OL8B1ZVLqSQ.

[4] "We're Losing the War against Bacteria, Here's Why | NYT," video, https://www.youtube.com/watch?v=OL8B1ZVLqSQ.

[5] "We're Losing the War against Bacteria, Here's Why | NYT," video, https://www.youtube.com/watch?v=OL8B1ZVLqSQ.

[6] "We're Losing the War against Bacteria, Here's Why | NYT," video, https://www.youtube.com/watch?v=OL8B1ZVLqSQ.

The rpoB gene encodes the beta-subunit of bacterial RNA polymerase, an enzyme critical for transcription. The E. Coli RNA polymerase (RNAP) is an enzyme composed of five subunits. (See image on the right).These subunits form the RNAP core enzyme responsible for RNA synthesis.[7] Rifampicin, an antibiotic, blocks transcription by binding to the RNA exit channel of RNA polymerase (i.e. the transcription pocket). Through such binding, rifampicin inhibits the bacterial cell from synthesizing proteins, thus causing the cell to die. For this reason, rifampicin is a bactericidal antibiotic. Given that rifampicin binds to the exit channel of RNA polymerase by making specific contacts involving the beta-subunit of RNA polymerase, and given that the rpoB gene encodes the beta-subunit of bacterial RNA polymerase, we know that mutations in the rpoB gene could potentially confer resistance as they would affect the binding of rifampicin. We were also able to empirically confirm this through virtual laboratory simulations and computational models. For this paper, we will be examining a Serine 511 to Phenylalanine 511 mutation occurring in the rpoB gene. The reason such a mutation is of interest is because a change in amino acid can confer antibiotic resistance by affecting the way rifampicin binds to the beta-subunit. Changes in amino acid structure produce steric hindrance which makes it more difficult for rifampicin to bind. And even though Serine 511 (mutated to become Phenylalanine 511) only interacts indirectly with rifampicin, the effects this change has with neighboring amino acids produces effects further downstream where rifampicin binds. More specifically, Phenylalanine has a much larger side chain compared to Serine. In fact, it contains an aromatic ring which is significantly larger and thus produces greater steric hindrance. We hypothesized that this Serine to Phenylalanine mutation results in greater steric hindrance (which would have a chain effect on the interactions of neighboring amino acids), making it more difficult for rifampicin to bind to the transcription pocket of RNAP and thus providing the bacteria with resistance to the drug.



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Methods

First, diluted E.Coli was plated onto LB plates and stock E. Coli was plated onto rifampicin plates. 0.1 ml of E. Coli was plated with a 0.001 serial dilution, so the bacteria colonies are countable (between 30 and 300). The concentration of rifampicin was 100 ug/mL. Then, the colony in the plate with the rifampicin was selected and lysed because the bacteria that survived on that plate would have the mutation that enabled them to resist the antibiotic.

A PCR and gel electrophoresis was conducted on the mutant to amplify the rpoB gene of the mutant bacteria and to look for a mutation in that gene that would explain its resistance to rifampicin.

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[7] NCBI, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6095464/.

The rpoB gene was selected because mutations in the rpoB gene make it difficult for rifampicin to bind to the transcription pocket of RNA polymerase, thus allowing the bacteria to be resistant.

Next, sequencing was done at the DNA Core Facility at UChicago to identify the mutant nitrogenous base and amino acid. Lastly, computer modeling with the Swiss PDB Viewer was used to see how the amino acids were interacting with each other in both normal and mutant bacteria.

Results:

Mutation Frequency:

Table 1. The number of colonies, dilution, volume plated, and the cfu per mL for each sample.

Sample	# colonies	Dilution	Volume plated	Cfu/ml
E.Coli from LB	130	10^-6	0.1 ml (or 100ul)	1.30 x 10^9
E.Coli from rif	99	NA	0.3 ml (or 300ul)	3.30 x 10^2

