CATASTROPHIC EVOLUTION OF TRIMETHOPRIM RESISTANCE

by

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CATASTROPHIC EVOLUTION OF TRIMETHOPRIM RESISTANCE

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ABSTRACT

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Keywords: Antibiotic Resistance, Trimethoprim, Trimethoprim Resistance, DHFR, Mutation, Protein Evolution, Morbidostat, Sequencing, SCA, FIS, f statistic

Understanding the molecular basis of antibiotic resistance is of great importance since antibiotic resistance is an ever growing public health problem. Pathogenic bacteria can accumulate resistance against antibiotics via horizontal gene transfer and spontaneous mutations. One of the most prevalent resistance mechanisms is increased antibiotic tolerance as a result of spontaneous mutations on the enzymes that are targeted by antibiotic molecules. Here, in this study, we investigated how ecological factors influence genetic trajectories that lead to antibiotic resistance. In a custom made continuous culture device that we call the Morbidostat, we evolved several wild type Escherichia coli populations against trimethoprim where six of these populations were continuously diluted with a mild dilution factor (~0.3 $hour^{-1}$) and remaining seven populations were continuously diluted with a strong dilution factor (~0.6 $hour^{-1}$).At the end of four weeks, all of the populations evolved to similar levels of trimethoprim resistance in a stepwise manner by accumulating three or four mutations on the promoter and coding regions of *DHFR* gene. The first mutation was almost always on the promoter region and the following first amino acid replacement on the protein was chosen from the sector regions we predicted by Statistical Coupling Analysis (SCA). Strikingly, evolutionary trajectories of the populations that evolved under strong dilution were far from predictability and population structures were highly heterogeneous. Prolonged clonal interference was abundantly observed in the populations evolving under strong dilution. Our results suggest that evolution of resistance highly depend on fitness constraints imposed by ecological factors

ÖZET

TRIMETOPRiM DiRENCiNİN KATASROFİK EVRİMİ

Tuğce Altınuşak
Biyoloji Bilimleri ve Biyomuhendislik Programı, Sabancı Universitesi,
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Anahtar Kelimeler: Antibiotik Direnci, Trimetoprim, Trimetoprim Direnci, DHFR, Mutasyon, Protein Evrimi, Morbidostat, Sekanslama, SCA, FIS, f testi

Antibiyotik direnci büyüyen bir sağlık sorunudur bu yüzden antibiyotik direncinin moleküler temelini anlamak çok önemlidir. Patojenik bakteriler yatay gen transferi yada mutasyon kazanarak antibiyotiklere dirençli hale gelirler. En yaygın bulunan direnç mekanizmaları ise antibiyotik moleküllerinin hedef aldığı proteinlerin genleri üzerinde meydana gelen mutasyonlardır. Biz bu çalışmada, antibiyotik direncine yol açan genetik gidim izlerinin ekolojik faktörlerce nasıl değiştini araştırdık. E.coli populasyonlarını "Morbidostat" diye adlandırdığımız cihazı kullanarak trimetoprime karşı dirençli hale getirdik. Altı kültürü hafif seyreltme faktörü(~0.3 $saat^{-1}$), diğer 7 kültürü ise kuvvetli seyreltme föktürü (~0.6 $saat^{-1}$) kullanarak evrimleştirdik. Dört haftanın sonunda bütün populasyonlar DHFR geni üzerinde yada promotorunda oluşan mutasyonlar kazanarak adım adım trimetoprim direnci kazandılar. İlk mutasyon çoğunluka promotor bölegisinde, sonraki mutasyonlar ise DHFR' da aminoasit değişimi olarak gözlemlendi. İlk aminoasit değişimi özellikle SCA analizi sonucunda bulduğumuz sektör bölgesinde görülüldü. Ayrıca,Kuvvetli seyreltilme ile evrimleştirilen kültür populasyonlarının evrimsel gidim izleri şarşıtıcı bir şekilde heterojen ve tahminden uzaktı. Uzun süreçli klonal karışma çoğunlukla kuvvetli seyreltilme ile evremleştirilen kültürlerde mevcuttu. Böylece, Trimethoprim direncinin evriminin ekolojik faktörlerce gelen fitnes kısıtlamasına bağlı olduğu sonucuna vardık.

To my family;

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LIST OF ABBREVIATIONS

TMP	Trimethoprim
CHL	Chloramphenicol
DHFR	Dihydrofolate reductase
PABA	Para-aminobenzoic acid
DHF	Dihydrofolic acid
THF	Tetrahydrofolate
OD	Optical Density
MIC	Minimum Inhibitory Concentration
SCA	Statistical Coupling Analysis
FIS	Functional Impact Score

ABBREVIATIONS OF MUTATIONS

M20I	Methionine to isoleucine transition in 20th amino acid of DHFR
P21Q	Proline to glutamine transition in 21st amino acid of DHFR
P21L	Proline to leucine transition in 21st amino acid of DHFR
A26T	Alaline to threonine transition in 26th amino acid of DHFR
D27E	Aspartic acid to glutamic acid transition in 27th amino acid of DHFR
L28R	Leucine to arginine transition in 28th amino acid of DHFR
W30C	Tryptophan to cysteine transition in 30th amino acid of DHFR
W30G	Tryptophan to glycine transition in 30th amino acid of DHFR
W30R	Tryptophan to arginine transition in 30th amino acid of DHFR
I94L	Isoleucine to leucine transition in 94th amino acid of DHFR
R98P	Arginine to proline transition in 98th amino acid of DHFR
F153L	Phenylalanine to leucine transition in 153rd amino acid of DHFR
F153S	Phenylalanine to serine transition in 153rd amino acid of DHFR
F153V	Phenylalanine to valine transition in 153rd amino acid of DHFR

1. INTRODUCTION

1.1 Antibiotics and Antibiotic Classes

Antibiotics are small organic molecules that are capable of killing microorganisms or inhibiting growth of microorganisms. The term and definition of antibiotic is firstly introduced by Selman Waksman who discovered several antimicrobial reagents such as streptomycin, actinomycin, streptothricin, gramicidin, andbacitracin. However, the dawn of antibiotic era is accepted as the discovery of penicillin by Alexander Fleming in 1928. While Fleming was screening *Staphylococcus aureus*, a bacterial species causing skin infections, food poisoning, and respiratory diseases, he noticed a contaminant mold which has been secreting antibacterial substance that have killed *staphylococcus aureus*. Following this observation, pure penicillin was produced and used to clear infectious diseases after 1940 [1, 2]. Since then, several novel antibiotics have been introduced to the market and used for medical purposes. Antibiotics are classified in five groups according to pathways that they inhibit. These pathways are (1) cell wall synthesis, (2) plasma membrane organization, (3) nucleic acid synthesis, (4) ribosomal function, and (5) folate synthesis (Figure 1) [3].

Cell wall synthesis has a vital role in survival of bacteria. Any damage or loss of bacterial cell wall can result in cell lyses and consecutive cell death. Cell wall components of gram negative and positive bacteria have some differences but peptidoglican layer is the common constituent, which is hence the target of cell wall synthesis antibiotics. Cell wall synthesis is carried on in three steps. First step is precursor synthesis that is the synthesis of UDP-MurNAc from UDP-GlcNAc with series of enzymatic reactions involving MurA to Mur F in cytoplasm. Fosmomycin is one antibiotic that targets MurA. Similarly, cycloserine is another drug that binds to both alkaline racemase and D-Alannyl-D-Alaline synthetase that are required in the last step of MurNAc synthesis. Second step involves transport of MurNAc to cytoplasmic

membrane by a lipid carrier. Lipid carrier formation is catalyzed by MraY and MurG links MurNAc to lipid molecules. Bacitracin, for instance, interferes with this step and blocks transfer. Final step is subunit polymerization and connection of new peptidoglican to cell wall. This step is also a target of β-lactam antibiotics such as penicilin, cephalaosporins, penems, carbapenems, and monobactams.

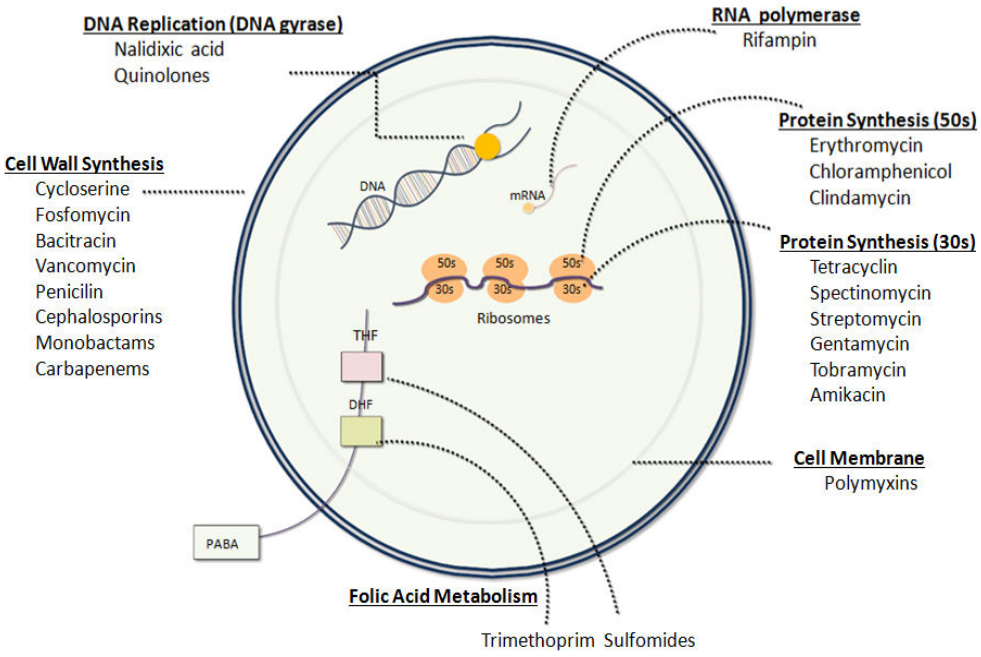


Figure 1: Classification of antibiotics. Antibiotics are classified in five groups according to mode of action. Figure is adapted from a reference [3]

Cell membrane is composed of lipid bilayer, proteins and lipoproteins. The main duty of cell membrane is regulating transport of ions and molecules. Many antibiotics target cell membrane. Polymyxins are one of these antibiotics which disturb the negative charge of gram negative bacteria found in lipid surface. This action results magnesium and calcium displacement resulting leakage of content of cell.

Many antibiotics inhibit nucleic acid synthesis via several mechanisms. Flucytosine stops thymidylate synthetase activity and causes thymine deficiency of cells. Acyclovoir interferes with thymine kinase and DNA polymerase of herpes virus

and Zidovudine inhibits reverse transcriptase enzyme of human immunodeficiency virus (HIV). Some intercalating agents are also used to impair DNA function. Although their antimicrobial action is debatable, chloroquine and miracil D kill plasmodia and schistosomoes. Rifamycin binds to cofactor binding site of RNA polymerase which is required for initiation of transcription. The other nucleic acids inhibition mechanism is impairing of DNA replication. Nalidixic acid, norfloxacin and ofloxacin belong to quinolones antibiotic classes and inhibit DNA gyrase which uncoils DNA during DNA replication [3].

Ribosomes synthesize proteins with sequential events of initiation, elongation and termination. Ribosome consists of two ribonucleoprotein subunits 30S and 50Swhich together form 70S initiation complex during protein synthesis. Unsurprisingly, both subunits are targets of protein synthesis inhibitors. Amino glycosides, a class of protein synthesis inhibitors, have free NH_4 and OH groups for binding to particular proteins of 30S subunit. For instance, streptomycin, kanamycin, and gentamycin bind to16S region of 30S subunit resulting 30S subunit depletion in pool and shutdown of protein synthesis. In the case of spectinomycin which is closely related to amino glycosides classes, it causes misreading of mRNA code and consecutive defective protein synthesis as a consequence of unstable binding of peptidyltRNA. Tetracyclin, which is another 30S inhibitor, binds transiently to aminoacyl-tRNA and blocks the access to ribosome. Chloramphenicol, erythromycin, and clindamycin are 50S subunit inhibitors. Chloramphicol affects both gram negative and positive bacteria by binding to peptidlyltransferase and stopping peptide bond formation. Erytromycin belongs to macrolides family and is generally more effective against gram positive bacteria. It interferes with peptidyltransferase reaction and translocation [3, 4].

In folic acid synthesis pathway, both trimethoprim and sulfomides impede tetrahydrofolate production which is an important precursor of DNA, RNA and some proteins [3, 4].

1.2 Antibiotic Resistance

Before antibiotics discovery, human suffering was enormous. Many people died because of infectious diseases from tuberculosis to pneumonia, to strep throat. After the discovery of antibiotics, all mankinds started to use antibiotics for every little disease symptoms and doctors prescribed antibiotics for every patient even their infection was not bacterial. Additionally, antibiotics were not only used by humans, there has been massive use of antibiotics in livestocks such as animal feed and water supply in order to prevent disease growth. Therefore, overuse of antibiotics revealed resistance [5].

Bacteria evolve many resistance mechanisms. The commonly seen mechanism is horizontal gene transfer of plasmids containing antibiotic resistant genes. The other most observable resistant mechanism is efflux pumps found in membrane of bacteria which is responsible for transport of antibiotics to out of cell. This mechanism is commonly seen in tetracycline resistance. Occasionally, an enzyme alters antibiotic molecules so antibiotics cannot bind to its target. In the case of spectinomycin, an enzyme chemically modifies spectinomycin molecule. Therefore, it can no longer bind to target site. Sometimes, an enzyme degrades antibiotics. For examples, b-lactamase enzymes bind to b-lactam ring of penicillin group and cleave rings and antibiotic cannot reach to its binding site (Figure 2) [6].

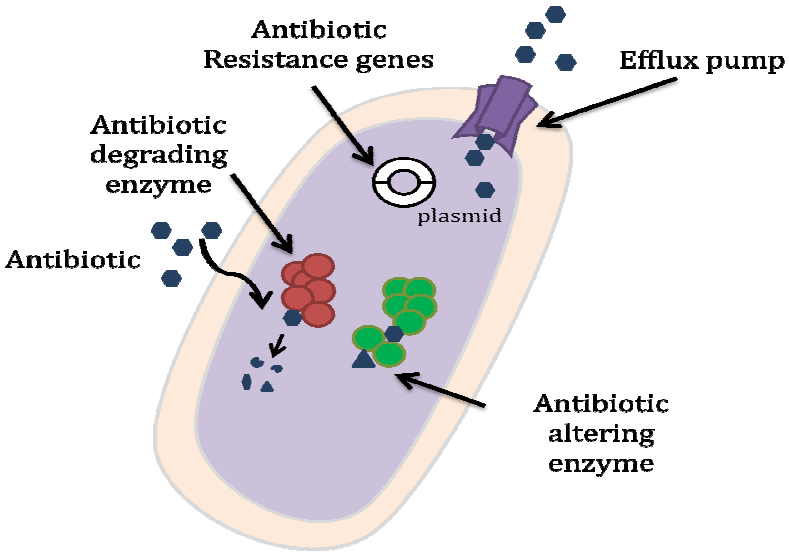


Figure 2: Antibiotic resistance mechanisms in bacteria

1.3 Mechanism of Action of Trimethoprim and Trimethoprim Resistance

Trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxybenzyl) pyrimidine] is a synthetic antibacterial agent that belongs to diamino pyromidines class. It interferes with folic acid pathway. Folic acid pathway begins with the formation of dihydropteroic acid from para-aminobenzoic acid (PABA) and pteridine by catalytic action of dihydropteroate synthetase. Dihydropteroic acid is reduced to dihydrofolic acid under favor of dihydrofolate synthetase. Dihydrofolic (DHF) acid is subsequently reduced to tetrahydrofolate (THF) with the help of cofactor NADPH by dihydrofolate reductase where TMP binds. Therefore, TMP impedes production of tetrahydrofolic acid which is crucial precursor of purines, tymidine, methionine, glycine and f-Met-tRNA generation. Consequently, DNA, RNA and proteins synthesis is blocked (Figure 3) [7].

