# Discovery of A Novel Mutation Conferring Rifampicin Resistance in *E. coli*

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Antibiotic resistance, the ability of bacteria to grow in the presence of an antibiotic, is a prevalent global health issue. Rifampicin is an antibiotic best known for its use in tuberculosis treatment, but it is also effective against *E. coli*. It targets RNA polymerase, specifically the β subunit. In *E. coli*, mutations between codons 500-575 of the gene encoding the β subunit, *rpoB,* are known to cause rifampicin resistance. The present study aimed to isolate rifampicin resistant *E. coli* and sequence this target region of *rpoB* in order to map the mutations that occur. Through this process, a novel nine base pair deletion of codons 515-517 was uncovered, in addition to seven previously reported point mutations. The growth of this deletion mutant was studied and found to have a doubling time of 33.1 minutes compared to the wildtype at 31.9 minutes, indicating that this mutation incurs a slight fitness cost in *E. coli*. The results presented here support previous findings and expand upon them by introducing a new mutation, which can further be characterized by investigating compensatory mutations and fitness costs.

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## Introduction

Among the top ten threats to global health lies antimicrobial resistance. A prevalent component of this issue is bacterial antibiotic resistance, which is estimated to be associated with 4.95 million deaths (World Health Organization, 2022). In the Center for Disease Control's 2019 *Report on Antibiotic Threats in the United States*, five strains of resistant bacteria are listed as urgent threats, with eleven listed as serious threats. In recent years, it has been reported that the Covid-19 pandemic worsened antibiotic resistance and the reasoning for this lies in the overuse of antimicrobials during this time. For instance, it is estimated that 72% of Covid-19 patients were treated with an antimicrobial medication; however, only 8% of these patients were found to actually have bacterial or fungal infections (Sulayyim et al., 2022). Furthermore, the abundant use of antimicrobials has led to the rise of multidrug-resistant (MDR) pathogens, termed "superbugs." These are microbes that have several resistance mutations that limit treatment options and often have increased virulence (Davies & Davies, 2010). A more thorough understanding of the mechanisms microbes exploit in order to achieve resistance is key on the journey to finding a solution to the health crisis that is antimicrobial resistance.

Bacteria can acquire resistance, the ability to grow in the presence of an antibiotic, in multiple ways. One avenue is through horizontal gene transfer where mobile plasmids pass resistance genes to other bacteria, primarily through conjugation (Li et. al., 2019). Another pathway is spontaneous mutations that are then passed on to daughter cells, which is the process of vertical gene transfer. Both horizontal and vertical gene transfer occur in nature and are contributors to the spread of antibiotic resistance. There are also several common mechanisms of resistance, although the specifics will alter depending on the strain of bacteria and antibiotic at play. Often, the mechanism of resistance will be to limit the ability of the antibiotic to enter or stay in the cell, alter the antibiotic's target protein, or directly inactivate the antibiotic (Jian et al., 2021). For example, fluoroquinolones are a broad-spectrum antibiotic used in human and veterinary medicine that functions by targeting DNA gyrase and DNA topoisomerase IV as a way of inhibiting replication in bacteria (Piddock, 1998). However, microbes have found multiple methods of resistance to fluoroquinolones. Often this is in the form of small mutations on the genes of the two targets, but it has also been found that there are mutations that enhance the efflux of the antibiotic (Mancuso et al., 2021). Thus, there are several ways for microbes to be resistant.

Rifampicin is an antibiotic that is most well-known for its treatment of tuberculosis, first administered in 1968. However, the high frequency of resistance mutations in bacteria quickly became apparent and the use of rifampicin was restricted (Campbell et al., 2001). Depending on the method and organism of interest, the rate of resistance has been found to be anywhere from $10^{-10}$ to $10^{-7}$ mutations per bacterium per generation (Goldstein, 2014). The antimicrobial effect of this drug comes by inhibiting DNA-dependent RNA polymerase (RNAP), either through reducing its affinity for RNA transcripts or by sterically obstructing the elongation on the 5'end of RNA thereby arresting RNA synthesis. As RNAP is highly conserved across species, rifampicin has been able to target a few gram-negative bacteria and a wide range of gram-positive cocci bacteria (Beloor et al., 2023). One of these gram-negative bacteria is *Escherichia coli*, which is also one of the species that has found ways of resisting rifampicin.

A method of evasion found in several microbes, including *M. tuberculosis* and *E. coli*, is point mutations in *rpoB*, the gene encoding the β-subunit of RNAP which is 4029 bp long, encoding 1342 amino acid residues. This subunit has key catalytic sites for the process of RNA synthesis, as well as a binding pocket for rifampicin (Sutherland & Murakami, 2018). Since *rpoB* is an essential gene, the mutations that confer resistance can often create fitness costs. In *E. coli*, compensatory mutations have been credited for improving the fitness of rifampicin resistant mutants (Reynolds, 2000). Additionally, a 2012 study, using *Salmonella enterica* as a model organism for *M. tuberculosis*, found that additional mutations can form in *rpoA*, *rpoB*, or *rpoC* which compensate for a loss in growth fitness (Brandis et al., 2012).