We used the following equation for our cfu/ml calculations:

$$cfu/ml = \#\ of\ colonies\ /\ (volume\ plated)(dilution\ used)$$

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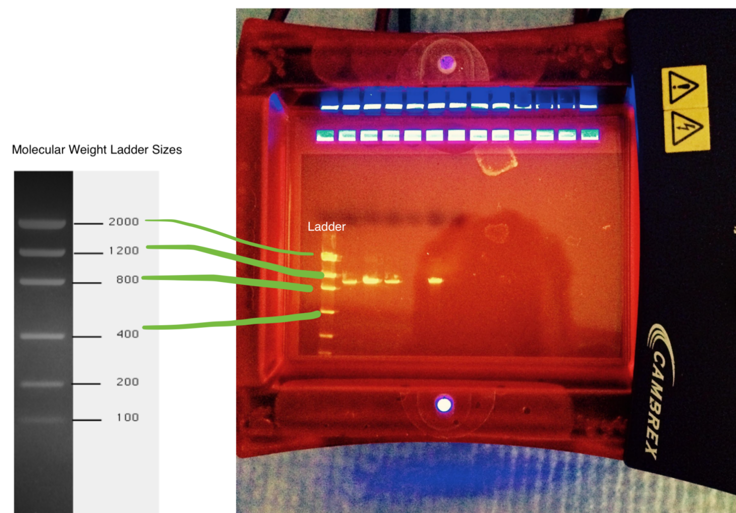
Using the data reported above, we found the frequency of the mutation (# of rifampicin colonies per the cfu/ml of the original stock of E. Coli) to be 7.6 x 10^-8. We then calculated the percentage of the population that is mutated (by dividing the cfu/ml of the rifampicin population by the cfu/ml of the total population) and found it to be 2.54 x 10^-5 %. (N.B. Given the entire class used the same dataset for analysis and the above calculations, our numbers reported above are equivalent to the class average.)

Gel electrophoresis:

Gel electrophoresis was performed to confirm the results of PCR performed on the sample of the rpoB gene. The image below shows the DNA ladder (LEFT) along with six student samples (RIGHT). Only four out of the six samples worked, however, the products that worked did match to the expected molecular weight (986 bp). See appendix for more details on PCR.



Sequencing :

Sequencing was done at the DNA facility at UChicago where we examined the nitrogenous bases of both samples to look for the mutation. We then determined the amino acid change as a result of the codon change. The reference E. Coli had a cytosine at base position 1535, while the mutant had a thymine instead. Using the surrounding nitrogenous bases and process of elimination, we concluded that the original codon, TCT, coded for serine, while the mutant, TTT, coded for phenylalanine instead.

Table 2. The differences between the reference and mutant E. Coli in nucleobase, codon sequence, and amino acid as a result of a mutation in a certain base position.

Item	Reference	Mutant
Nucleobase #	1535	1535
Nucleobase	C	T
Sequence before/after mutation site	GT TCA	GT TCA
Codon # (approximate)	511	5111
Codon Name	Serine	Phenylalanine
Codon Sequence	TCT	TTT
Codon Sequence before/after mutation site	SQL QFM	SQL QFM
Amino Acid Description	Polar uncharged side chain	Hydrophobic side chain
Direct/Indirect Interaction with Rifampicin	Indirect interaction	Indirect interaction
Previously discovered mutation from Campbell et al research?	Yes	Yes

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Protein Modeling:

Protein modeling was done with the Swiss PDB Viewer to view the amino acid interactions and electron clouds.

The serine in normal E. Coli has a polar uncharged side chain, while the phenylalanine in the mutant E. Coli has a hydrophobic side chain. However, the H-bonds with amino acid residues and rifampicin remained the same. Their interaction with rifampicin also remained indirect.

Steric hindrance and hydrogen bonds for rotamers and amino acids were observed as well. All amino acids had indirect interactions with rifampicin.

N.B. We lined up our E. Coli sequence with Thermus Aquaticus which is the reason for the numbering disparities in the following tables (compared with the numbering in Table 2- see above)

Table 3. The characteristics of the amino acids and their H-Bonds in normal vs. mutant E. Coli.

Sample	Amino Acid	Type of Amino Acid Side Chain (from table)	H-Bonds w/amino acid residues;	H-Bonds w/rifampicin; strong/weak	Indirect or Direct Interaction with rif
E.Coli	serine	polar uncharged	Ser 411, Arg 405, Arg 405, Gln 633, Glu 397, Leu 413, Thr 566, Arg 409, Glu 397, Lys 395	Gln 393, Phe 394, His 406	indirect
Mutant	phenylalanine	hydrophobic	Ser 411, Arg 405, Arg 405, Gln 633, Glu 397, Leu 413, Thr 566, Arg 409, Glu 397, Lys 395	Gln 393, Phe 394, His 406	indirect

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Table 4. The characteristics of the amino acid interactions and steric hindrance for each serine 392 rotamer (unmutated E. Coli).
(Angstrom setting = 6)