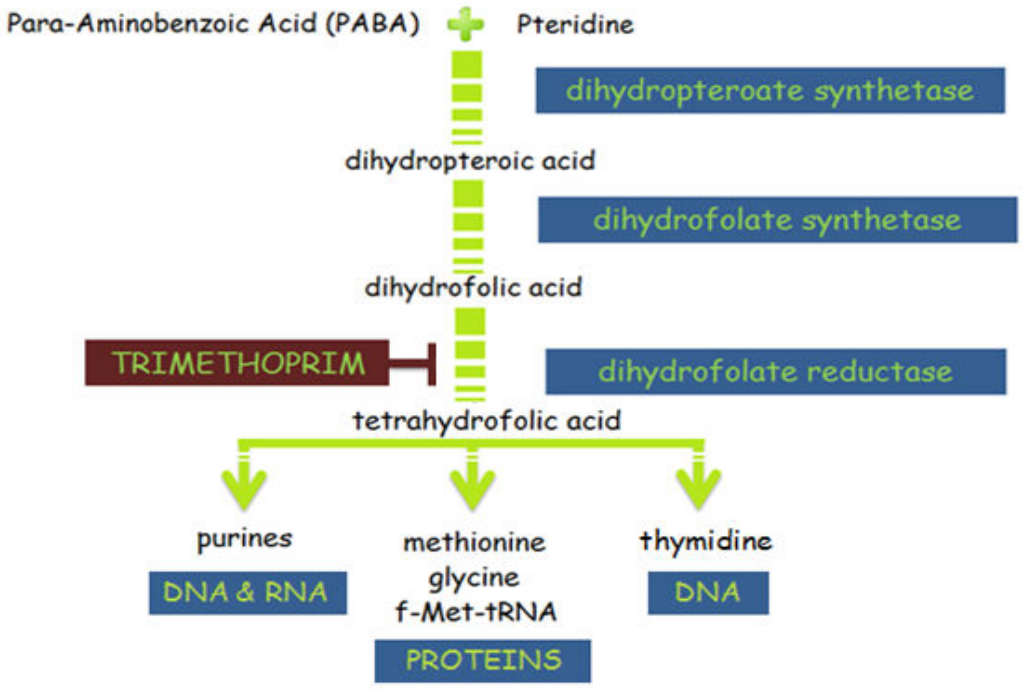


Figure 3: Folic Acid pathway adapted from reference [7]. Trimethoprim inhibits DNA, RNA and protein synthesis

Chemical composition of Trimethoprim (TMP) allows the drug fit well to the active site of dihydrofolate reductase (DHFR) enzyme which is encoded by *FolA* gene. Trimethoprim is structurally similar to folic acid, the natural substrate of DHFR and it competes with dihydrofolate for binding DHFR (Figure 4). Trimethoprim is suitable for human use since it binds to prokaryotic DHFR with 10000 times more affinity than mammalian DHFR [3, 8]. Therefore, TMP selectively binds its target and it is safe and efficient folic acid pathway inhibitor.

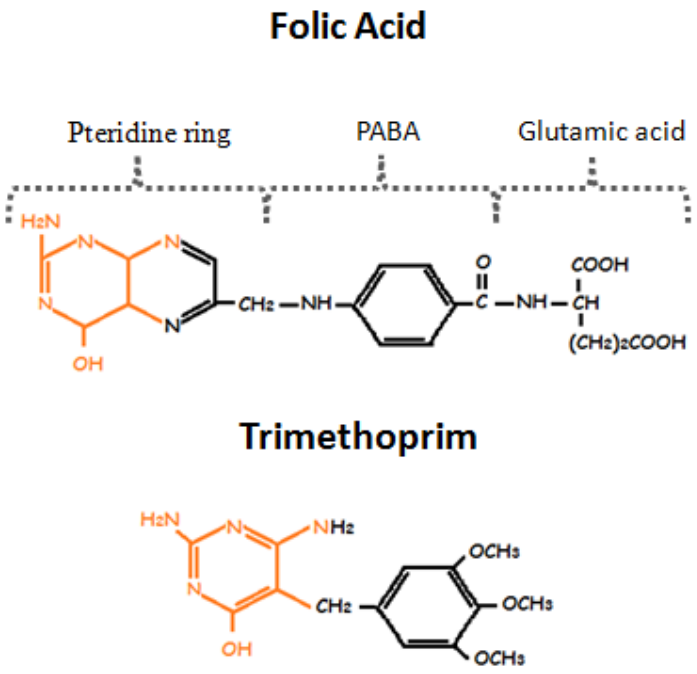


Figure 4: Comparison between structure of Trimethoprim and folic acid. Folic acid is the ligand of DHFR whereas Trimethoprim is the competitive inhibitor of DHFR. They share similarity colored in orange [9]

TMP was initially used in Proteus septicemia treatment in 1962. After the discovery of synergy between sulfonamides, combinations of these drugshave been in clinical use against various kind of infections since 1968 in the United States and United Kingdom [10, 11]. Latterly, although sulfomides and Trimethoprim combinations have been relatively inexpensive to single usage of one, TMP alone was tried to cure urinary tract infection in Finland in 1972 due to side effects of sulfonamides. Consequently, TMP

was used alone in urinary and respiratory tract infections in several European countries and United. Now, TMP is one of the most commonly used antibiotics in the world and worldwide utilization of TMP reveals TMP resistance as a significant health problem [11].

Trimethoprim (TMP) resistance can be either acquired or intrinsic (Figure 6). Some organisms are naturally more resistant to TMP .Cell wall and membrane impermeability and together with the efflux pumps are main reasons of natural resistance to TMP. For instances, *pseudomonas aeruginosa* and other pseudomonas types are intrinsically invulnerable to TMP because they posses robust cell wall and mexABoprM drug efflux system [11]. Furthermore, gram negative bacteria tend to be more resistant to several antibiotics than gram positive bacteria due to cell membrane structure differences. Although gram positive bacteria has thicker peptidoglican layer than gram negative, additional outer lipid membrane of gram negative bacteria provides protection from drug penetration. Other intrinsic resistance may be originated from having insensitive DHFR against TMP as TMP is specifically designed to inhibit bacterial DHFR. Mammalian DHFR has approximately 30% similarity with bacterial DHFR (Figure 5). Therefore, they are intrinsically resistant TMP. *Bacillus Anthracis* and *Lactococcus lactis* have also insensitive DHFR and they are innately resistant to TMP [12, 13]. The last intrinsic resistance mechanism is thymidylate bypass. It is very common in folate autotrophic species such as *Leishmania*, a parasitic protozoan. These microorganisms have a novel pteridine reductase enzyme which can reduce folate and unconjugated pteridines. Hence, DHFR inhibition by TMP cannot affect *Leishmania* species [14].

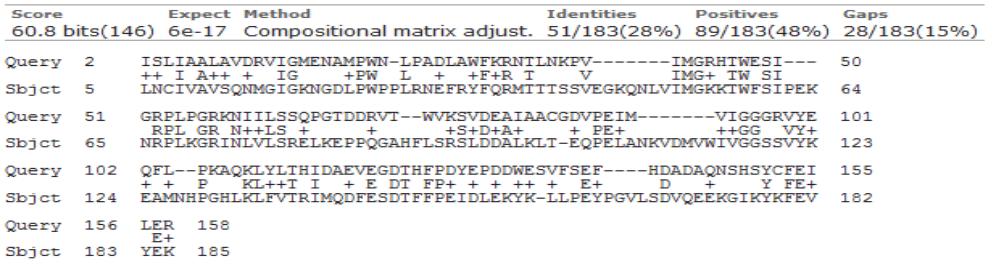


Figure 5: Protein blast of human and bacterial (MG1655 E.coli) DHFR. They share 28% identities.

Acquired resistance may occur due to production of insensitive DHFR protein by acquisition of plasmid from outside or resistant *folA* gene cassette can be found in transposable elements of microorganisms. In nature, there are approximately twenty different transferable element such as transposons, plasmids and integrons containing resistant folA gene [7]. Such DHFR variants are generally much more inefficient than normal enzyme. Most of them have sacrificed electrostatic or conformational components for sake of gaining resistance to TMP [15]. Especially, horizontal gene transfer of DHFRI and DHFRII variant provides nearly 1000 times MIC (minimum inhibitory concentration) value [10]. In addition to those mechanisms, TMP resistance may be acquired by spontaneous mutation or gene amplification under selective condition. These mutations may be either on efflux pumps so that drugs would be expelled before it reaches target or on its actual target DHFR. Promoter or ribosome binding site (Shine Dalgarno sequence) mutations in target or multi drug efflux pumps genes have regulatory role in transcription and translation. This causes overproduction of intracellular DHFR or efflux pumps expression on surface of membrane. Increased expression of DHFR or multidrug efflux pumps leads to high levels of resistance against trimethoprim (Figure 6).

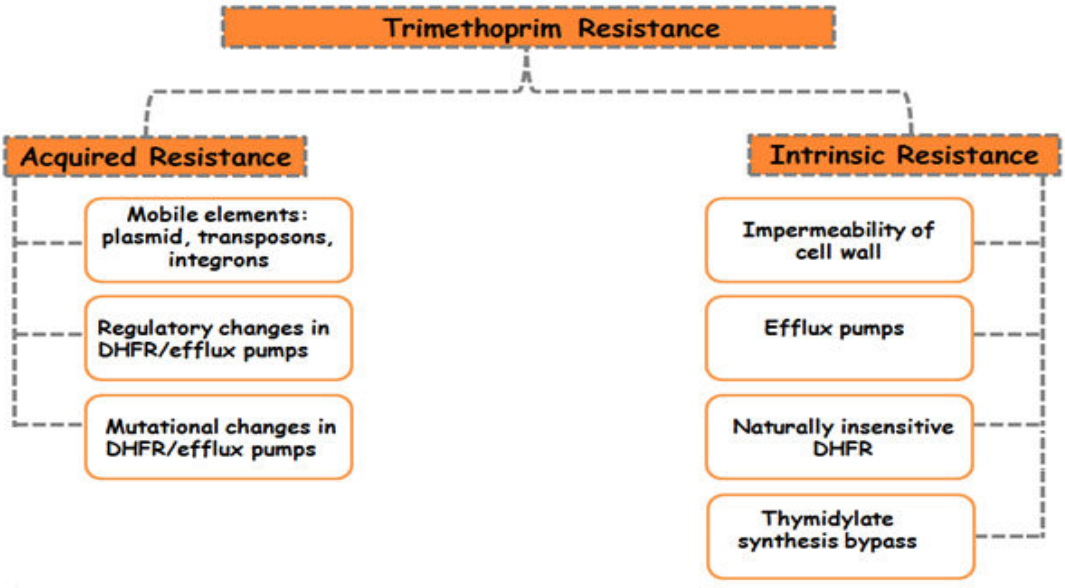


Figure 6: Trimethoprim Resistance Mechanism is divided in to two parts: acquired and intrinsic.

1.4 Structure of Dihydrofolate reductase

Folic acid pathway inhibitors have been used for a long time in both prokaryotes and eukaryotes. Since folic acid pathway is very essential for cells, DHFR is targeted by anti malarial agent pyrilmethamine, trimethoprim and as well as anticancer drug methotrxate. Long term usage of all these drugs reveals DHFR dependent resistance [16].

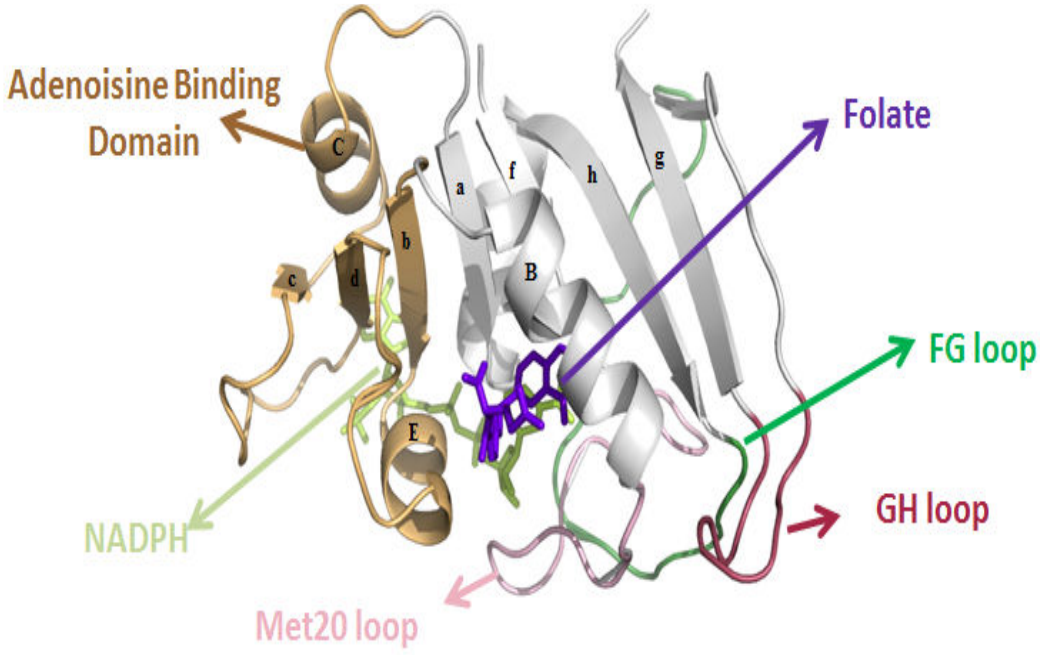


Figure 7: 3D structure of dihydrofolate reductase (DHFR) complexed with dihydrofolic acid (folate) and cofactor NADPH .Enzyme active site is located between loop I (met20 loop), α helix B and β sheets a, e, b.
α helices are represented with upper case letter and β sheets are represented with lower case letter [17, 18]. DHFR is composed of eight β sheets (a, b, c, d, e, f, g, h) and four α helices (B, C, D, F)

To be familiar with DHFR dependent resistance, structural understanding of DHFR is critical. DHFR is ubiquitously found in all prokaryotes and eukaryotes; however, there is a great deal of sequence diversity in DHFR while conserving some regions on the protein structure that are vital for enzymatic activity and protein

stability. For example, all variants of DHFR contain four α helices: two α helices for substrate binding and two α helices for coenzyme binding and also Loop 1 and cis peptide bond between two lysine are common structures.(Loop1 is situated between β sheet A and α helix B and cis peptide bond is located between β sheet e and α helix F shown in figure 7). In DHFR catalysis, 2 processes are mainly important. These are protonation of substrate and transfer of hydrate ion. The aspartic acid (D) residue at position 27 (position 30 in human DHFR) in e. coli is responsible in protonation of substrate dihydrofolic acid and also determines ligand specificity [17, 19]. In hydrate transfer, Methionine residue at position 20 in e. coli provide electrostatic stabilization [19]. In order to analyze which residues are vital, several mutagenesis studies have been completed and Ala9, Asp27, Leu28, Phe31, Arg44, His45, Thr46, Leu54, Tyr100, Thr113, Gly121 and Asp122 are shown to have huge impact catalytic cycle [20]. These residues are also conserved in evolutionary constraint according to detailed SCA analysis of DHFR gene. SCA (Statistical Coupling Analysis) is method based on Multiple Sequence Alignment (MSA) to reveal long range evolution record. All proteins found in different organism evolve from ancestral origin and most of residues evolve independently. However small proportion of residues evolves together and the network of coevolving residues called sector. Sectors are generally related to tertiary structure of protein. Frequency of reiteration of residues in distinct organism displays importance of residue for protein functioning [21].