The mutations in *E. coli* have been well studied and found to primarily occur between amino acid residues 500 and 575 (Landick, Stewart, & Lee, 1990). A recent study looked at 760 individual residue mutants throughout the entire binding site for rifampicin on the β-subunit of RNAP (Yang et al., 2023). Through this analysis they were able to identify specific mutations that cause high resistance, fitness costs, or even hypersensitivity to the antibiotic. Notably, the importance of residues 516, 526, and 572 in rifampicin binding were recognized as a majority of mutations in these amino acids led to high levels of resistance. They were also able to find that substitutions in 529, 566, and 570 cause the greatest loss in fitness and residues 521 and 525 will lead to heightened rifampicin sensitivity when changed. However, many of the amino acid residue mutations attempted required more than one nucleotide substitution by site-directed mutagenesis and are therefore less likely to occur in nature. Some of these same mutations have

been observed to occur in high stress environments as well. For example, when under thermal stress, parallel mutations to those in rifampicin resistance arose in residue 572 of the RNAP β-subunit (Rodríguez-Verdugo, Gaut, & Tenaillon, 2013).

Another study was able to isolate 16 individual rifampicin resistant *E. coli* mutants in a laboratory setting through exposure to the antibiotic (Wu & Hilliker, 2017). All were point mutations except for a 9 base pair deletion across residues 506-509 that effectively replaced the amino acids FGSS with cysteine in an α-helix near the rifampicin binding site. Similarly, many of the point mutations were found to affect residues adjacent to the binding site, with the most frequently found being a histidine to tyrosine mutation at residue 526. Many of these point mutations have also been seen in *M. tuberculosis* cultured from patients (Kapur et al., 1994). The mutation of H526Y (aligned with the *E. coli* codon numbering system) was the most frequently observed in this study as well.

The following research aims to isolate naturally occurring rifampicin resistant *E. coli* and sequence the target region of *rpoB* codons 500-575 in order to map the mutations that occur. Several mutations were identified, including a novel one, the growth of which was investigated as a way to evaluate any fitness costs. The following overall supports and expands upon the understanding of spontaneously occurring mutations that confer rifampicin resistance in *E. coli*.

## Materials & Methods

*Preparing Rifampicin Stock Solution and Media*

To 12 mL of DMSO, 0.3 grams of rifampicin was added and vortexed until completely dissolved to create a 25 mg/mL stock. It was then sterile-filtered through a 0.2 micron PVDF membrane, and stored in the dark at -20°C.

Prepared solidified LB agar was heated up in a water bath until molten and then cooled to 50°C**.** Once cooled, the agar was aliquoted in four sterile bottles at volumes of 300 mL. The 25 mg/mL stock of solubilized rifampicin was added to these 300 mL aliquots to achieve the desired concentrations (see Table 1). Approximately 20-25 mL of the agars was poured into each plate in a laminar flow hood and let to cool before being stored in a covered box to block light at 4°C.

**Table 1. Rifampicin Concentrations**

| Desired Concentration µg/mL | Volume Rifampicin (µL) |
|---|---|
| 25 | 300 |
| 50 | 600 |
| 100 | 1200 |
| 200 | 2400 |

The volumes of a 25 mg/mL stock of rifampicin needed to achieve the desired concentrations in 300 mL of LB agar.

*Evolving Antibiotic Resistant* E. coli

The stock of wild type DH5α *E. coli* (Strain NEB 5-α, New England Biolabs, Catalog #C2988J) was streaked out on a plate of LB agar and incubated at 37°C overnight, then saved at 4°C. A single colony was inoculated with a pipette tip into 5 mL of LB liquid medium in a sterile 15 mL conical tube. This culture was then incubated at 37°C overnight, shaking at an RPM of 200. After this incubation period, the culture was diluted by adding 5 µL of culture to 5 mL of LB medium in a 15 mL conical tube. Five new sterile 15 mL conical tubes of 5 mL LB medium were inoculated by adding 5 µL of the diluted culture (1:1000 dilution) to achieve approximately 2 x $10^4$ cells/mL. These five cultures were then incubated in parallel at 37°C overnight, shaking at 200 RPM. These cultures were each spread on plates with different concentrations of

rifampicin (i.e., 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml). For each pair of rifampicin concentration and *E. coli* culture, one plate received 20 µL of culture and 180 µL of sterile water and another received 200 µL of culture. Both were spread by adding beads to the plate and shaking. This was done for every combination of rifampicin concentration and culture, all of which were incubated upside down at 37°C overnight, then left at room temperature for another 24 hours to increase colony size.

In addition, the five liquid cultures were spun down for 5 minutes at 5000 x *g* to form a pellet (after being stored at 4°C for two weeks). The pellet was resuspended in 400 µL of LB medium. To a 100 µg/ml rifampicin plate, 100 µL of culture was added and spread by the momentary addition of beads and shaking. This was repeated for another 100 µg/ml rifampicin plate and two 200 µg/ml rifampicin plates per culture, all of which were incubated upside down at 37°C overnight.