Rotamer Number	Amino acid Interactions	Steric hindrance, H bonds, atomic interactions description	Steric Hindrance Score	Indirect or Direct Interaction with rif
1	Phenylalanine385, Arginine388, Serine389, arginine134	Strong hydrogen bonding interactions with Phe385, Arg388 and Arg134	-4	indirect
2	Serine389, arginine134	Strong hydrogen bonding interactions with Ser389 and Arg134	-4	indirect
3	Phenylalanine385, Arginine388, Serine389, arginine134	Strong hydrogen bonding interactions with Phe385, Arg388, Arg134 and Ser389	-3	indirect

Table 5. The characteristics of the amino acid interactions and steric hindrance for each phenylalanine 392 rotamer (mutated E. Coli).
(Angstrom setting = 6)

Rotamer Number	Amino acid Interactions	Steric hindrance, H bonds, atomic interactions description	Steric Hindrance Score	Indirect or Direct Interaction with rif
1	Phe 385	Rings on both Phe residues interact and form steric hindrance at 4 sites	10	indirect
1	Ile 136	2 sites of steric hindrance		indirect
2	Phe 385	6 sites of steric hindrance between both rings, 1 site with carbon, 2 sites with oxygen group	30	indirect
2	Ile 136	2 sites of steric hindrance		indirect
3	Phe 385	6 sites of steric hindrance with ring	10	indirect
4	Arg 134	3 sites of steric hindrance with ring (one with Nitrogen group, 2 with carbon atoms)	4	indirect
5	Arg 134	H bond with N atom of Arginine; steric hindrance between Arginine Nitrogen and ring	56	indirect
5	Arg 388	2 sites of steric hindrance (Arginine Carbon and Phe ring)		indirect
5	Ser 389	steric hindrance		indirect

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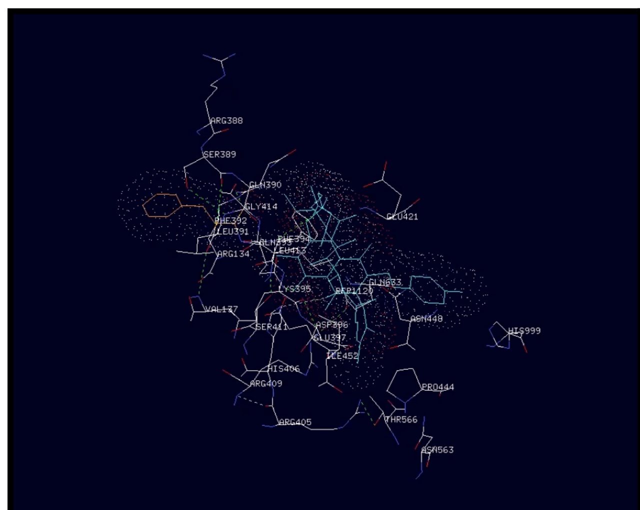
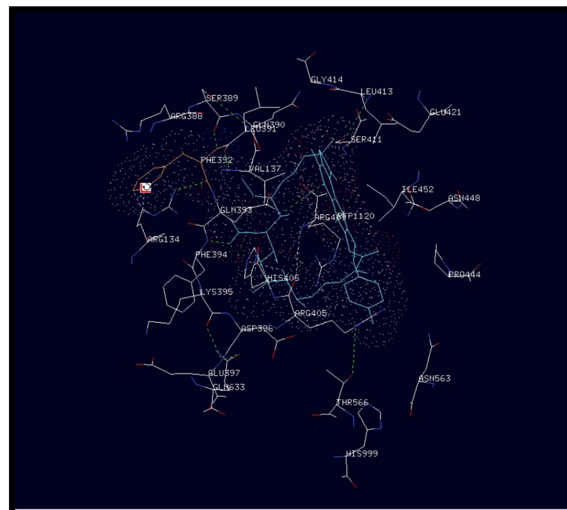
Below are images of Phenylalanine rotamers 1 (left) and 4 (right). The Phenylalanine molecule is depicted in orange.