In previous SCA of e.coli DHFR, residues 15, 21, 27, 28, 31, 32, 35, 37, 42, 44, 51, 54, 55, 57, 59, 63, 77, 81, 94, 113, 121, 125, 133 are determined on sector based on p=0,005 probability density cutoff and 3, 11, 13, 15, 21, 22, 27, 28, 31, 32, 35, 37, 39, 40, 42, 44, 47, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59, 63, 64, 77, 81, 90, 94, 100,111, 113, 121, 122, 125, 126, 133, 153 are coevolved DHFR residues based on p=0,010 probability density cutoff according to student's t distribution. They comprise 14% and 25% of DHFR, respectively [21]. These residues are thought to be hot spots in allosteric control and enzymatic function of DHFR and they should be the most affected residues during drug selection.

1.5 Morbidostat

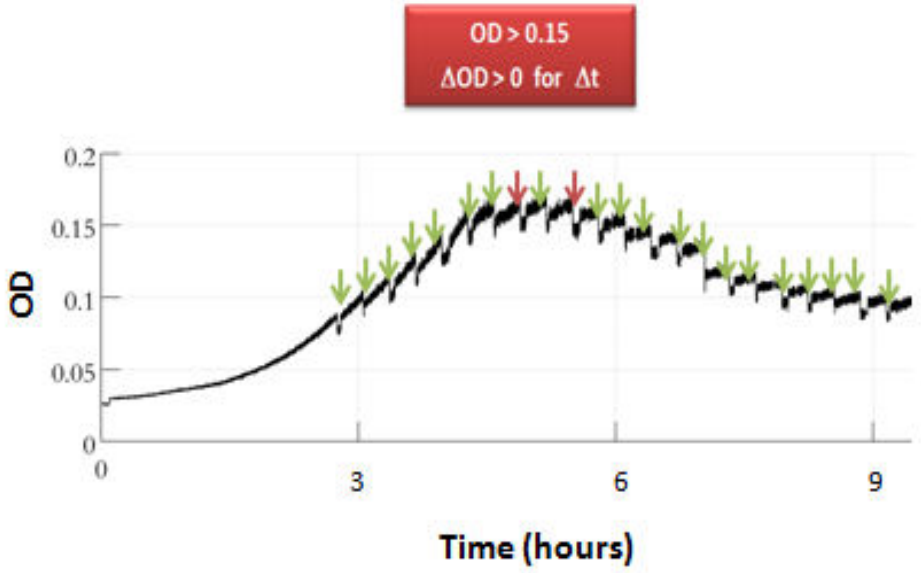


Figure 8: Morbidostat working principle. X axis shows OD and y axis shows time in hours. Each cycle dures Δt time. Drug or medium addition is decided according OD value after Δt time. Green arrows correspond to media dilution and red arrows represent drug injection. Drug is added only if OD>0.15 and ΔOD>0.

Evolution acts on an organism by obtaining continuous adaptive mutations in protein sequences. These adaptive mutations are necessary for fitness of the organism to the environment and determine population dynamics [22]. In order to understand population dynamics and evolution of organism, computer controlled selection device "morbidostat" is built. Bacteria cultures growth is monitored over time in morbidostat. It enables to keep experimental condition under controlled selective pressure by using controlled algorithm. In Morbidostat, The optical densities of cultures are recorded in fixed time period (Δt). Growth rate (ΔV/V) is calculated and device decides to add fresh media or drug. Bacterial growth is controlled with dilution and drug inhibition. Whenever the OD of culture is equal or exceeds ODthr (OD threshold) or growth rate surpasses dilution rate, drug is added

in predetermined dilution time (Figure 8). So, drug concentration is arranged to let bacterial population to expose to fixed growth of inhibition. Therefore, more reproducible and also resistant bacteria population may be selected among other parallel population [23].

2. AIM OF THE STUDY

Dihydrofolate reductase (DHFR) enzyme has been a target for many drugs since it is a fundamental precursor of purines biosynthesis, thymidylate and some amino acids. Trimethoprim is the one of these drugs that targets bacterial DHFR and its long-term and widespread usage reveals resistance. Although there are various mechanism, accumulation of spontaneous mutation in target is expected and inevitable scenario. However, there is little knowledge about preference of position, fitness, order or nature of spontaneous mutation. Selection of advantageous spontaneous mutation having higher fitness is the origin of resistance.

In the first part of this study, we aimed analyze the origin of resistance by shedding light on mutational choice. Finding advantageous mutation types, their compatibility with each other and in what order they were accumulated might provide an answer for defeating TMP resistance. Secondly, we purposed to gain more insight about dynamics of population and final destination of mutational choice against different selection types. For that reason, we arranged our experimental condition to let bacteria populations grow only if their fitness was above a certain threshold and we used mild dilution for 6 cultures and strong TMP dilution for 7 cultures to generate distinct selection environment. Finally, we aimed to establish a connection about preferences of mutation by researching network of evolutionary coevolved residues called sectors with Statistical Coupling Analysis. So we asked that sectors were more likely to be hit against selective pressure. Consequently, with this study, the next step of mutational choice may be predicted. New synthetic TMP analogs may be designed for a particular mutation types in future.

3. MATERIALS & METHODS

3.1. MATERIALS

3.1.1 Chemicals & Media Components

Chemicals for Media Components	Supplier Company
Agar-Agar	Merck, Germany
DMSO	Biochem, Germany
Choloramphenicol	Sigma, Germany
Ethanol	Merck, Germany
Glucose	Sigma, Germany
LB	Merck, Germany
Magnesium Sulfate (MW: 246,48)	Sigma, Germany
M9 Minimal Salts 5X	Sigma, Germany
Protein Hydrolysate Amicase	Fluka, Germany
Calcium chloride (MW: 147,02)	Applichem, Germany
Trimethoprim	Sigma,Germany

Table 1: Chemicals that are used in Media

3.1.2 Antibacterial Agents

Chloramphenicol and Trimethoprim stock solutions were prepared as 50 mg/ml dissolved in 100% ethanol and DMSO respectively. Solutions were stored at -20^{0}C

3.1.3 Growth Media

M9 minimal salt solution (1X, autoclave sterilized) was supplemented with 4% glucose (autoclave sterilized), 0.2% amicase and MgSo4 and CaCl2 was added to solution to have final concentration 2mM, 100uM respectively. Solution was sterilized with corning cellulose Acetate membrane 0.22 micron bottle top filters and stored at room temperature. Chloramphenicol was added to media to obtain last concentration as 25ug/ml before use in experiment

LB-Agar 20ml per plate was used as solid medium for bacteria growth

3.1.4 Bacteria Strains

AttP21-PR-Mcherry Chlaramphenicol resistant MG1655 strains from Tobias Bergmiller, IST were used in whole experiment.

3.1.5 Software

Matlab program was used in morbidostat part of experiments and clc main workbench was used to analyze DHFR sequencing result

Equipment	Company
Autoclave	Priorclave, UK
Balance	Schimadzu, TW423LV, Japan Sartorius, BP610, Germany
Distilled Water	Millipore, Elix S, France
Shaker Incubator	New Brunswick Sci., Innova 44, USA
Spectrophotometer	Amersham Biosciences, UK
Incubator	Memmert, Modell 300, Germany
Laminar Flow	Heraeus, Germany
Microliter Pipettes	Gilson, Pipetman, France
Microscope	Olympus, CK40, Japan Olympus, CH20, Japan Olympus, IX70, Japan
Plate Reader	TECAN Infinite F200 pro TECAN Infinite M200 pro
Pinner	V&P Scientific,USA
Plate Shaker Incubator	Heidolph, Germany
Deep Freeze -20	Regal, Turkey
Deep Freeze -80	New Brunswick Sci.,U410,USA
Refrigerator +4	Regal, Turkey
Vortex	VWR,USA

Table 2: Equipments are used in this study.

3.2 METHODS

3.2.1 Morbidostat

Initially, 100 ul frozen wt isogenic bacteria cultures were added to 13 sterile culture tubes containing 12 ml M9 minimal media having 25ug/ml CHL. Before tubes were placed to tube holder of incubator, drug A, B and media flow and pumps were checked and matlab code was started. Neither media nor drugs were injected to tubes in order to let bacteria adapt to the environment in first hours. When the OD of cultures surpassed 0.03, injection started and continued 1 min for strong selection and 30 min for mild selection in every 18 min. At the end of this cycle waste pumps were functioned to keep all cultures at same volume and for avoidance of overflow. Each pump was set to have flow rate as 1ml/min.

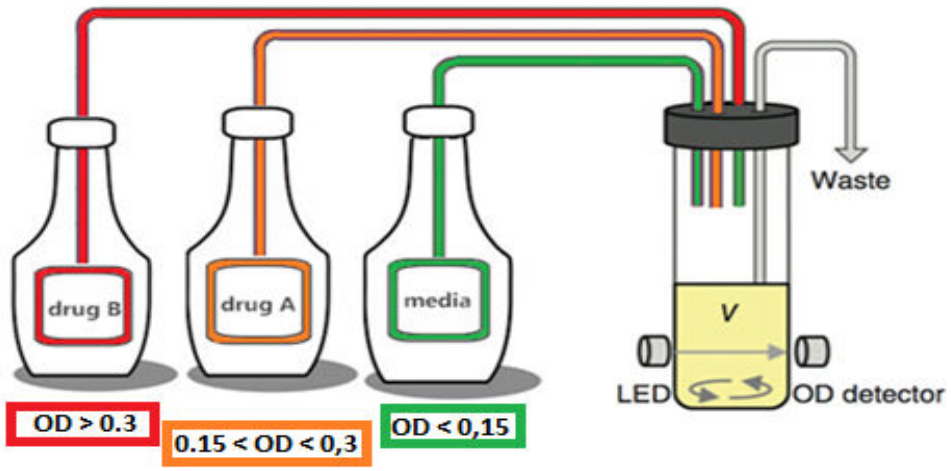


Figure 9: Morbidostat experiment working mechanism. Drug A is added if OD is between 0.15 and 0.3 and culture has positive trend in growth. Drug B is added if OD is greater than 0.3 and concentration in tube is higher than 60% of drug A. Otherwise media is added.

According to growth rate of cultures, computer algorithm determined whether Drug A or B or fresh media injection. If OD was smaller than 0.15, media was added. If it was between 0.15-0.3 or growth rate was exceeding the dilution rate, Drug A was added. If it was greater than 0.3 or the concentration of drug in culture tubes was greater than 60% of drug A, drug B was added (Figure 9). In addition, Pumps of morbidostat were set for mild and strong selection. Drugs were added during 30 second for mildly diluted cultures (culture 1-2-3-4-6-7) and 60 second for strongly diluted cultures. Therefore, dilution rates were arranged to a certain thresholds which were 0.3 $hour^{-1}$ and 0.6 $hour^{-1}$ in mildly and strongly diluted cultures respectively. In both systems, bacterial growth was restricted but mild dilution system was more tolerable than strong dilution, which provides survival of more bacteria population. (Figure 10) After 20-24h, cultures were frozen by using 15% glycerol at $-80^{0}C$ and following experiment was started from frozen samples.

The concentration of drug bottle used in experiments is shown in following table:

days	drug A	drug B
day 1	10ug/ml	50ug/ml
day 2	10ug/ml	50ug/ml
day 3	10ug/ml	50ug/ml
day 4	10ug/ml	50ug/ml
day 5	10ug/ml	50ug/ml
day 6	10ug/ml	50ug/ml
day 7	10ug/ml	50ug/ml
day 8	50ug/ml	250ug/ml
day 9	50ug/ml	250ug/ml
day 10	50ug/ml	250ug/ml
day 11	50ug/ml	250ug/ml
day 12	250ug/ml	1250ug/ml
day 13	250ug/ml	1250ug/ml
day 14	250ug/ml	1250ug/ml
day 15	250ug/ml	1250ug/ml
day 16	250ug/ml	1250ug/ml
day 17	250ug/ml	2000ug/ml
day 18	250ug/ml	2000ug/ml
day 19	250ug/ml	2000ug/ml
day 20	400ug/ml	2000ug/ml
day 21	400ug/ml	2000ug/ml
day 22	400ug/ml	2000ug/ml
day 23	400ug/ml	2000ug/ml
day 24	1000ug/ml	50ug/ml
day 25	1000ug/ml	50ug/ml

day 26	1000ug/ml	50ug/ml
day 27	1000ug/ml	50ug/ml

Table 3: Drug A and B concentration in each days of experiment. Drug B is generally 5 fold of concentration of drug A

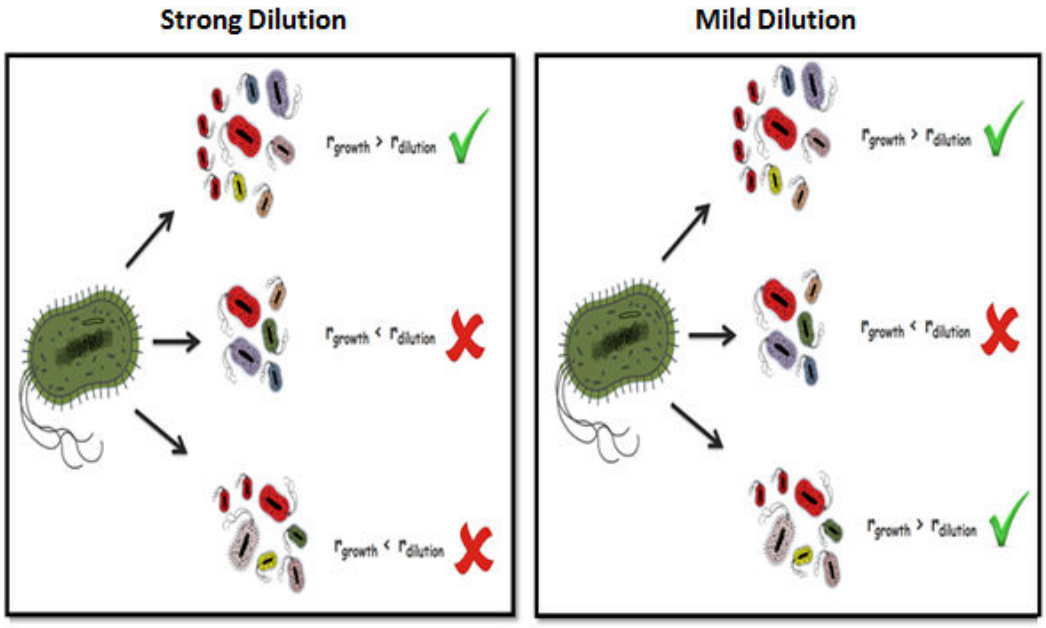


Figure 10: Comparison of strong and mild dilution in morbidostat. Dilution rate is more restricted in strongly diluted cultures than mildly diluted cultures. Colors and sizes represent that population is very mixed. Arrows show three imaginary conditions which are same in each rectangle. Red colored (mutant) bacterium in first arrow has higher reproducibility than in third arrow which also has higher reproducibility than second arrow. Two Bacteria populations having less fitness than other are eliminated from cultures in strongly dilution case while one bacteria population is eliminated from culture in mild dilution case.

3.2.2 Measurement of Growth Rate

96 well plates containing 150ul M9 minimal media (25ug/ml CHL) was prepared and then bacteria were seeded with pinner from master plate (appendix a). They have grown at 30C with shaking and OD measurement was done in every 15 minutes with TECAN for 24 hours. Growth rate is calculated with $\ln (OD_2/OD_1) / t_2-t_1$ formula according to exponential phase of each day of population.

3.2.3 Determination of Minimal Inhibitory Concentration (MIC)

MIC values of each day of each culture were determined. 18 different concentration of TMP was tested (3000, 2500, 2000, 1500, 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 ug/ml). 96 well plates comprising 150 ul M9 minimal media with 25ug/ml CHL and different concentration TMP solutions were prepared. Bacteria were added with pinner from master plates (appendix a). They have grown at 30C with shaking for 24 hours. OD of each well was measure with the help of TECAN. The result was analyzed with Matlab code

3.2.4 Single Colony Selection and Sample Preparation for Sequencing

Mix population was streaked into LB agar from daily frozen samples of morbidostat. They were grown at 37C over night. Then single colonies were chosen randomly. They were grown in M9 minimal media with 25ug/ml CHL overnight and frozen with 15% glycerol.