*Thermal Stress Culture*

From the wild type DH5α *E. coli* plate, one colony was selected and dislodged in 5 mL of LB liquid medium in a sterile 15 mL conical tube. This was then incubated at 42.2°C overnight, shaking at 200 RPM. For ten additional days, the culture was split back by adding 5 µL of the incubated culture to 5 mL of fresh LB medium to achieve approximately 20 generations a day. After the tenth split of the culture, and eleven days from the start, the culture was each spread on LB plates with different concentrations of rifampicin (25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml). For each rifampicin concentration culture, one plate received 100 µL of culture and 100 µL of sterile water and another received just 200 µL of culture. Both were spread around by momentarily adding beads to the plate and shaking. This was done for every combination of rifampicin concentration and culture, all of which were incubated upside down at 37°C overnight, then left at room temperature for another 24 hours.

*Preparing the Primers*

The primers were designed after a study done by Wu and Hilliker in 2017, with the sequences seen in Table 2, to amplify nucleotides 1417-2160 of *E. coli rpoB*. The desiccated forward and reverse primers (from Eton Bioscience) were spun down for one minute and resuspended in the proper amount of nuclease-free water, 430 µL for the forward and 390 µL for

the reverse, to make 100 µM stocks. Stocks of 10 µM were also made by mixing 90 µL of nuclease-free water with 10 µL of the proper stock. All primer stocks were stored at -20°C.

**Table 2. Primer Sequences**

| | **Sequence** |
|---|---|
| **Forward** | 5´- CGT GCG GTG AAA GAG CGT CTG TCT - 3´ |
| **Reverse** | 5´- ACG TTT AGC TAC CGC AGT TAC ACC -3´ |

The forward and reverse primers used in PCR to amplify a 744 nucleotide region of *rpoB* in *E. coli* (Wu & Hilliker, 2017).

*PCR*

On ice, 150 µL of One*Taq* 2X Master Mix with Standard Buffer (New England Biolabs, Catalog #M0482S), 30 µL of the 10 µM forward primer, 30 µL of the 10 µM reverse primer, and 90 µL of nuclease-free water were combined and thoroughly mixed by pipetting up and down. To each PCR tube, 20 µL of this reaction master mix was aliquoted. For each colony identified as of interest, half of it was picked and swatched on a patch plate, and then the other half was picked and dislodged in the PCR tube. PCR was run on a thermocycler for a total of 35 cycles, with 94°C for 30 seconds, 57°C for 30 seconds, and 68°C for 1 minute, with an initial denaturation at 94°C for 5 minutes and a final extension of 68°C for 5 minutes.

*Agarose Gel to Check PCR Products*

A 1% agarose gel was prepared by combining 0.5 grams of agarose and 25 mL of 1X TAE buffer. This was microwaved until bubbling, then removed and swirled until completely in solution. Once in solution 25 mL more of 1X TAE was added along with 5 µL of 10,000X SybrSafe. This was poured into the gel cast with two combs and left to set for 20 minutes, afterwhich the gel was realigned and 1X TAE was added to fill the reservoir and cover the gel. To separate wells, 5 µL of a DNA ladder was added and 2 µL of each of the PCR products. This was run at 100 V for 20 minutes before being imaged on a Bio-Rad Gel Doc XR, with Filter 1 and automatic exposure for intense bands.

*Sequencing and Alignments*

The PCR products were sent to Quintara Biosciences for Sanger sequencing with the 10 μM forward primer. The sequence files received were uploaded to Benchling and used to create alignments with the known wildtype *rpoB* sequence. The alignment program used was MAFFT on auto with adjust direction enabled accurately and a gap open penalty of 1.53. The results of these alignments were manually confirmed through analysis of the chromatograms.

*Growth Curves*

Liquid cultures were created of the wild type DH5α *E. coli*, one of the point mutation colonies (colony #15), and one of the 3 AA deletion colonies (colony #20) by dislodging a picked isolated colony in 5 mL of LB medium. These cultures were incubated overnight, shaking at 37°C. The next day, a spectrophotometer was used to measure the optical density of these cultures when diluted tenfold using LB medium as a blank. The actual OD600 was calculated and used to determine the amount of culture needed to reach an OD600 of 0.025 in a 125 mL shake flask with 50 mL of LB medium for each culture. These were then placed back in the incubator and the OD600 was measured by spectrophotometer at given time points over twenty-four hours, diluting as needed to obtain OD600 readings < 1.0.

Another growth curve was developed on a microplate scale. The process of creating the liquid cultures above was repeated, but this time with colony #22 to represent the 3 AA deletion and a one-hundred-fold dilution after the first night of incubation after which they were incubated at 37°C for another 24 hours. The next day, a spectrophotometer was used to measure the optical density of these cultures when diluted tenfold using LB medium as a blank. The actual OD600 was calculated and used to determine the amount of culture needed to acheive an OD600 of 0.01 in 5 mL of LB medium for each culture. These cultures were used to seed five wells each in the center of a 96-well plate, with the surrounding wells being filled with LB medium to act as blanks. This plate was placed in a BioTek Synergy H1 Multimode Plate Reader set to read the OD600 of every well every ten minutes for twenty-four hours at 37°C and a fast agitation rate. Doubling times were calculated from both growth curves using the following equation: $\frac{\ln(2)}{\frac{\ln(OD_2)-\ln(OD_1)}{T_2-T_1}}$, where OD is the OD600 reading, and T is the time in minutes.