Discussion:

Our mutation sample circumvented the effects of rifampicin on RNA-polymerase mainly through the increased steric hindrance resulting from the Phenylalanine side chain. Though both Serine (and the mutated Phenylalanine) do not interact directly with rifampicin, this change is nevertheless significant given the chain of effects increased steric hindrance has on the interactions of neighboring amino acid residues. Hydrogen bonds are conserved across the original form and the mutant, and thus they are not a factor of great importance when considering how this mutation inhibits the binding of rifampicin. Serine contains a polar uncharged side-chain while Phenylalanine contains a hydrophobic side chain, but the main difference arises from the increased electron cloud that comes from the large benzene ring on Phenylalanine's side chain. This increased electron bulk is responsible for the steric hindrance that ultimately inhibits rifampicin binding. This difference in steric hindrance between the original and the mutant is shown through the large difference in the steric hindrance scores between the rotamers of serine 392 and phenylalanine 392 in tables 4 and table 5. As a result, rifampicin cannot bind effectively to the RNAP as the steric hindrance changes conformations downstream by altering interactions upstream. Rifampicin binds to the exit channel of RNA polymerase by making specific contacts involving the beta-subunit of RNA polymerase, and the rpoB gene encodes the beta-subunit of bacterial RNA polymerase, and so we know that mutations in the rpoB gene are responsible for rifampicin resistance. Any bacteria that survive in the presence of rifampicin necessarily have such a mutation that allows them to survive.

When RNAP functions, it allows transcription of DNA into RNA which subsequently allows protein synthesis to occur. Proteins are essential for carrying out tasks within a cell thus steric hindrance caused by phenylalanine is key for E. Coli survival in response to rifampicin.

Many additional studies have corroborated similar mutations in the rpoB gene (our specific mutation was also previously discovered from Campbell et al research). The NIH performed a study on the basis of the observation that rifampicin resistance arose in the *absence* of the antibiotic during an evolutionary experiment. After 2000 of generations of thermal stress, it was found that 13 of the 114 E. Coli clones exhibited rifampicin resistance. Twelve of thirteen clones involved a mutation in codon 572 of the rpoB gene, with three different mutations noted in that codon (that were previously known to confer rifampicin resistance)[8]. Additionally, these three mutations arose independently in more than one population, providing evidence – by the principles of evolutionary convergence – that the mutations provide some survival advantage. This relationship between mutations providing resistance in the rpoB gene and survival is one of interest (as usually such mutations would decrease the fitness of a bacterial population in the absence of antibiotics), and we hope to further investigate this relationship.

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[8] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3598500/

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Conclusion:

Through our examination of the rpoB gene and the manner by which rifampicin binds to the transcription pocket of RNAP, we were able to glean valuable information regarding the mechanisms by which certain mutations can confer antibiotic resistance. Through various modes of experimentation, we were able to observe the way certain hydrogen bonds could facilitate or inhibit the binding of rifampicin as well as the effects of steric hindrance. The most noteworthy interactions regarding the Serine 511 to Phenylalanine 511 mutation were those involving steric hindrance, as hydrogen bonds were largely conserved from the original to the mutant. Unlike Serine, Phenylalanine contains a benzene ring as a component of its side chain. The increased electron density of such a functional group results in greater steric hindrance with surrounding amino acid residues, thus causing a chain effect of structural changes even further downstream where rifampicin binds. Overall, our results did support our initial hypothesis; the increased steric hindrance from the Phenylalanine side chain instigated a change in amino acid interactions thus inhibiting rifampicin from binding to the exit channel of RNAP and providing E. Coli with resistance to the drug.

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Appendix:

Rifampicin concentration 100ug/ml
Plate 100ul on LB
Plate 300ul on Rifampicin

Thermocycling Conditions (Bacterial Lysis):
6 minutes at 65C
2 minutes at 98C

Thermocycling Conditions (PCR)
PCR was performed to amplify the rpoB gene from the mutant sample. Then, gel electrophoresis was performed to confirm the results through verifying whether the gel presented the molecular weight of around 986bp.[9] The thermocycling conditions and steps were as follows:

Hold at 95C for 4 minutes
Denature at 95C for 30 sec
Anneal at 60C for 30 sec
Extension at 72C for 60 sec
Repeat steps 32 times
Hold at 72C for 10 minutes

[9] Elizabeth A. Campbell et al., "Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase," *Cell* 104, no. 6 (2001): pp. 901-912, https://doi.org/10.1016/s0092-8674(01)00286-0, 902.

Store at 4C

Primer Information: Sequence and Concentration (1 uM)

5’-TCGAAGGTTCCGGTATC CTGAGC-3’ (forward rpoB1240F)

5’-GGATACATCTCGTCTTCGT TAAC-3’ (reverse rpoB2226R)

PCR Recipe

“Ready-to-go” beads (contain Taq polymerase, nucleotides, dehydrated buffer)
Forward & reverse primer mix
DNA template (derived from lysed E.Coli)

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