1ml Agar stabs were prepared for sample preparation and 20 ul bacteria from frozen single cells were seeded on to agar stab and kept in +4.

3.2.5 Sequencing

SNP Discovery/Mutation Discovery sequencing was performed for bacterial samples. FolA gene of E. coli (K12 MG1655) region was sequenced by using following primers:

Toprak28-5 GGGAACCGAAGAAGGTAAACA
Toprak28-3 GCGTCTTAAACACAGCCTGAT

Sequencing primers for SNP located in 34 nucleotides upstream of folA gene designed specifically to target position from 50136 to 50535 of e.coli genome.

3.2.6 Sequencing Analysis

Pair wise sequence alignment were done my using mclab tools and mutations were analyzed by using clc main workbench

3.2.7 Statistical Coupling analysis (SCA)

SCA analysis was performed with MSA of 4166 sequence by using promals 3D software. RMDS is root mean square deviation and calculated with following formula:

RMDS=$\sqrt{\sum_{t=1}^{n} \frac{(K_t - Y_t)^2}{n}}$ where K is Kimberly Reynold's alignment and Y is our alignment.

3.2.8 f Statistic Calculation

F_{RS} was calculated with following formula:

$F_{RS}=(H_R-H_S)/H_R$ where H_R is regional heterozygosity and separately calculated for two regions which are strong and mild selections and H_S is subpopulation heterozygosity and calculated for each day with following formula:

$H_S=1-\sqrt[n]{\prod_{i=1}^{n} Pi}$ P is frequency and n is number of sub clones. Detailed calculation is shown in figure 9.

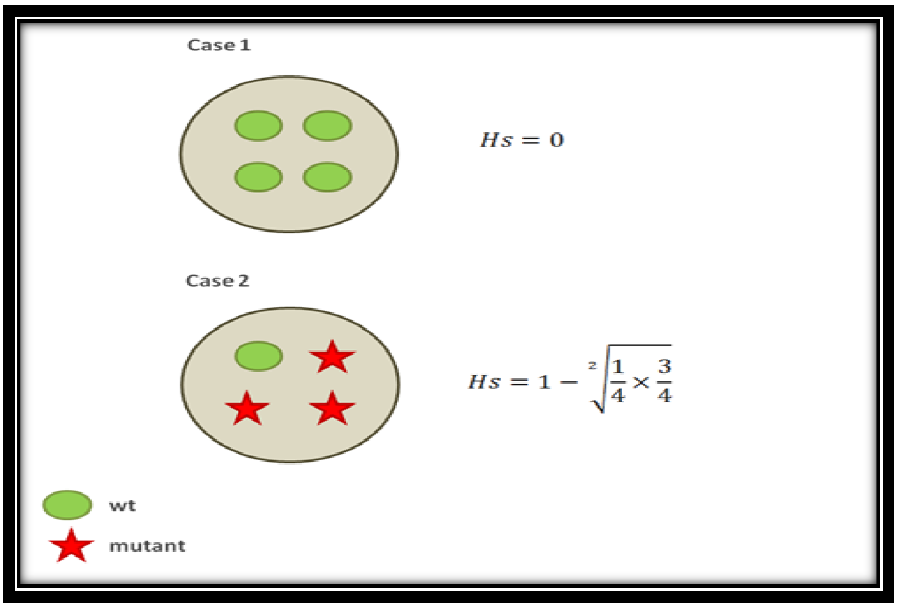


Figure 11: Calculation method of Heterozygosity of subpopulation. Green circles represent wild type and red stars represent mutant genotypes.

3.2.9 Mutation Assessor

FI scores were calculated by using program that are found in website (mutationassessor.org)

4. RESULTS

In our experiment, we evolved 13 Chloramphenicol resistant Mcherry MG1655 E. coli strains by using morbidostat. We carried out experiment during 23 days (504 hour). We tried two different selective pressures by setting trimethoprim dilution strong and mild. First six cultures were determined to be exposed to mild dilution and other seven were prescribed as strong selection. We took daily stock from each day of each culture tubes. After experiment was completed, we sent samples to sequencing from day 1, 4, 7, 10, 13, 16, 19, 23. If we noticed any complexity to understand mutation order, we sent samples from other days as well. Thereafter, we continued to experiment four days more in order to analyze whether cultures gain another mutation or not.

4.1 Final Genotypes of cultures

Although there were similarities between mutation positions, we observed nine different genotypes out of thirteen cultures. We detected that some mutations have not been involved in some mutational combinations due to epistatis. For instances, A26T was not observed with D27E in any genotypes. This mutation was mostly placed after L28R or W30 residue mutations. Additionally, D27E was always accumulated after or before F153 residue mutations.

Promoter mutations also accumulated next mutation according to some preference. For instance, g-31 was observed in only three genotypes and acquired L28R and A26T as final genotype (Figure 12).

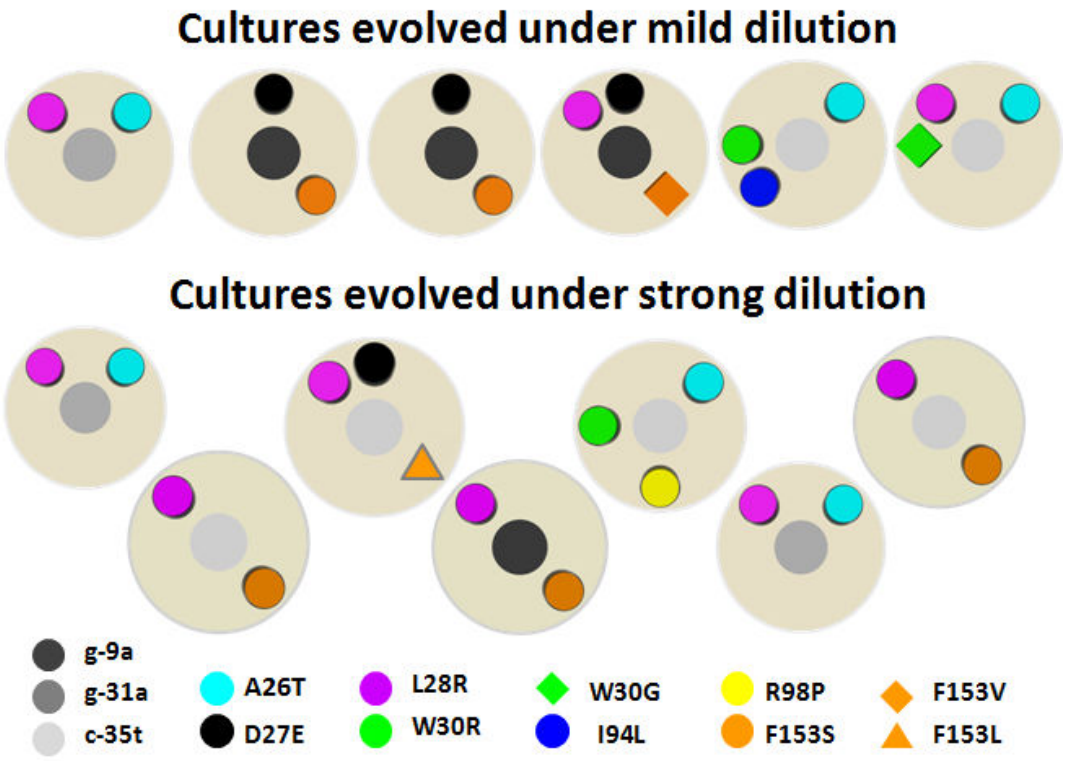


Figure 12: Final genotypes of cultures evolved under mild and strong dilution. Promoter mutations are found in the middle of circles and symbolized by gradient of grey colors.

4.2 Mutation Trajectories

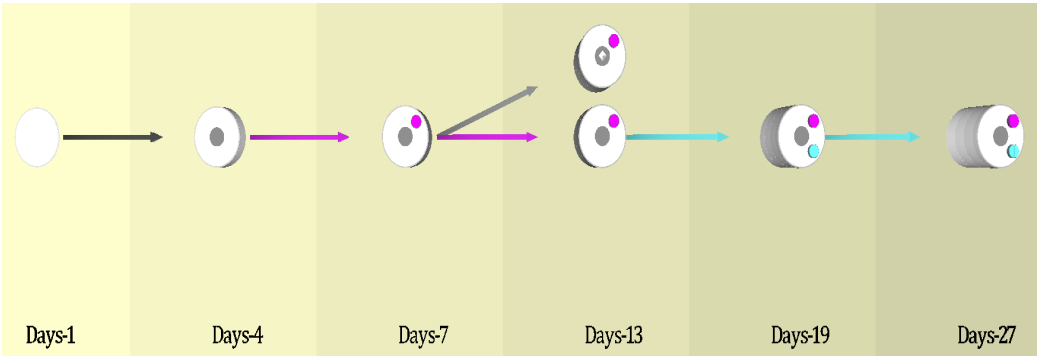


Figure 13: Mutation trajectories of culture 1. Grey, magenta and cyan circle correspond to g-31a, L28R and A26T, respectively. White diamond represents c-35t mutation at

day 13. ¼ of population have both g-31a and c-35t promoter mutation. The order of mutation is g-31a→L28R→A26T

The first mutation acquisition of culture 1 is promoter mutation at position -31 which is g to a transition. Second mutation is L28R which is Leucine (Leu) to Arginine (Arg) amino acid transition at 28th amino acid as a result of t →a change in 83rd nucleotide position at day 7. A26T Alaline (Ala) to Threonine (Thr) change is the third and last SNP that we observed at day 19. The possessions of these three SNPs are conserved until day 27 of the experiment. The sequencing result of culture 1 is highly ordered except at the day 13. One single colony has 2 promoters: g-31a and c-35t mutation with A26T at this day but this combination is not observable at day 19 (Figure 13).

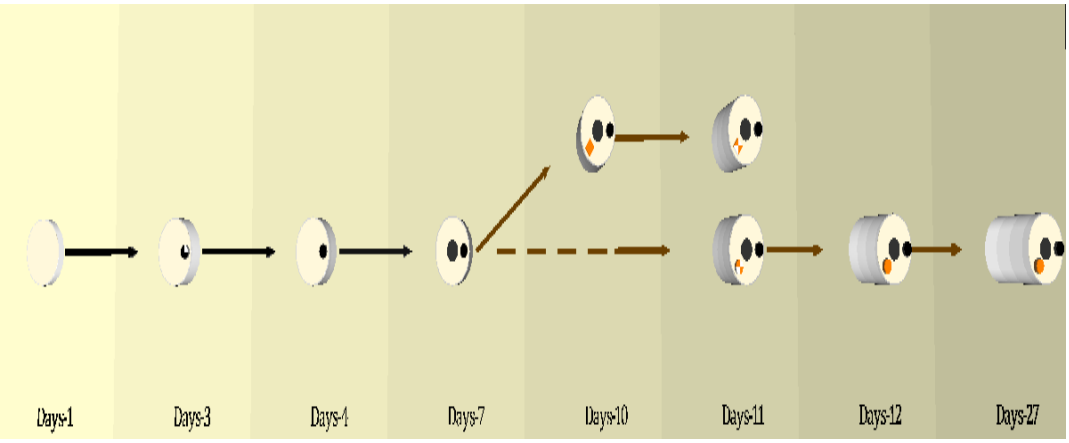


Figure 14: Mutation trajectories of culture 2. Black circle represents D27E. g-9a is shown with dark grey circle located in the center of the cylinder surface. Orange diamond and orange circle correspond to F153V and F153S, respectively. Mutation acquisition orders are D27E→g-9a→F153V and D27E→g-9a→F153S

In the case of culture 2, the first mutation suprisingly is not a promoter mutation. Negatively charge Aspartic acid (D) residue at 27th residue is firstly changed in to again negatively charged Glutamic acid (E) because of t→g transition at 81st nucleotide, and secondly, g-9a promoter mutation is accumulated approximately at day 7. Finally, t→a transition in 456th nucleotide of DHFR results Phenyalaline (Phe) to valine (Val)

change in 153rd residue at day 10. Although all sequencing result of day 11 shows that 100% of populations have F153V, the percentage diminishes to half and at day 12 because of F153S accumulation (t→c in 457th position). F153S is over dominated the population. D27E, g-9a and F153S SNPs are rested until the final day of experiment. Sequencing result of culture 2 clearly showed that F153S is capable of eliminating F153V (Figure14).

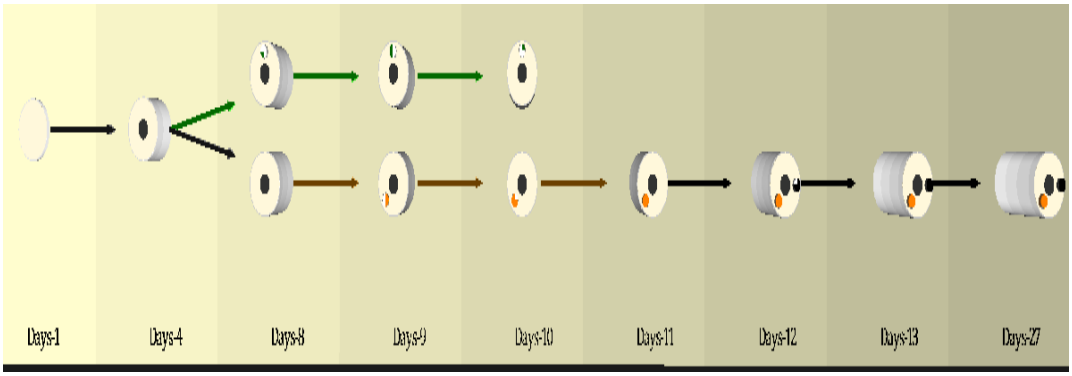


Figure 15: Mutation trajectories of culture 3. Dark grey circle located on the center is g-9a promoter mutation. Green, orange and black circle represent W30C, F153S, D27E, respectively.Mutation order is g-9a→W30C<F153S→D27E in culture 3

Culture 3 is again initiated with promoter mutation (g → t) at 9 nucleotides downstream of ribosome binding site of fol A gene. At day 4, W30C is accumulated in ¼ of culture population and ratio increases to ½ of population at day 9. However, newly acquisition of F153S starts to compete with W30C with same ratio. We can basically differentiate of which mutation's fitness is stronger by glancing at sequencing results of day 10 since ¾ of population now turns to g-9a + F153S and rest of the population is g-9a+W30C and at day 11. Complete dominance of F153S can be easily observed. New mutation D27E occurs at day 12 with ¾ ratios. This combination does not change until the final day of experiment. Similar to F153V, W30C has lower fitness effect on population than F153S (Figure 15)

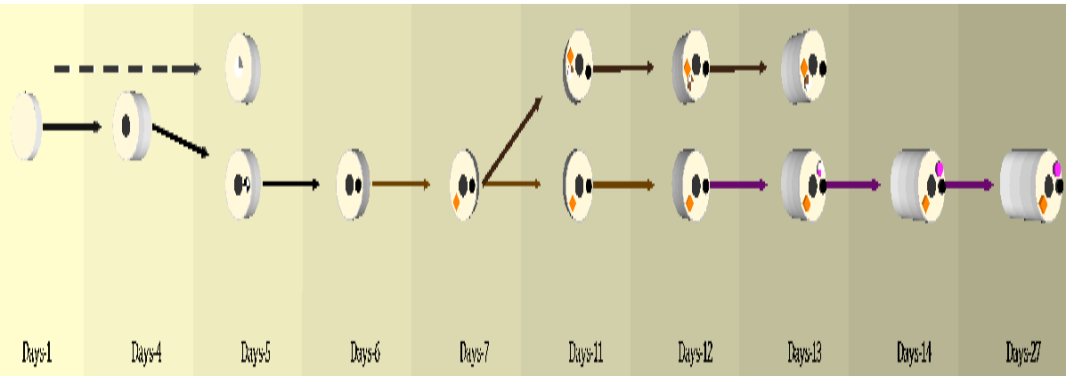


Figure 16: Mutational trajectories of culture 4. Dark grey and light grey circle found in the middle of cylinder surface are promoter mutation g-9a and c-35t, respectively. Orange diamond signifies F153V whereas black, brown and magenta circles correspond to D27E, M20I and L28R SNPs. g-9a→ D27E→ F153V→ M20I<L28R is sequential acquisition of mutation in culture 4