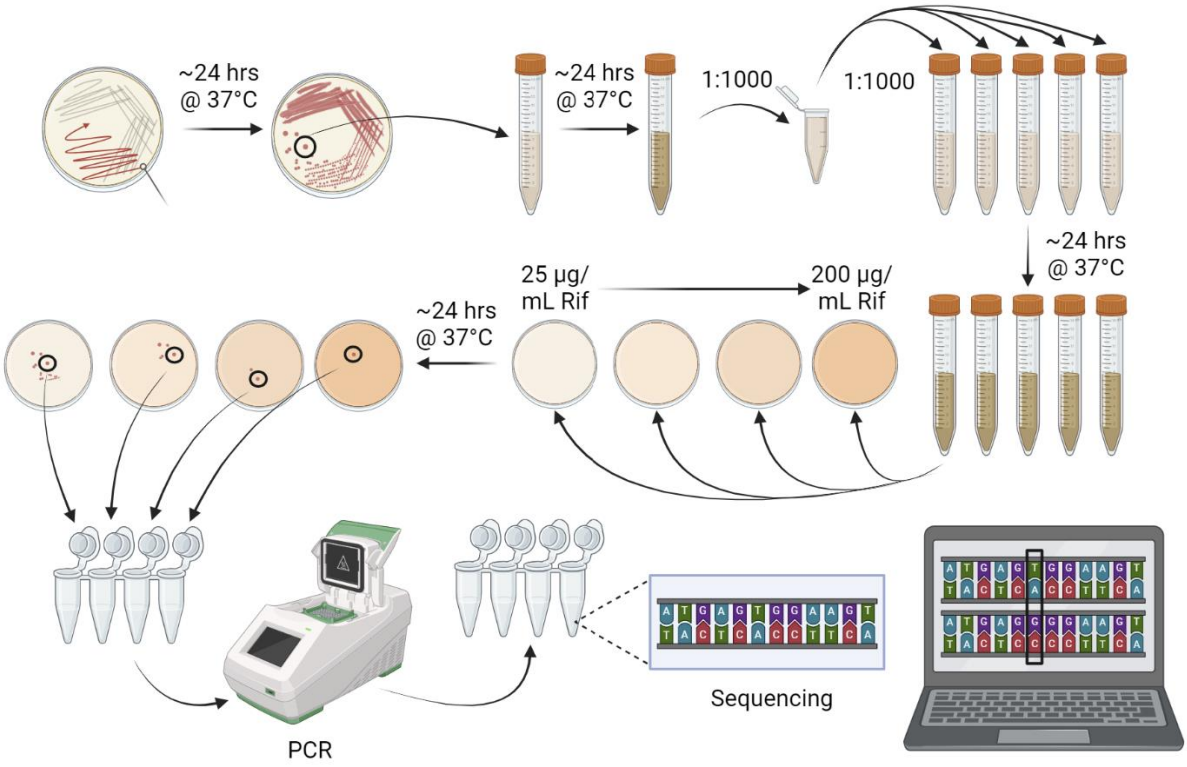


**Figure 1. Basic Workflow.** This is a visual of the initial work done to get rifampicin resistant *E. coli* colonies and determine their mutations.

## Results & Discussion

The five DH5α *E. coli* cultures that were grown yielded differing amounts of colonies when plated against the four concentrations of rifampicin. None of the plates that received 20 µL of culture had any colonies present. Of the twenty plates that received 200 µL of culture, twelve plates had at least one colony, with 21 colonies isolated in total (see Table 3). The most colonies grew on the lowest concentration of rifampicin with ten colonies overall, tapering down to the highest concentration which only one colony was able to withstand from culture C. The least number of resistant colonies came from culture A, which only led to one, while the most were six resistant colonies from cultures C and D. At least one colony from each culture was chosen to be sequenced (see Table 3) for mutations in *rpoB* within the hotspot region of codons 500-575. All eleven polymerase chain reactions performed before sequencing were determined to be successful by the presence of clear bands for all isolates on a gel between the 1kb and 0.5kb markers (see Figure 2). The target amplicon was 744 bp long.

**Table 3. Colonies from the First Round of Plating**

| RIF (µg/mL) | Culture A | Culture B | Culture C | Culture D | Culture E |
|---|---|---|---|---|---|
| **25** | 1 | 2 | 1 | 5 | 1 |
| **50** | 0 | 2 | 1 | 1 | 2 |
| **100** | 0 | 0 | 3 | 0 | 1 |
| **200** | 0 | 0 | 1 | 0 | 0 |

Number of colonies obtained from plates with varying concentrations of rifampicin (RIF) with colonies chosen for sequencing highlighted in green.