Sequencing result of Culture 4 on day 4 proves that mutation accumulation begins repeatedly with promoter mutation g-9a with 100% but other promoter mutation c-35t is observed in 25% of culture. Wilt type bacteria population increases DHFR expression by two promoter mutation. On day 6, 100% of population possesses g-9a +D27E mutation. After, F153V is added and whole population possesses g-9a, D27E and F153V on day 7. On day 11, new mutation M20I arises in ¼ of cultures and increases to ½ on day 12. The rest of the population on these days does not acquire any mutation. While they are competing with each other, g-9a, D27E and F153V population gains L28R mutation on day 13 and expel population having g-9a,D27E, F153V and M20I. By analyzing this competition, we could result M20I fitness effect is lower than L28R if population contains g-9a, D27E and F153V as background mutations. (Figure 16)

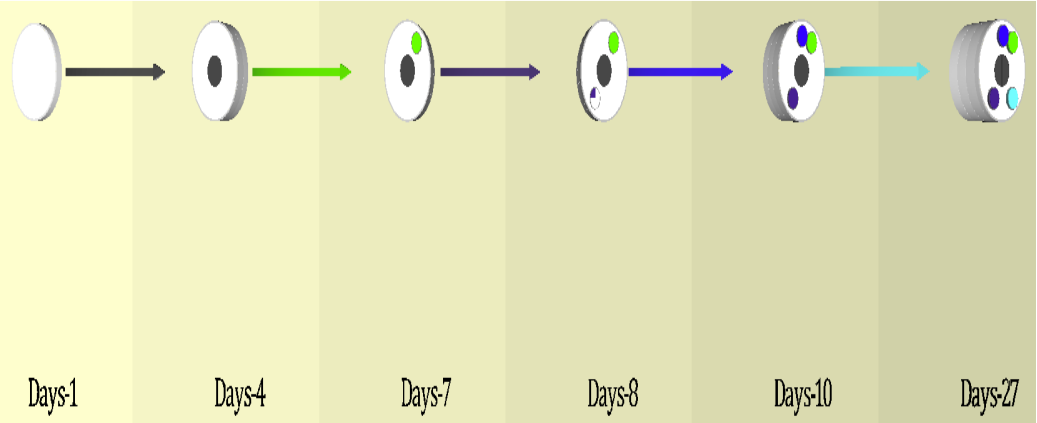


Figure 17: Mutation trajectories of culture 6. Dark grey, green, purple, navy blue, cyan circle correspond to c-35t, W30C,c+34t, I94L, A26T, respectively

Culture 6 has five sequential mutation accumulations. Initial SNPs is a promoter mutation c→t in 35 nucleotides downstream of Shine Dalgarno sequence of folA. Second mutation is W30R which is detected in 100% of population on day 7. Third mutation is interestingly not found in coding region, instead is located 34 base pairs upstream of folA gene. +34 position was repeatedly sequenced with other primer pair in order to be sure of its existence. Acquisition of c+34g mutation is initiated on day 8 with ¼ ratios but whole culture population contains this mutation on day 10 . Fourth mutation is isoleucine to leucine change at 94th residue is acquired in same day. The final fifth mutation is A26T. c-35t, W30R, c+34, I94L and A26T combination pursue their existence until 27th day of experiment. Mutation acquisition order of this culture is very clear and ordered. (Figure 17)

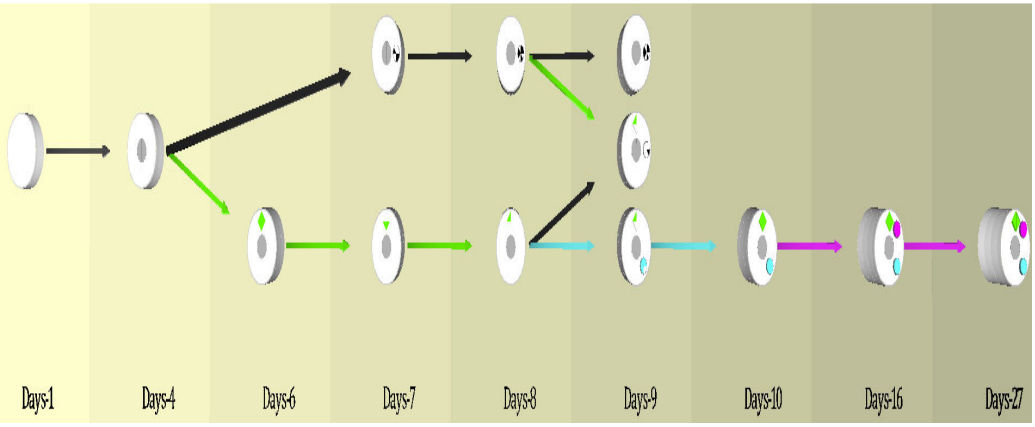


Figure 18: Mutation trajectories of culture 7. Light grey circle represents c-35t promoter mutation. Black, magenta, cyan circles correspond to D27E, L28R, A26T, respectively. Green diamond signifies W30G.

c-35t promoter mutation is first arising mutation of culture 7. On day 6, W30G mutation is added on c-35t and occupies 100% of population. However, on day 7, population is divided into 2 genotypes. 50% of population carries c-35t+W30C and others have c-35t+D27E. On day 8, 5/8 of population has c-35t+D27E and 3/8 carries c-35t+W30C. We could make an inference that D27E is better mutation than W30C in the case of reproducibility and fitness only if their background is same. On day 9, culture 7 population are divided in 3 genotypes. 1/8 has still c-35t+D27E and 1/8 has c-35t+D27E+W30C. These two combinations are washed out in later days. The rest of population carries c-35t+W30C+A26T which is occupied by 100% of population at day 10. L28R is lastly accumulated on c-35t+W30C+ A26T. (Figure 18)

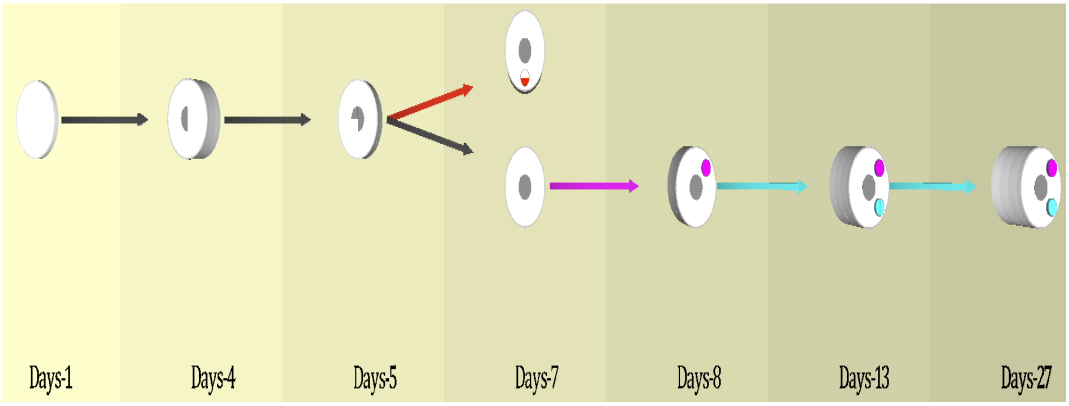


Figure 19: Mutation trajectories of culture 8. Light grey, red, magenta and cyan circles correspond to g-31a, P21Q, L28R and A26T, respectively. The orders of mutation acquisitions are g-31a→p21L and g-31a→L28R →A26T

After the g-31a promoter mutation acquisition on day 4 and 5 of culture 8, 50% of population P21Q accumulates on g-31a. Nonetheless, L28R prevails over P21Q and A26T is accrued on g-31a+L28R. This composition lasts to day 27. (Figure 19)

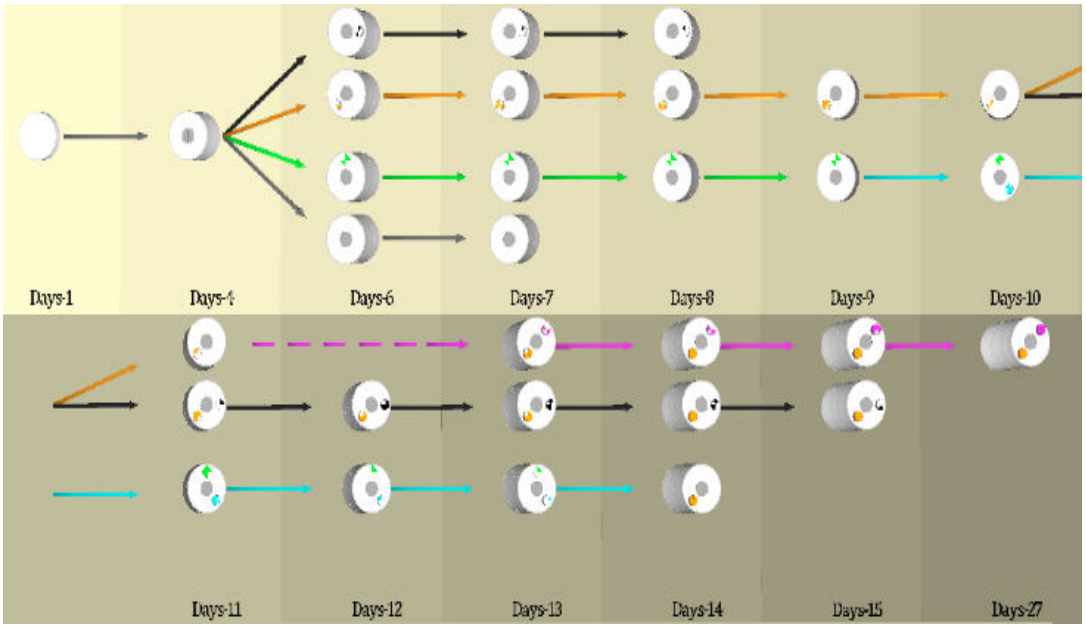


Figure 20: Mutation trajectories of culture 9. Light grey, black, magenta and cyan circles represent c-35t, D27E, L28R and A26T, respectively. Orange diamond, green diamond and orange circle correspond to F153V, W30G and F153S, respectively.

Dynamic of population is very complex in culture 9. First mutation c-35t is observed on day 4 of experiment. On day 6, 3/8 of population gains W30G, 2/8 of population acquires D27E and 1/8 of population accumulates F153S on promoter mutation. On day 7, c-35t+W30G and c-35t+F153S populations share same ratio which is 3/8. The rest of bacteria culture has c-35t+D27E. On day 8, c-35t+F153S mutation is found in ½ of population, 3/8 of population consist of c-35t+W30G and 1/8 of population contains c-35t+D27E which is excluded from culture in later days. On day 10, 7/8 and 1/8 of culture carries c-35t+F153S and c-35t+W30G, correspondingly. Nevertheless, c-35t+W30G group makes an attack to c-35t+F153S by acquiring A26T and dominates culture with 6/8 ratio. c-35t+F153S group attacks again by starting D27E accumulation with 1/8 ratio on day 11 and is predominated with 6/8 ratio on day 12. c-35t+W30G+A26T is started to be eliminated from culture on day 12-13. However, survival of c-35t+F153S+D27E group is disrupted by L28R mutation. Competition between c-35t+F153S+D27E group and c-35t+F153S+L28R group maintains during next 4 days. Finally, c-35t+F153S+L28R group wins. From this competition, we could make three assumptions: (1) F153S>W30G>D27E if strains have c-35t promoter

mutation background (2) L28R>D27E if they have c-35t + F153S background (3) F153S+D27E couple is stronger than W30G+A26T couple. (Figure 20)

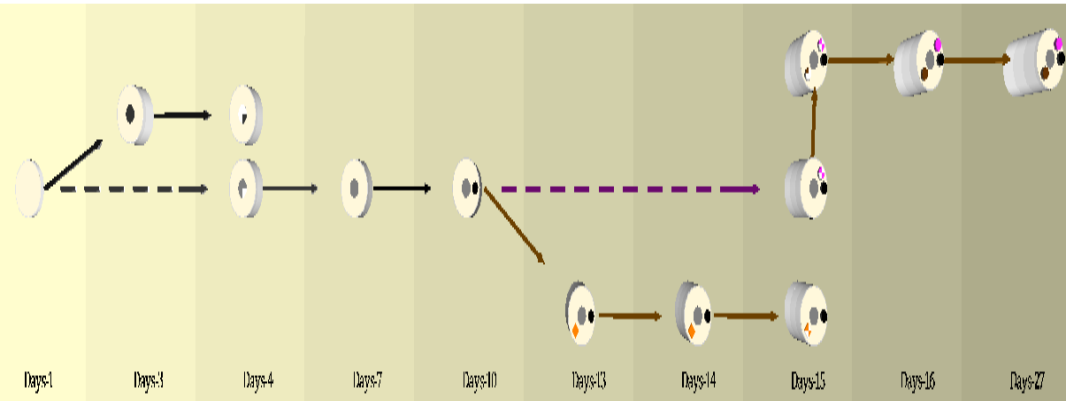


Figure 21: Mutation trajectories of culture 10. Dark grey and light grey colors correspond to promoter mutation g-9a and c-35t, respectively, Black and Magenta colored circles represent D27E and L28R mutations. Orange diamond signifies F153V and brown circle represents F153L.

Culture 10 commences with g-9a promoter mutation on day 3 but percentage is dropped into 25% on day 4 by dint of c-35t promoter mutation acquisition, which proves the c-35t is capable of annihilate g-9a promoter mutation. D27E is acquired on day 10 and F153V is acquired on day 13. However, Genotype of population turns into c-35t+D27E+F153L+L28R because c-35t+D27E firstly accumulates L28R and then F153L on day 15 and this configuration is final destination of culture 10. (Figure 21)

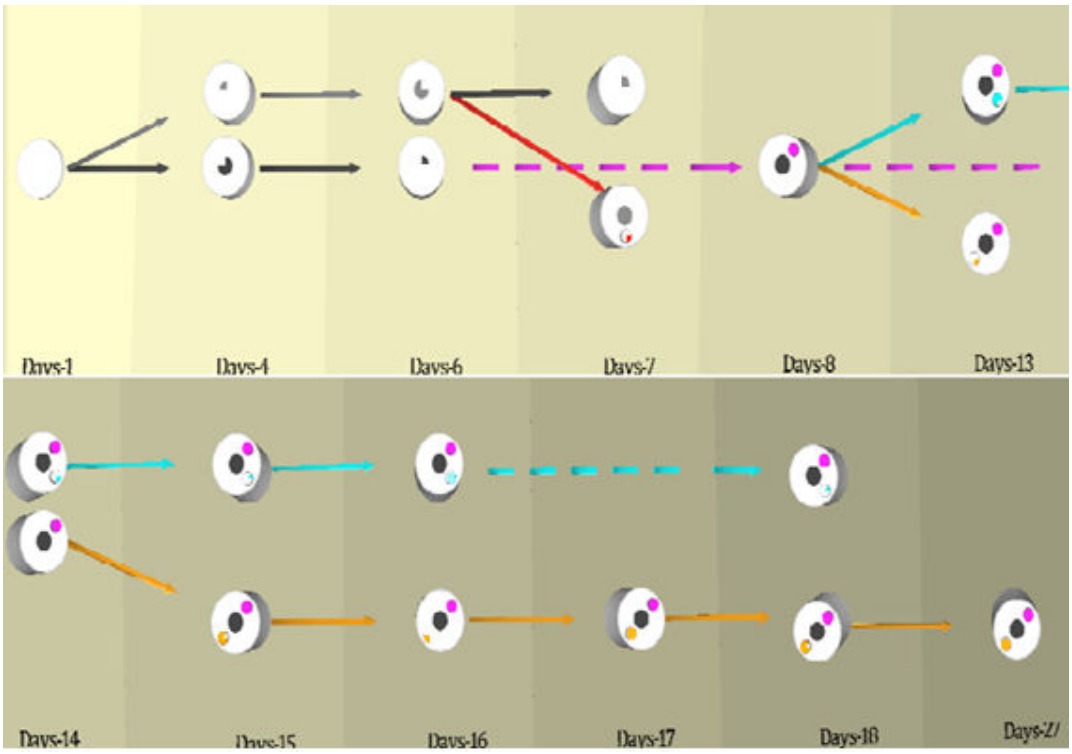


Figure 22: Mutation trajectories of culture 11. Light grey and dark grey circles signify promoter mutation g-31a and g-9a, correspondingly. Magenta, Cyan and orange filled circles represent L28R, A26T, F153S. The mutation order is g-9a→L28R→A26T<F153S

Culture 11 follows promoter mutation path which consists of ¾ g-9a and ¼ g-31a on day 4, per contra, ratios of promoter mutation becomes exact opposite on day 6 proving that the fitness effect of g-9a is superior than g-31a. However their conflict continues with addition of new mutations. g-31a acquires P21L on day 7 with the ratio 1/3 whereas g-9a is accumulates L28R which dominates the whole culture on day 8. Laterly, g-9a+L28R population is divided into 2 with gaining F153S with 1/8 ratio and A26T with 7/8 ratio on day 13. These two populations fight each other to survive during day 13-18. On day 19, g-31a+L28R+F153S finally overwhelms with g-31a+L28R+A26T group. However, we may result that the fitness effect of F153S is higher than A26T but very close because scrambling time dures very long and the winner is exactly opposite for some days. (Figure 22)

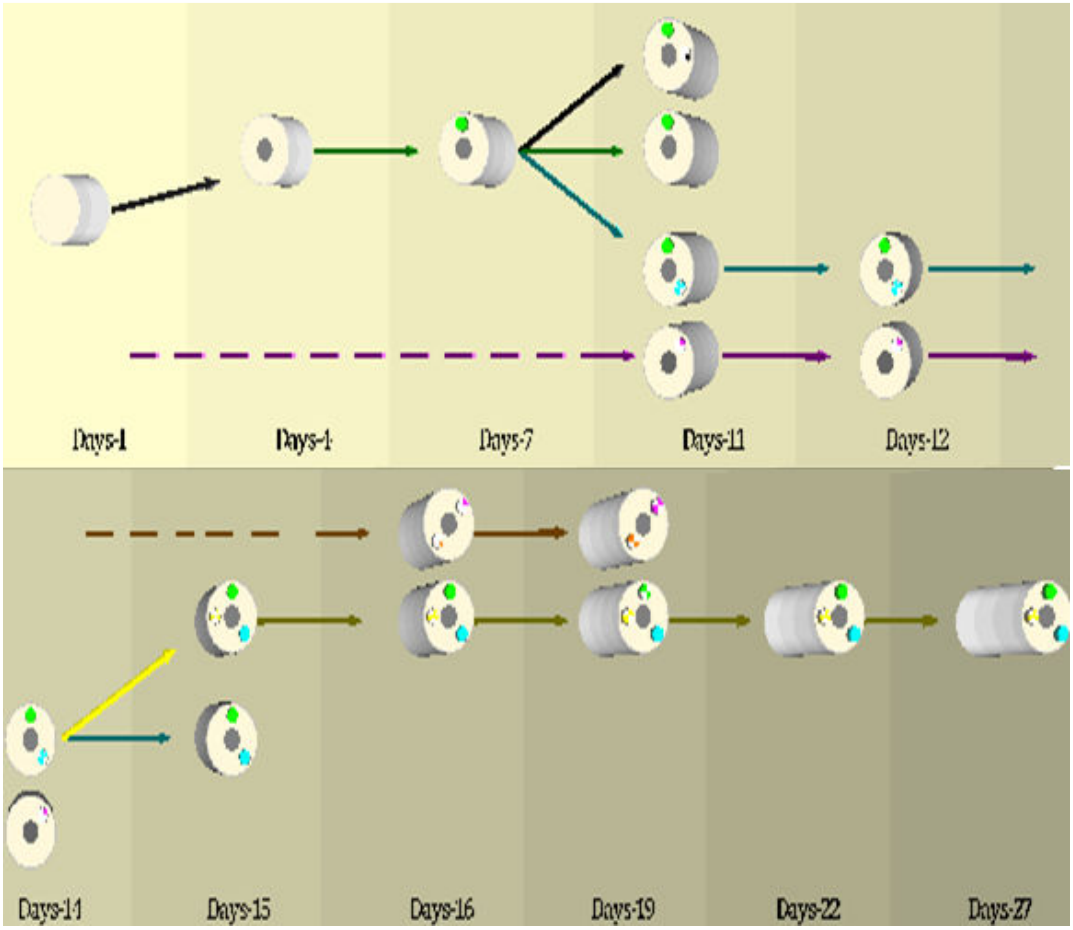


Figure 23: Mutation trajectories of culture 13. Light grey, green, magenta, cyan and yellow filled circles correspond respectively to c-35t promoter mutation, W30C, L28R, A26T, R98P.

As usual, promoter mutation comes first in culture 13. W30C is secondly gained c-35t on day 7. Nevertheless, four different genotypes are observed on day 11. Two of which are population that get hold of new mutation on c-35t+ W30C. c-35t +W30C + D27E occupies 1/8 of population whereas c-35t+ W30C+A26T is included in half of population. 1/8 of culture does not accumulate any new mutation and the other 1/8 of population gains L28R mutation right on the promoter mutation on day 11. On day 12, most of the population becomes c-35t+ W30C+A26T. R98P mutation is accumulated by population on day 15 and maintained until the last day experiment. The mutation acquisition orders are determined as c-35t→W30C → D27E →R98P and c-35t→W30C → A26T →R98P. (Figure 23)

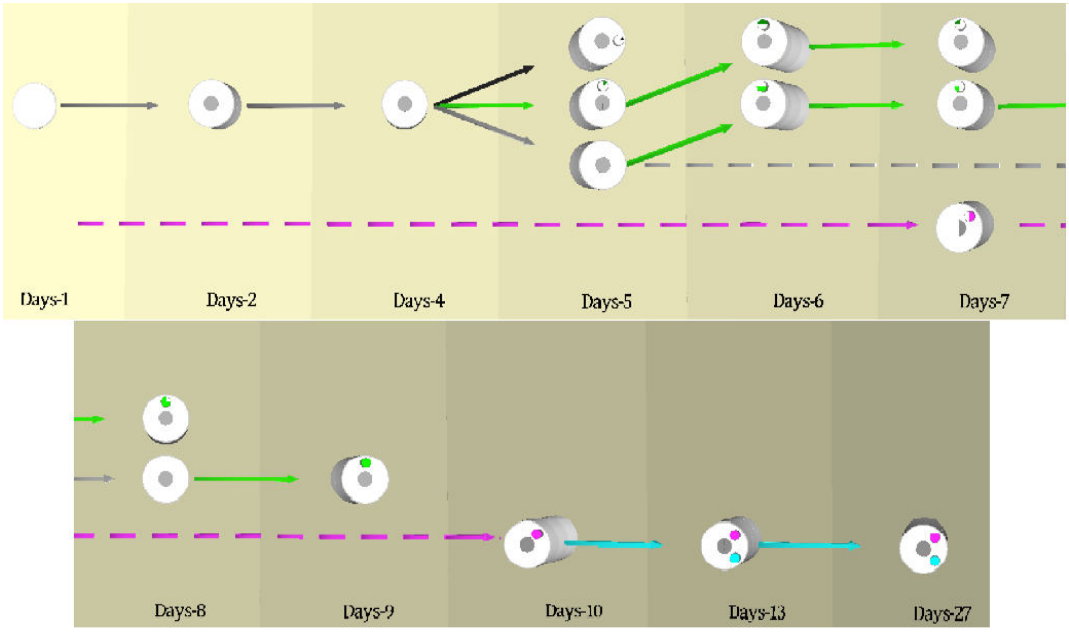


Figure 24:Mutation trajectories of culture 14. Light grey and grey circles correspond to c-35t and g-31a. D27E is shown with black circle. Dark green and light green filled circles represent W30C and W30R. A26T and L28R are shown respectively with Magenta and cyan circles.

A mutation trajectory of culture 14 begins with c-35t promoter mutation. 1/8 of population accumulates D27E and other 1/8 of population gains W30C on fifth day of experiment. c-35t+D27E is not observed once again. 3/8 of population has W30R and the rest of population has W30C on the day 6. However, on day 7, undetectable promoter mutation g-31a acquires L28R and this group spans 5/8 of population by beating W30R and W30C. By looking sequencing result, we can compare the fitness effect of W30C and W30R because W30C is directly eliminated from population but W30R fights against g-31a+L28R. g-31a+L28R fails on day 10. g-31a+L28R gains A26T on day 13 and genotype of population becomes g-31a+L28R+A26T (Figure 24)

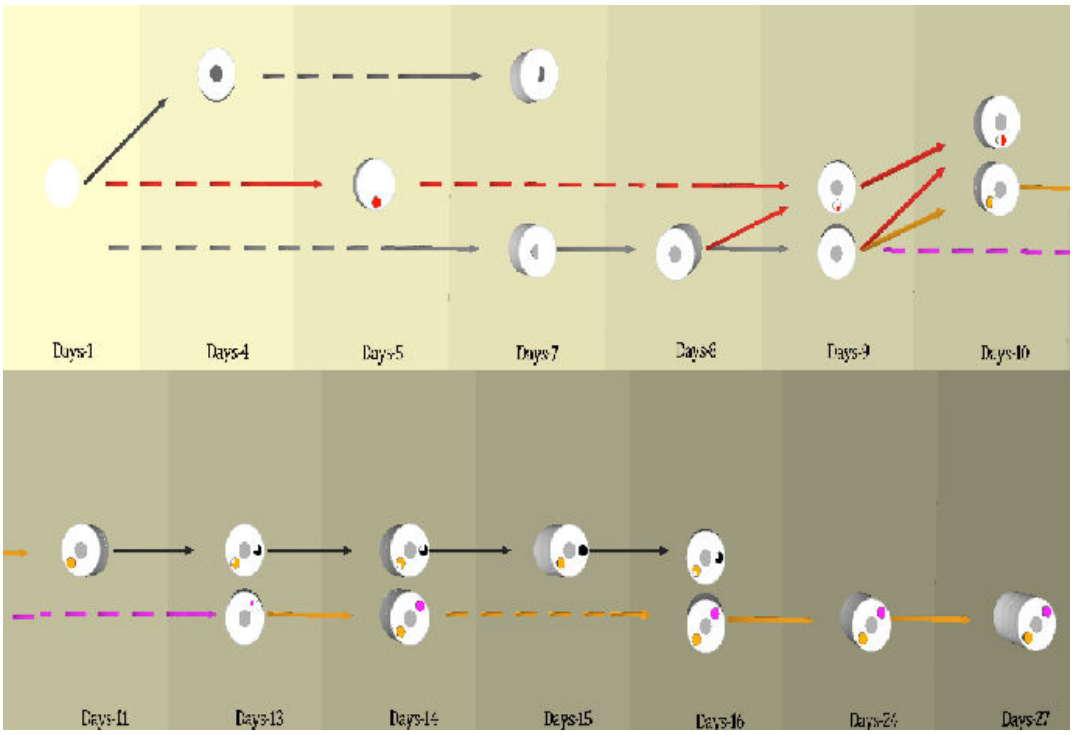


Figure 25: Mutation trajectories of culture 15. Dark grey and light grey symbolize promoter mutation c-15gand c-35t respectively. Black, Orange, magenta and red circles correspond to D27E, F153S, L28R and P21Q, correspondingly.

c-15g novel promoter mutation is firstly observed in culture 15 on day 4. However, all genotypes of population are P21Q at day 5 but it is eliminated from cultures. c-35t is occupied by half of the population on day 7 and defeats c-15g on day 8. Population then acquires P21Q with 1/4 ratio on c-35t at day 9 and occupies half of the population on day 10. c-35t + P21Q group competes with c-35+F153S on day 10 and fails. c-35+F153S accumulates D27E on day 13 with 5/8 ratio, the rest of culture posess c-35t+L28R .Laterly, c-35t+L28R gains F153S on day 14 and it is eliminated on day 15 but this group reattacts during day 16-23 and defeats c-35t+F153S+D27E. As a result of culture 15 trajectories, L28R has higher fitness than D27E as observed in culture 9 (Figure 25)

4.3 Minimum Inhibitory Concentration Measurement

After completion of morbidostat experiment, minimum inhibitory concentrations (MIC) of daily stocked cultures were measured. Both cultures evolved under mild and strong cultures gained similar level of trimethoprim resistance in stepwise manner (Figure 26, Figure 27). All MIC results are shown in appendix B.

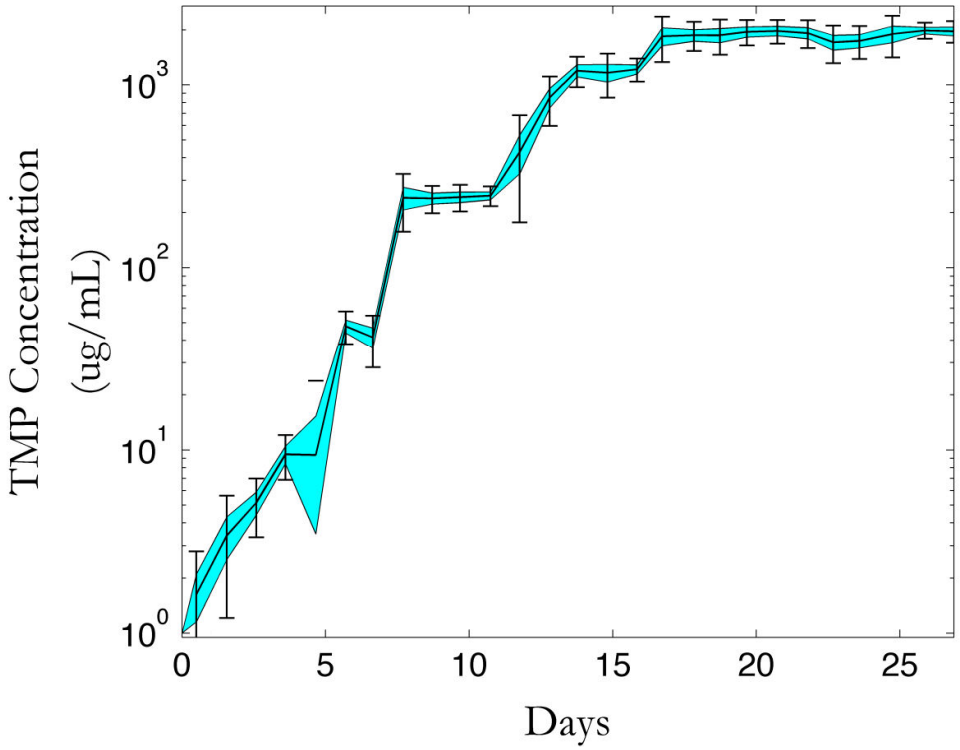


Figure 26: Minimum inhibitory concentration of culture 1 as an example of mildly evolved culture

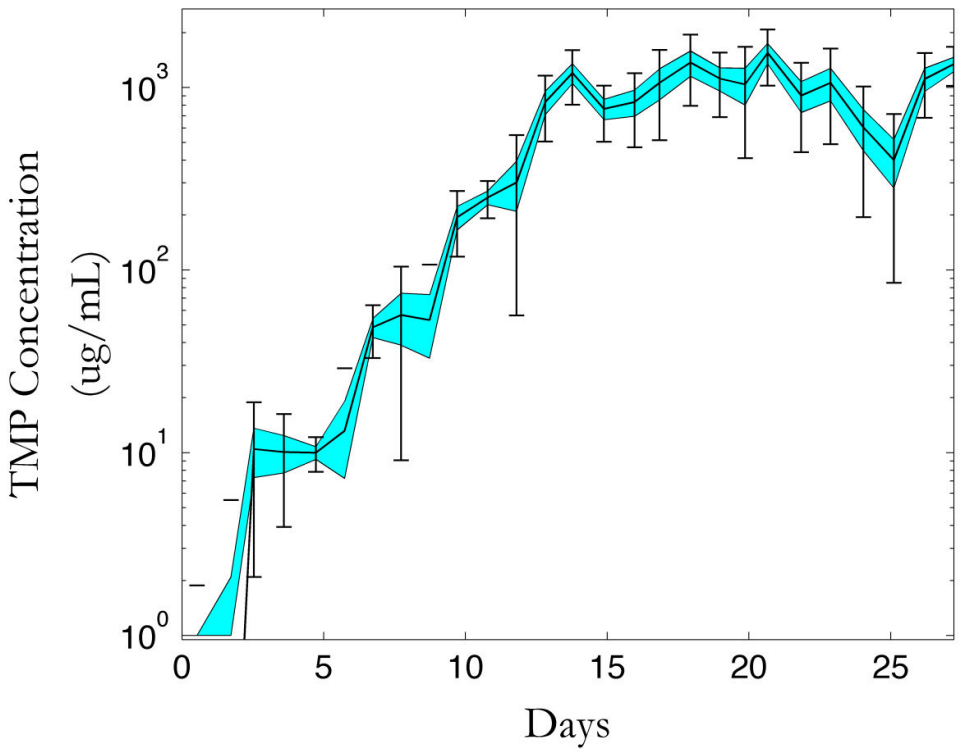


Figure 27: Minimum inhibitory concentration of culture 9 as an example of strongly evolved culture

4.4 Growth Rate Measurement

After the morbidostat experiment completed, growth rate of daily population of each culture were measured with TECAN and calculated according to exponential phases. According to growth rate measurement, both growth rates of strongly diluted culture and mildly diluted samples were suited to predetermined dilution factors. (0.3 for mild and 0,6 for strong dilution). Neither of cultures was dropped under the dilution factors (Figure 24-25).