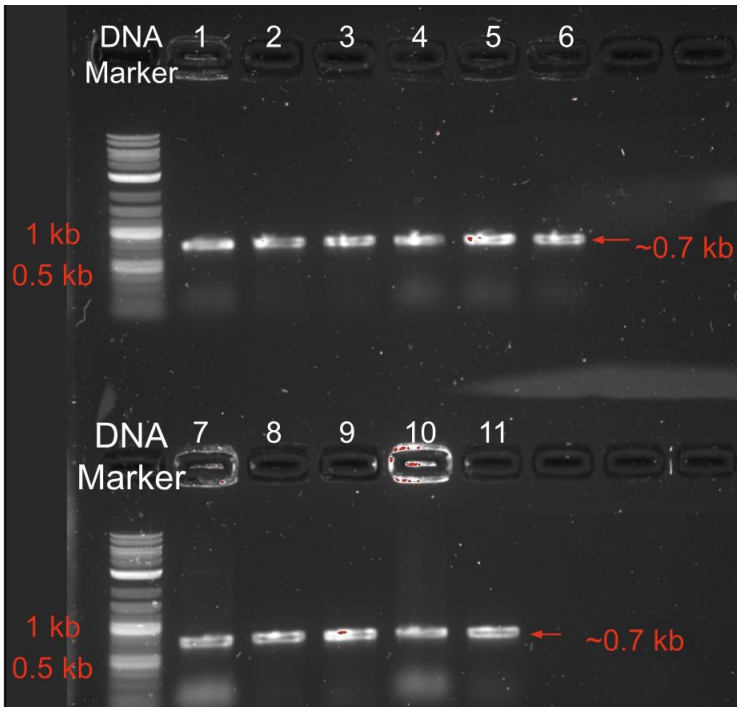


**Figure 2. PCR Products from the First Round of Plating.** A 1% agarose gel run for 20 minutes at 100V with two DNA ladder makers on the left and the 11 PCR products, amplifying a segment of *rpoB* from the rifampicin resistant *E. coli* isolates. The expected bands are indicated around 744 base pairs.

After two weeks of storage at 4℃, the remaining volumes of cultures A-E were plated on the two highest concentrations of rifampicin. After 24 hours at 37℃ these plates were found to have grown eighteen colonies total, with the most coming from culture E with nine (see Table 4). The only culture which failed to result in any colonies was culture B. From all of the colonies found only those from culture A and the 200 µg/mL rifampicin plates of culture E were chosen for sequencing as there were significantly more colonies reported at these concentrations than there were in the previous plating. The same PCR protocol was followed for these eight isolates to identify mutations within that same *rpoB* region. Once again these were all deemed successful by the presence of a clear band on a gel between the 1kb and 0.5kb markers (see Figure 3).

**Table 4. Colonies from the Second Round of Plating**

| RIF (µg/mL) | Culture A | Culture B | Culture C | Culture D | Culture E |
|---|---|---|---|---|---|
| **100** | 2 | 0 | 4 | 1 | 3 |
| **200** | 0 | 0 | 2 | 0 | 6 |

Number of colonies obtained from plating the remaining culture on the two highest concentrations of rifampicin, with colonies chosen for sequencing highlighted in green.

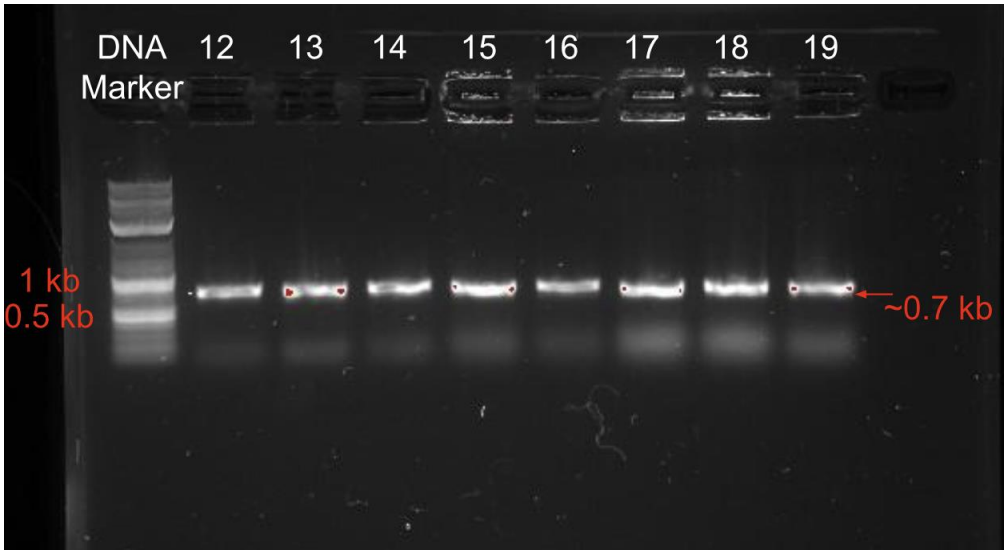


**Figure 3. PCR Products from the Second Round of Plating.** A 1% agarose gel run for 20 minutes at 100V with a DNA ladder maker on the left and the eight PCR products, amplifying a segment of *rpoB* from the rifampicin resistant *E. coli* isolates. The expected bands are indicated around 744 base pairs.

In addition, one culture was thermally stressed at 42℃ for approximately 200 generations and plated against all four concentrations of rifampicin at two different plating volumes, 100 and 200 µL. These plates revealed a total of ten colonies, three of which were from the 100 µL volume with the remaining seven from the 200 µL volume (see Table 5). The five colonies that grew at the two highest concentrations of rifampicin were chosen for sequencing, and the PCR products were all confirmed by the presence of a clear band on a gel between the 1kb and 0.5kb markers (see Figure 4).

**Table 5. Colonies from Thermally Stressed Culture**

| RIF (µg/mL) | 100 µL Culture | 200 µL Culture |
|---|---|---|
| **25** | 2 | 2 |
| **50** | 0 | 1 |
| **100** | 0 | 1 |
| **200** | 1 | 3 |

Number of colonies obtained from plating the two volumes of the thermally stressed culture on the four concentrations of rifampicin, with colonies chosen for sequencing highlighted in green.

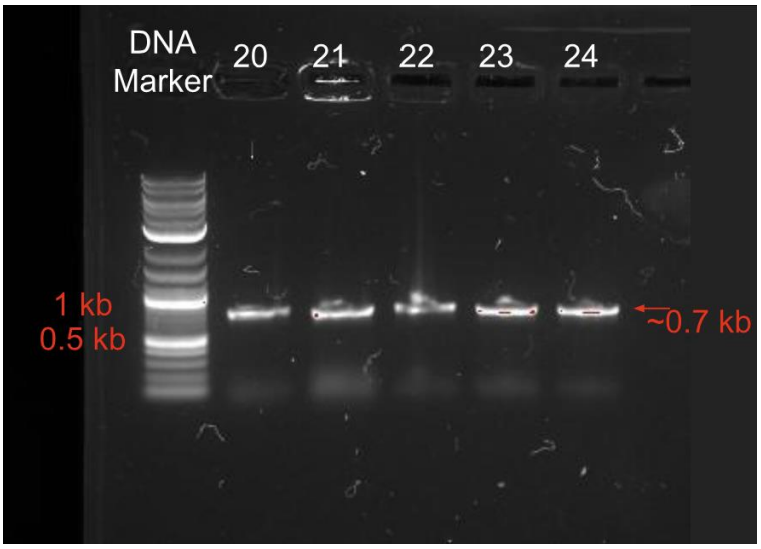


**Figure 4. PCR Products from the Thermally Stressed Colonies.** A 1% agarose gel run for 20 minutes at 100V with a DNA ladder maker on the left and the five PCR products, amplifying a segment of *rpoB* from the thermally stressed *E. coli* isolates. The expected bands are indicated around 744 base pairs.

From three rounds of plating, PCR, and sequencing, eight individual mutations were observed (see Table 6). Each colony exhibited exactly one mutation within the amplified region, all of which are thought to impact the ability of rifampicin to bind due to their location within the binding pocket. The majority of these were point mutations, with the exception of one deletion. There were three individual point mutations seen at residue 516 that each resulted in different amino acid residue changes. The normal aspartic acid residue was changed to either asparagine, glycine, or valine with a collective frequency of 28.3%. All of these changes result in the loss of

the reactive hydroxyl group. Two different changes occurred at residue 526, where the histidine residue was shifted to either tyrosine or aspartic acid. Both of these mutations eliminate a positively charged moiety in this position, but mutation H526Y was seen significantly more than H526D and was the most frequently found mutation overall at 41.7%. The final two point mutations were both serine to phenylalanine changes occurring at residues 522 and 531 that effectively removed the reactive hydroxyl groups. All of these aforementioned point mutations have been previously reported in literature.

**Table 6. Mutations Found in *rpoB***

| Nucleotide Position | Original Nucleotide | New Nucleotide | Codon | Original AA | New AA | Colony #'s | Frequency (%) |
|---|---|---|---|---|---|---|---|
| 1546 | G | A | 516 | Asp | Asn | 6 & 7 | 8.3 |
| 1547 | A | G | 516 | Asp | Gly | 2, 3, 9 & 24 | 15.8 |
| 1565 | C | T | 522 | Ser | Phe | 1, 12, & 14 | 12.5 |
| 1576 | C | T | 526 | His | Tyr | 4, 8, 10, 11, 15-19, & 21 | 41.7 |
| 1576 | C | G | 526 | His | Asp | 5 | 4.2 |
| 1592 | C | T | 531 | Ser | Phe | 13 | 4.2 |
| 1547 | A | T | 516 | Asp | Val | 23 | 4.2 |
| 1543-1551 | ATG GAC CAG | — | 515-517 | Met-Asp-Gln | — | 20 & 22 | 8.3 |

The mutations found in the alignments of the sequenced isolates, how they affect the amino acid residues, which isolates they occurred in, and their frequencies.

The final mutation was exhibited in two of the colonies from the culture that was thermally stressed. This mutation is a nine base pair deletion of codons 515-517 that eliminates the residues methionine, aspartic acid, and glutamine. This is the first known record of this mutation occurring. In order to confirm the presence of this mutation, PCR was repeated on these two colonies (20 & 22) and they were sent out for sequencing with the reverse primer. The result for colony #20 was inconclusive, but sequencing both strands using the forward and reverse primers for #22 was able to successfully confirm this mutation (see Figure 5).