Furthermore, nearly growth rate of all cultures decreased after first mutation accumulation. Since almost all cultures firstly accumulated promoter mutation, we could conclude that expression level change of DHFR have been some costs for

bacteria. We did not make any comments on other drop or increment in growth rate because of clonal interference.

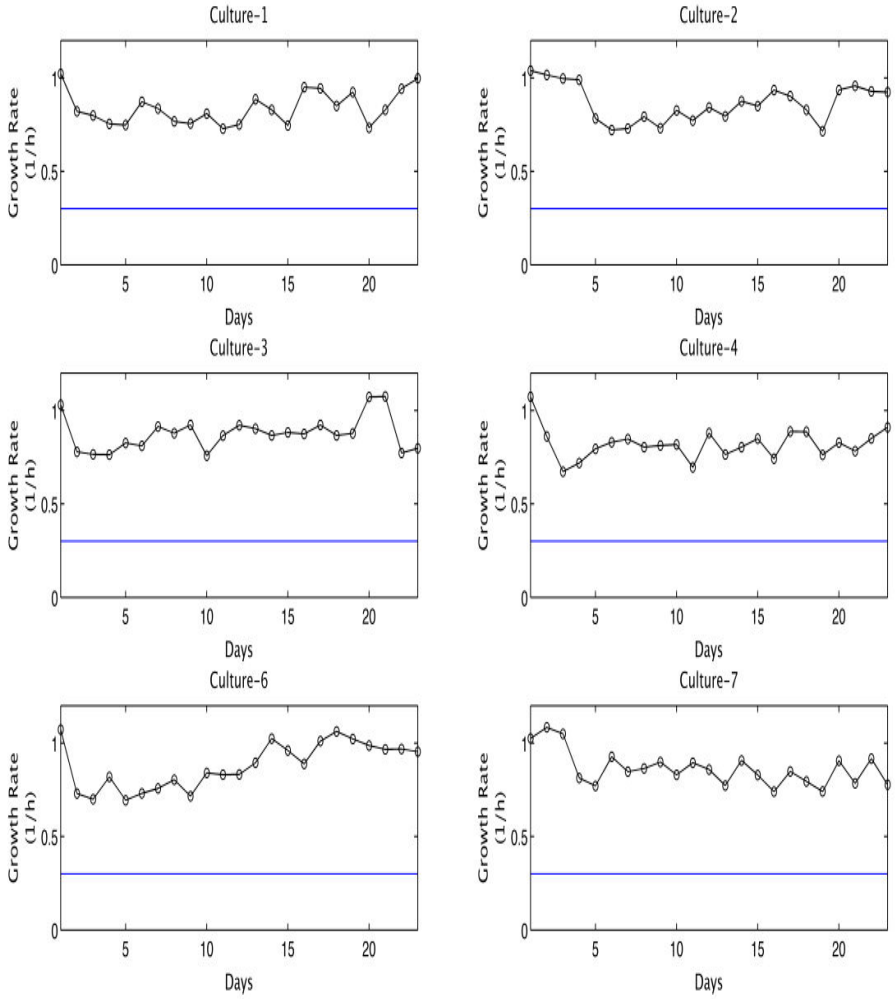


Figure 28: Growth Rate of mild selection cultures versus days.

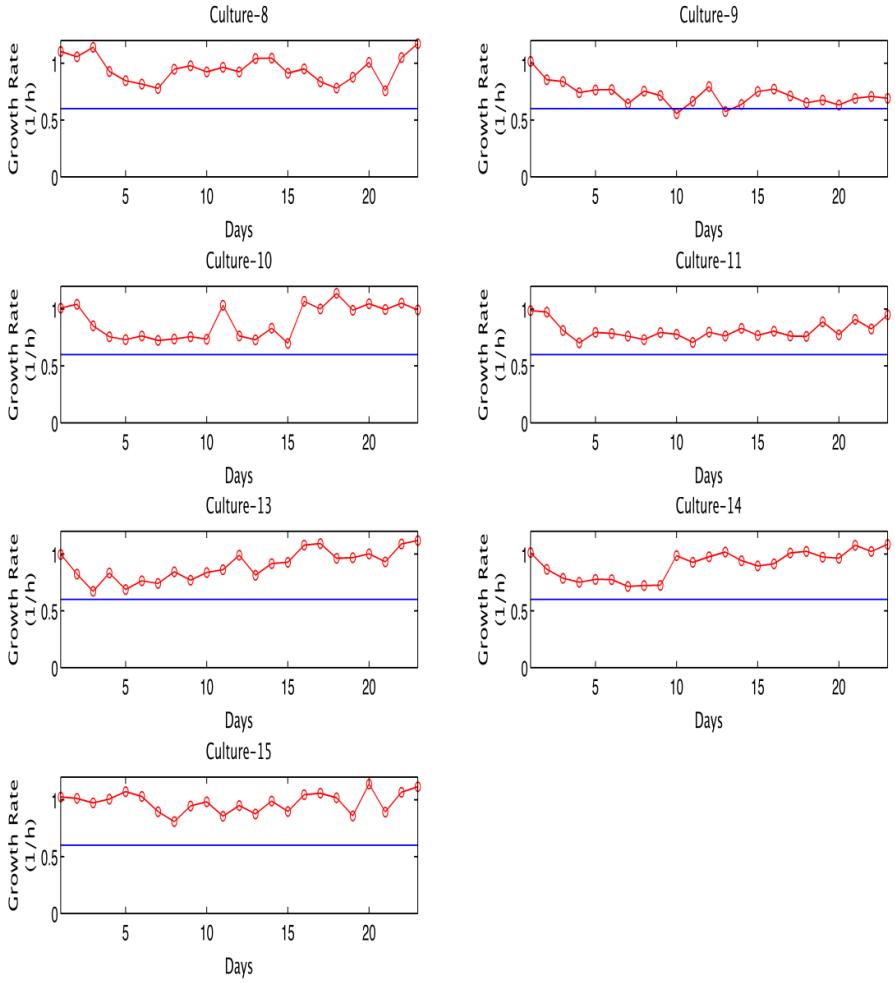


Figure 29: Growth Rate of strong selection cultures versus days.

4.5 Statistical Coupling Analysis

Statistical coupling analysis is bioinformatics technique based on multiple sequence alignment used to characterize to evolutionary constrained amino acid in protein family. More specifically, this method quantifies how much amino acid distribution at one position is altered when the amino acid distribution of at another position is changed. If distribution is different from mean of distribution which is the expected amino acids distribution (generally, 20 different amino acids are expected to

be found at same frequency), some degree of conservation exists and amino acids are coupled, which is called sectors. Global analyses of coupled residues are analyzed in a weighted correlation matrix. In order to characterize residues of DHFR, We performed multiple sequence alignment consisting of 4166 sequence of DHFR variants in statistical coupling matrix. Position specific conservation scores were calculated as function of entropy (D). If D value is greater than one, residue was highly conserved and it is shown in matrix with red color (Figure 30). Number of conserved residue of DHFR and conservation scores of each position are shown in figure 31 and 32 respectively. Pairwise conservation scores were calculated and sectors were analyzed with p=0.0135-0.0225 cutoff. Residues 7, 11, 14, 15, 18, 21, 22, 23, 24, 25, 27, 31, 32, 35, 37, 39, 42, 44, 49, 53, 54, 55, 57, 59, 71, 81, 90, 94, 107, 113, 121, 122, 125, 126, 133, 153, 158 were found in sector region. To test our consistency, we also compared our SCA result with SCA of Ranganthan Lab. We found that they are highly consistent (Figure 33).

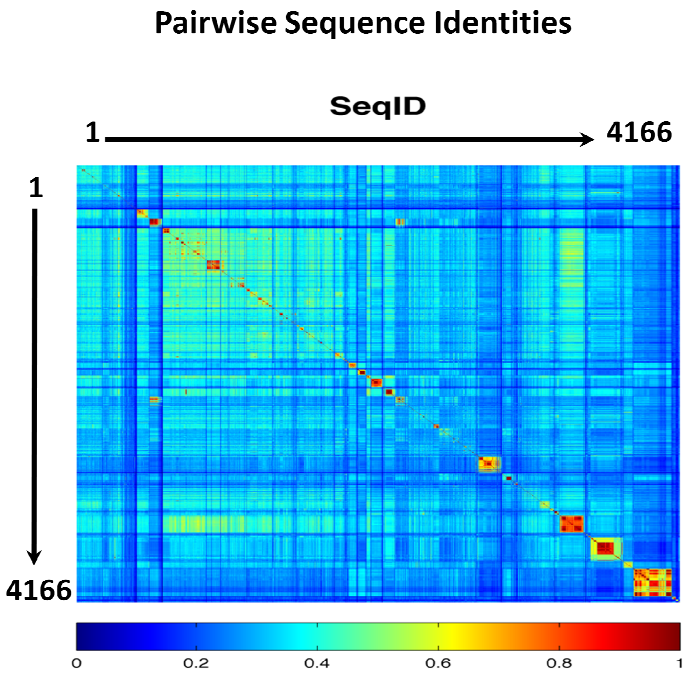


Figure 30: The Statistical Coupling Matrix: a weighted correlation matrix consisting of 4166 sequence of DHFR variants. Blue color represents less conserved whereas red color signifies more conserved residues.

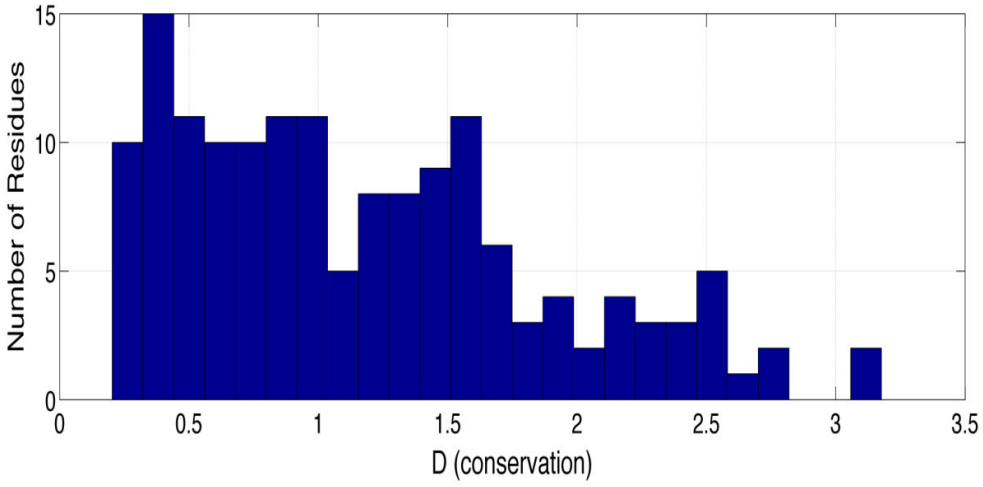


Figure 31: Number of conserved residues in DHFR versus conservation scores. Scores that are higher than 1 is accepted as conserved residue.

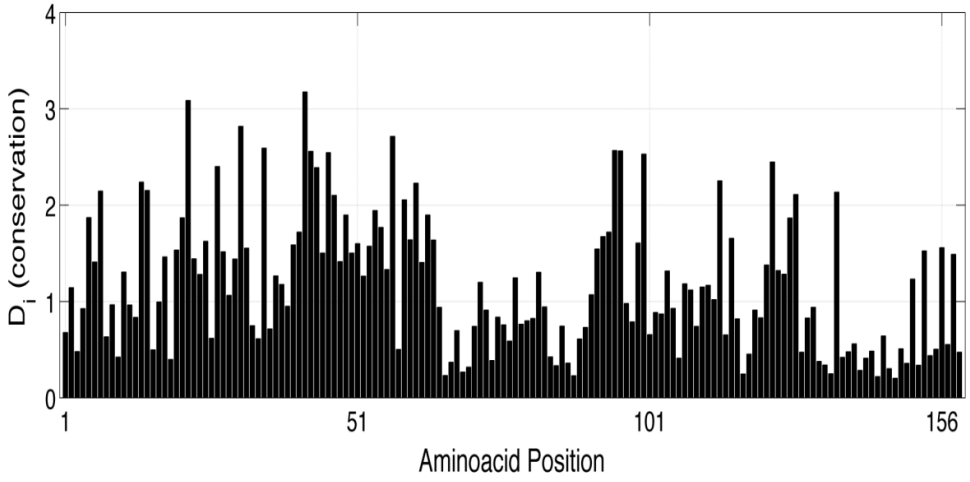


Figure 32: Conservation Score of each residue of DHFR. DHFR contains 156 amino acids.

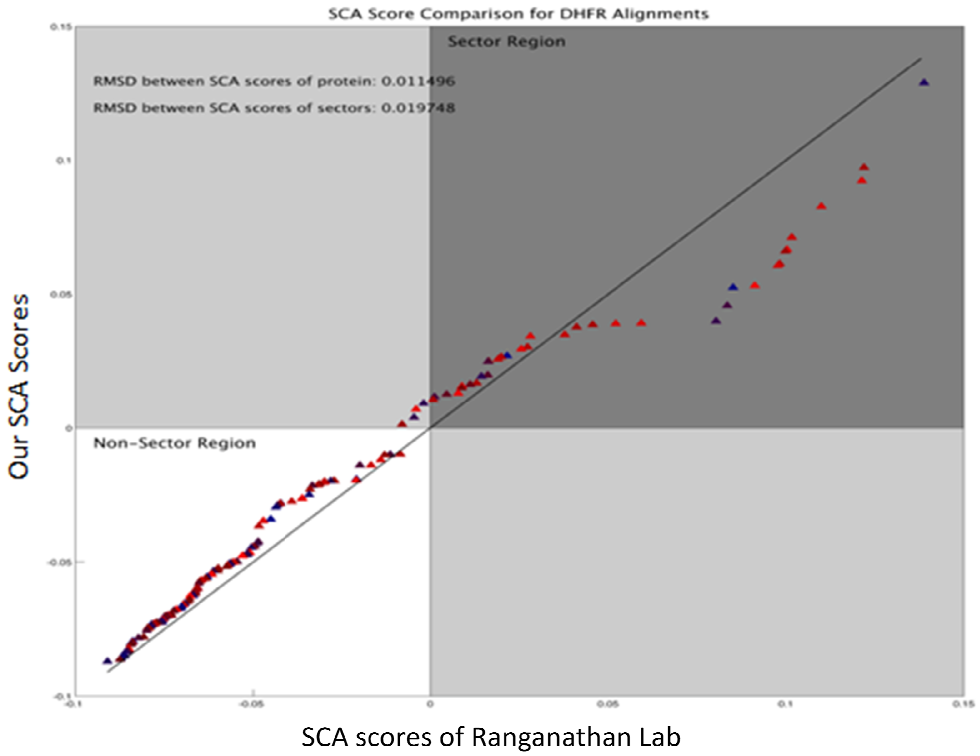


Figure 33: Previous MSA alignment (alignment of Kimberly Reynolds from Ranganathan Lab, Texas, USA) versus our alignment. Kimberly Reynolds used 418 sequences whereas we used 4166 sequence of DHFR in MSA to find SCA score of residues. RMDS is root mean square deviation and correspond to magnitude of variation between two results. Residue numbers are symbolized with gradient color from orange to navy blue.