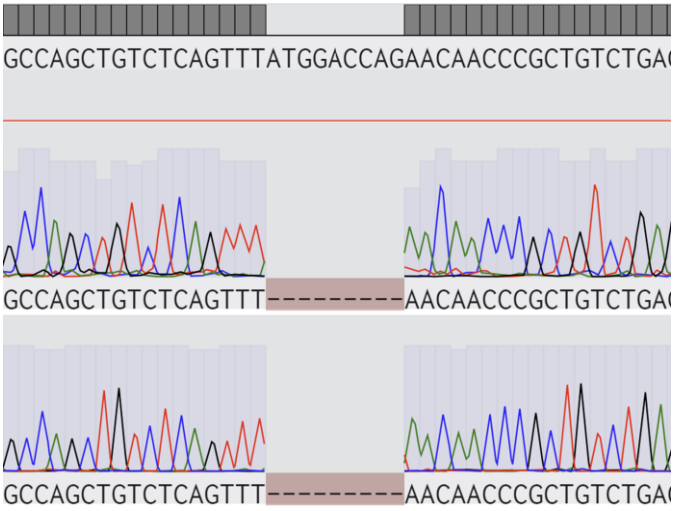


**Figure 5. Nine Base Pair Deletion Alignment.** The forward and reverse sequences of colony #22 aligned against the WT template sequence, showcasing the nine nucleotides ATG GAC CAG are deleted.

Due to the novel nature of the deletion mutant, a growth study was done to determine if it resulted in any fitness costs (see Figure 6). This was first done with 50 mL culture sizes with the wildtype, the most frequently occurring point mutant (H526Y), and the deletion mutant (Δ515-517). As this was done before the confirmation of the deletion sequence, the colony that was used to create the culture was #20, which was unable to be confirmed with complete confidence. However, this growth curve was able to exhibit a clear trend. The deletion mutant seems to exhibit a longer lag in the beginning of the experiment, and also does not reach a terminal optical density as high as both the point mutant and the wildtype. The doubling times were calculated from slopes in the 2 to 2.5 hours time window for the wildtype, mutant H526Y, and mutant

Δ515-517 which were 37.8, 38.2, and 36.3 minutes, respectively. This suggests the mutations may not impact the doubling time during the exponential phase of growth.

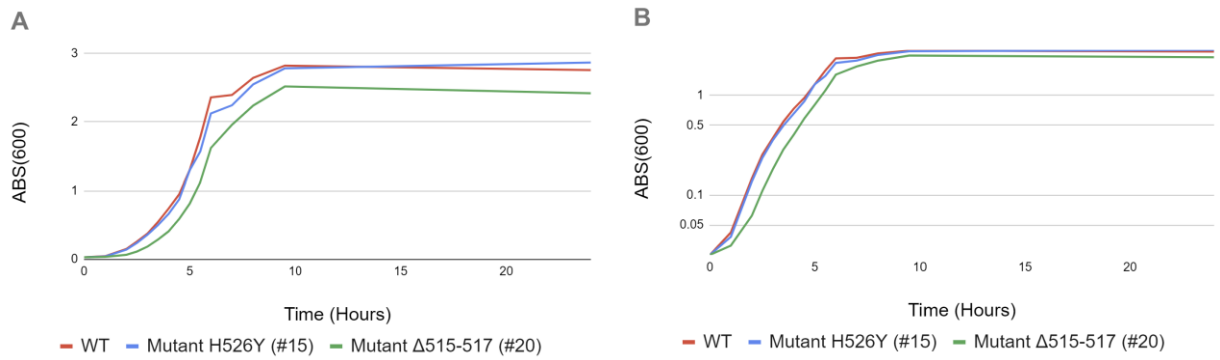


**Figure 6. Growth Curves by Spectrophotometer.** The OD600 values of 50 mL cultures in 125 mL shake flasks of WT *E. coli*, mutant H526Y (colony #15), and mutant Δ515-517 (colony #20) measured over 24 hours at 37°C using a spectrophotometer (A) on a log-based scale (B).

For the purpose of facilitating data collection and analysis from replicates, the growth study was repeated in a 96-well plate (see Figure 7). This time colony #22 was used for the deletion mutant as this mutation had been properly confirmed by the reverse sequence. The doubling times were calculated from the approximately 30 minute range where the cultures passed through absorbance readings of 0.05 to 0.1. For the wildtype, mutant H526Y, and mutant Δ515-517 this resulted in doubling times of 31.9, 33.9, and 33.1 minutes, respectively. This again shows that the mutations likely do not significantly impact the doubling time during the exponential phase of growth. Unlike what was seen in Figure 5, the deletion mutant was able to reach the same terminal optical density as the others, but overall the terminal densities in this study were not as high. The point mutant and the deletion mutant followed similar growth trends throughout the run. This includes a slightly longer lag in the beginning of the run and an earlier decrease in growth rate. Overall, signs point to a slight fitness cost in the deletion mutant compared to the wildtype.

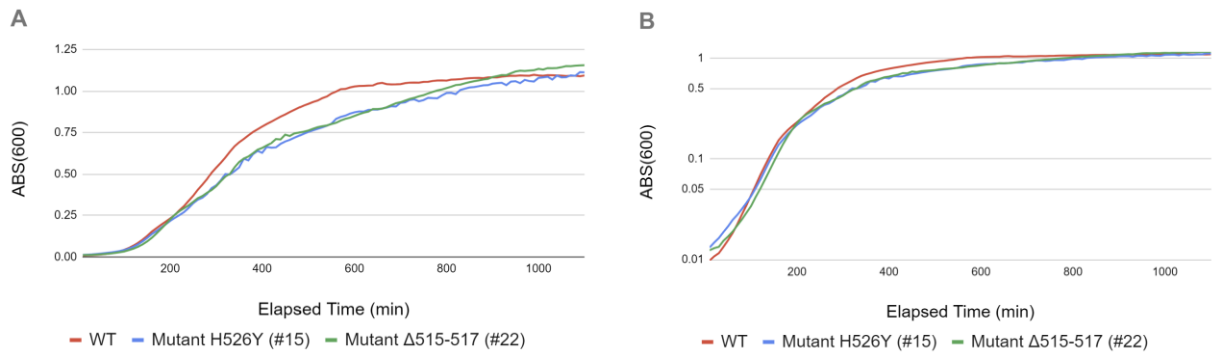


**Figure 7. Microplate Growth Curves.** The average OD600 values of 5 replicates WT *E. coli*, mutant H526Y (colony #15), and mutant Δ515-517 (colony #22) measured over 17 hours at 37°C using a plate reader (A) on a log-based scale (B).

## Conclusions & Future Directions

Overall, 55 rifampicin resistant colonies were cultured, 24 of which were chosen for sequencing. This process revealed eight mutations within the amplified region of *rpoB*, including one novel mutation. The 9 bp deletion of codons 515-517 has not been previously reported in literature and as seen in the growth curves, this deletion mutant experiences a slight fitness cost compared to the wildtype. Not only do these findings reinforce the presence of naturally occurring point mutations within codons 500-575 of *rpoB* in conferring rifampicin resistance in *E. coli*, but they also expand upon the scope of mutations possible by introducing a new 9 bp deletion.

One limitation of this study is the lack of an investigation into mutations occurring outside the target region of *rpoB.* The data supports that mutations for rifampicin resistance were occurring within this region, but nothing can be said about the remainder of *rpoB* or other genes. Additional mutations may be occurring outside the region investigated since *rpoB* is an essential gene where mutations often incur fitness costs. These fitness costs have been seen to be improved by compensatory mutations in *E. coli* (Reynolds, 2000). Furthermore, *S. enterica* is known to exhibit additional mutations in *rpoA, rpoB,* and *rpoC* that function to compensate for loss of fitness in rifampicin resistant mutants (Brandis et al., 2012). A simple way this could be investigated in the isolated mutants is through whole genome sequencing. Aligning the results of this sequencing with the wildtype would make it possible to assess the whole scope of mutations occurring within the mutants' genomes at once.

Moreover, the fitness costs of the isolated rifampicin resistant mutants are not well understood, although the growth curve results suggest their existence. The extent of the fitness for each mutation conferring rifampicin resistance could be better understood by replicating the growth curve study done for mutant H526Y and mutant Δ515-517 for all isolates, including those that represent the same mutation. These growth curve studies may also be performed while manipulating the conditions outside of standard ranges in an attempt to better understand the parameters influencing the fitness. For example, the effect of a lower temperature on fitness has been previously studied for two rifampicin resistant *E. coli* mutants not found in this study. In liquid medium, the strains exhibited relative growth rates of 20% and 40% of WT when at 20°C, compared to 70% and 67% of WT when at 37°C (Kurepina, 2022). Other stress conditions may also be studied, such as high temperatures, oxygen deprivation, and carbon starvation. This

compounded with the knowledge of mutations occurring outside of the amplified region of *rpoB*, especially those thought to be compensatory, would aid in creating a bigger picture of rifampicin resistant *E. coli*.

Finally, the workflow of this study will be modeled in a new course at Worcester Polytechnic Institute, BB 1801 Foundations of Biology and Biotechnology Laboratory, that will be required for all Biology and Biotechnology (BBT) majors. This will introduce basic techniques such as how to pipette, perform dilutions, and culture bacteria while simultaneously necessitating conceptual understanding of what is happening at a molecular level. This applies both to techniques such as PCR and the mechanism of the antibiotic and mutations being investigated. The course has the potential to allow freedom to explore the scientific process as well as provide a foundational experience for BBT students.

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# Appendix

## Appendix A. Origin of Every Isolate

| Isolate # | Culture of Origin | [RIF] µg/mL | *rpoB* Mutation |
|---|---|---|---|
| 1 | A | 25 | S522F |
| 2 | B | 50 | D516G |
| 3 | B | 50 | D516G |
| 4 | C | 100 | H526Y |
| 5 | C | 100 | H526D |
| 6 | C | 100 | D516N |
| 7 | C | 200 | D516N |
| 8 | D | 50 | H526Y |
| 9 | E | 50 | D516G |
| 10 | E | 50 | H526Y |
| 11 | E | 100 | H526Y |
| 12 | A | 100 | S522F |
| 13 | A | 100 | S531F |
| 14 | E | 200 | S522F |
| 15 | E | 200 | H526Y |
| 16 | E | 200 | H526Y |
| 17 | E | 200 | H526Y |
| 18 | E | 200 | H526Y |
| 19 | E | 200 | H526Y |
| 20 | TS | 200 | Δ515-517 |
| 21 | TS | 200 | H526Y |
| 22 | TS | 200 | Δ515-517 |
| 23 | TS | 200 | D516V |
| 24 | TS | 100 | D516G |

The assigned number for each colony chosen for PCR, the culture they resulted from (TS = Thermal Stress), what concentration of rifampicin they were on, and what mutation was found.