In addition, we compared detected SNPs with our SCA scores and we found that nearly all mutants are inside sector region. Culture 1 is firstly hit in L28 residue. Culture 2, 4, 10 firstly accumulates D27. Culture 8 and 11 hit P21 and L28. Culture 15 has first coding mutation at P21 and F153 residues. Culture 3 accumulates W30 and F153 together. Culture 6 and 13 firstly acquire W30 mutation. Culture 7 accumulates W30 and D27. Culture 9 acquires D27, F153 and W30. Culture 14 acquires W30, D27 and L28 as first coding mutation. Since all first coding mutation such as P21, D27, L28, F153 are situated in sectors only exception with W30, we can conclude that first mutational choice of culture are almost in sector regions. Generally, second mutations are also found in sectors according to our analysis but we cannot generalize them

because epistatic interaction between SNPs also effects on preference of second coding mutation in bacteria.

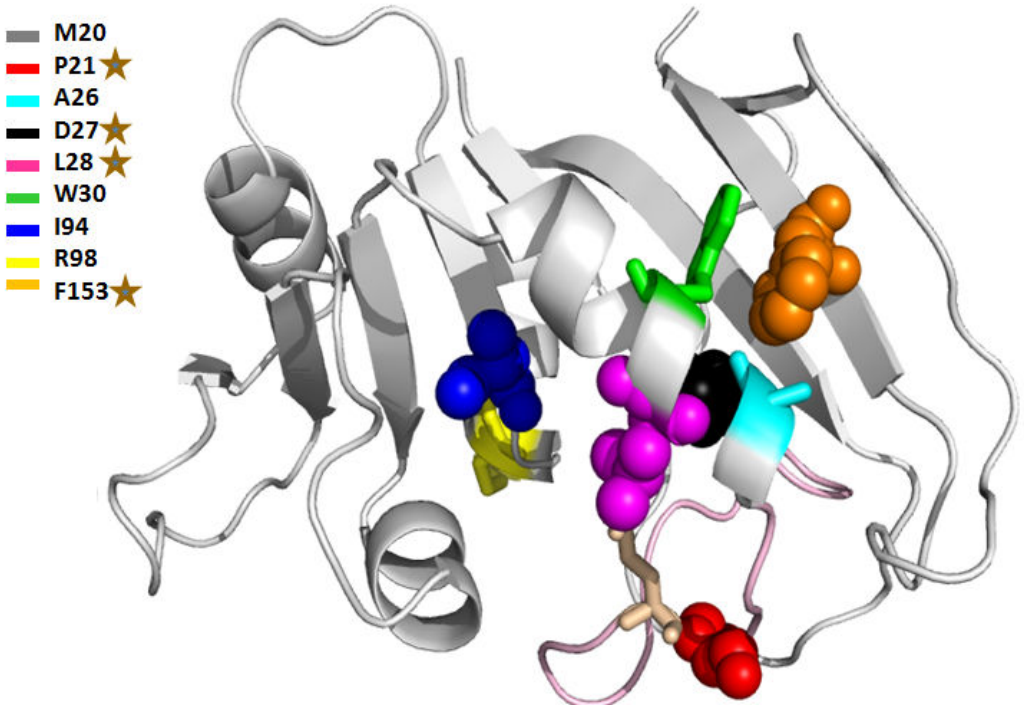


Figure 34: Mutated residues located on sector region. All mutated residues are shown in 3D structure of DHFR. Mutations found in sectors are shown with ball shape. P21, D27, L28, I94, F153 are found in sector. If mutation found in sector is preferred as first coding mutation, it is labeled with star.

4.6 Functional Impact Score and Mutation Assessor

Microorganism develops new strategies to survive against wide use of drugs and the most observable strategy is acquiring of spontaneous mutation. Such mutation-based resistance, however, is not only particular to microorganisms. Cancerous cells may also develop resistance by mutation acquisition against chemotherapy and even the origin of the cancer cells is due to an amino acid change resulting to oncogene activation or inhibition of tumor suppressor gene. This kind of mechanisms make researchers to find out nature of mutations, many new bioinformatics software are newly introduced to literature. Mutation Assessor is the one of the software program that calculates mutation

impact on proteins function such as stability, protein- protein interactions, catalytic activity or protein expression based on FIS (Functional impact score). It is mainly developed for cancerous mutation. It uses evolutionary information coming from conserved patterns. As in Statistical Coupling Analysis (SCA), FIS uses multiple sequence alignments of protein families and sub families to reveal the mystery of residues playing important role on protein function and also cancer progression. As a result of alignment, conservation score and specificity score is calculated. Conservation score is computed based on entropy differences between original residue and variant residue by analyzing entire protein family. Entropy differences are high if residue is conserved across the protein family. Entropy term reflects the physical and chemical effect of residue on protein. Specificity score is calculated based on conservation in sub family, by this way, diversifications are lowered whereas the specificity of residues increases the sum of the conservation score and specificity score gives the Functional Impact Score (FIS). After the FIS calculation, Validation test is performed by using Uniprot database containing 60,041 neutral and disease associated variants. FIS scores are differentiated as disease associated or unaffected mutant. As a result, phenotypic consequences of polymorphism are detected by this method which is also called mutation assessor. [24]

Mutation assessor has been mostly utilized to find cancer and disease associated mutation. However, we applied this technique to detected DHFR SNPs so as to analyze the impact on protein function because DHFR have found in all organisms as an essential enzyme and shared approximately 70% similarity with human DHFR. Additionally, DHFR enzyme was not completely irrelevant with cancer topic, on the contrary, it has been used as a drug target for cancer cells. In our analysis, we detected almost all mutants have medium or high impact.

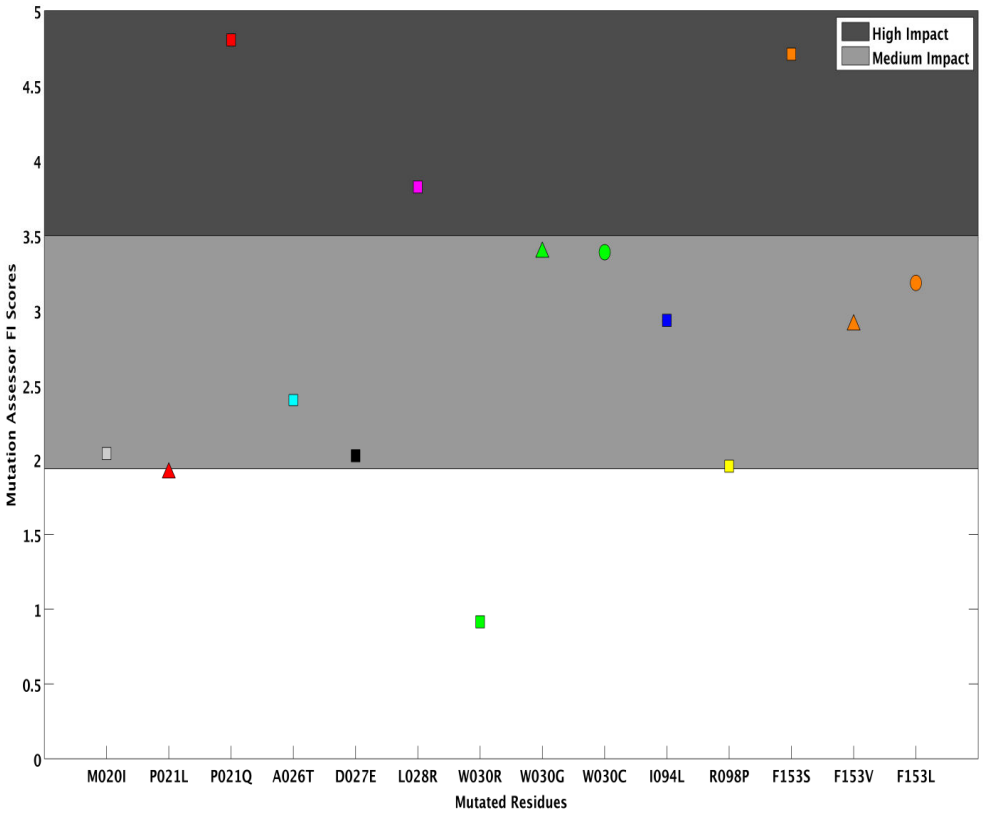


Figure 35: Mutation Assessor FI scores of mutant residue. Dark grey region represents high impact whereas light grey region corresponds to medium impact mutation. If FI score is lower than 2, such mutant has lower effect on protein structure. If score is between 2 and 3.5, it is accepted as medium impact and if score is higher than 3.5, mutant is highly effective.

According FIS analysis, P21L has 1.91 FI score and W30R has 0.915 FI score. Therefore they have low impact. However, P21L is very close to 2 and we can assume that it has medium impact. Besides, P21Q is counted as high impact mutant that has 4.805 FI score. The other residues that have high impact are L28R and F153S with score 3.825 and 4.71, correspondingly (figure 35). Consistently, these two mutations are situated on nearly all final destination of cultures together or alone except culture 9 and culture 13.

FI scores of W30G and W30R are also very high although they are placed in medium impact area of graph. F153V, F153L and I94 L are also found in medium

impact area with score 2.905, 3.185 and 2, 9 respectively (figure 35). From that result, we can conclude that F153 residue is very important for protein stability or catalytic activity and also 6/13 of culture harbor one of F153 variant in their final destiny. M20I, A26T, D27E, R98P have also medium impact FI score. They are all found in final destination except M20I.

4.7 F statistic and dynamics of cultures

F statistic is very helpful to analyze evolutionary process and genetic variation in population. Therefore we calculated F_{RS} score of each day of each culture so as to find sub population divergence from regional populations. Region corresponds to dilution types in our study. By this way, diversity of mildly and strongly diluted cultures may be compared. Indeed, Diversity of strongly diluted cultures is higher than the mildly diluted cultures. For instance, if we compare H_S of culture 2 (mild culture) with culture 15 (strong culture), two days of culture 2 include diversification whereas culture 15 has durable diversity which continues thirteen days. (Figure 36a and 36b) However, if whole diversity score is analyzed, there are also some exceptions such as culture 8, 10 and 14 from strong cultures having low diversification. (Appendix C)

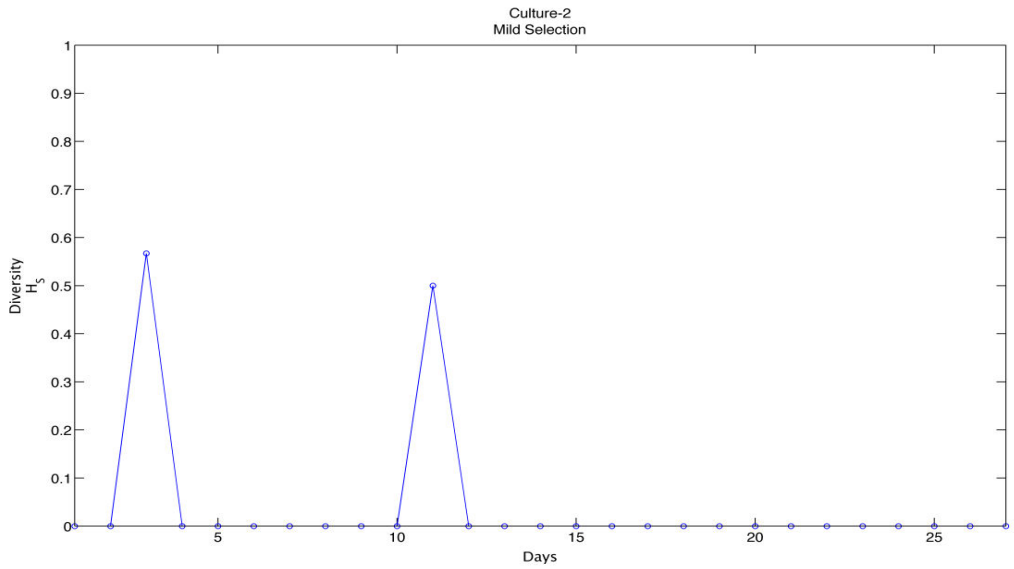


Figure 36a: Heterozygosity of subpopulation of mild selection exemplified by culture 2. Day 3 and day 11 of culture 2 comprise dissimilar subpopulations. if Hs=0; there is no diversity

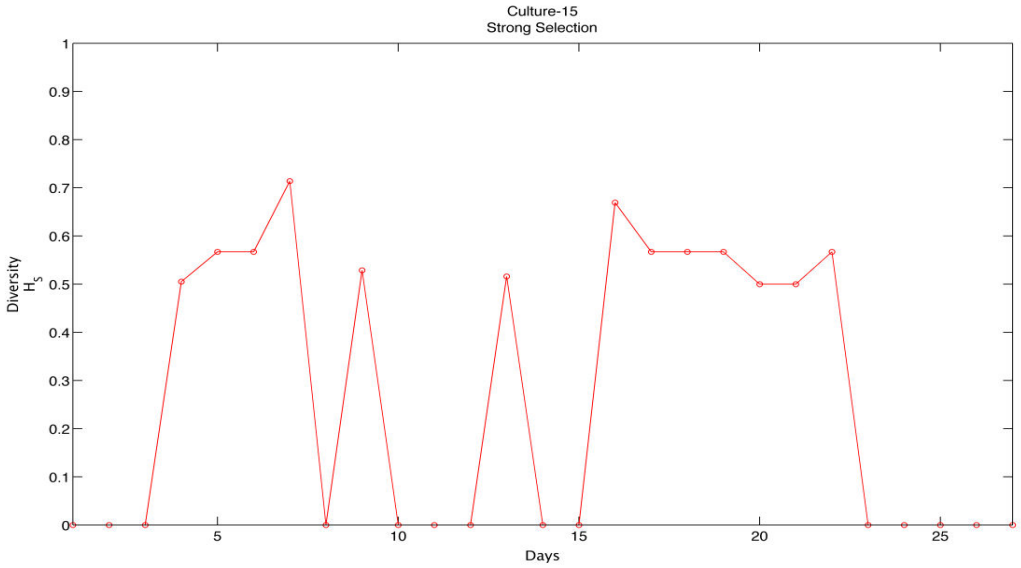


Figure 36b: Heterozygosity of subpopulation of strong dilution is exemplified by culture 15. Day 4, 5, 6, 7, 9, 13, 16, 17, 18, 19, 20, 21, and 22 of culture 15 comprise dissimilar subpopulations. If Hs=0; there are no diversity.

Furthermore, Mean Duration of strongly diluted cultures is 6.42 days and nearly 3 fold of mildly selected cultures which is 2.66 days. The standart deviations are 1.21 for mildly diluted cultures and 4.5 for strongly diluted cultures. According to t test that we performed p value is 0.036 and it is not statistically significant. Therefore, diversification is higher in strongly diluted cultures than mildly diluted cultures. (Figure 37)

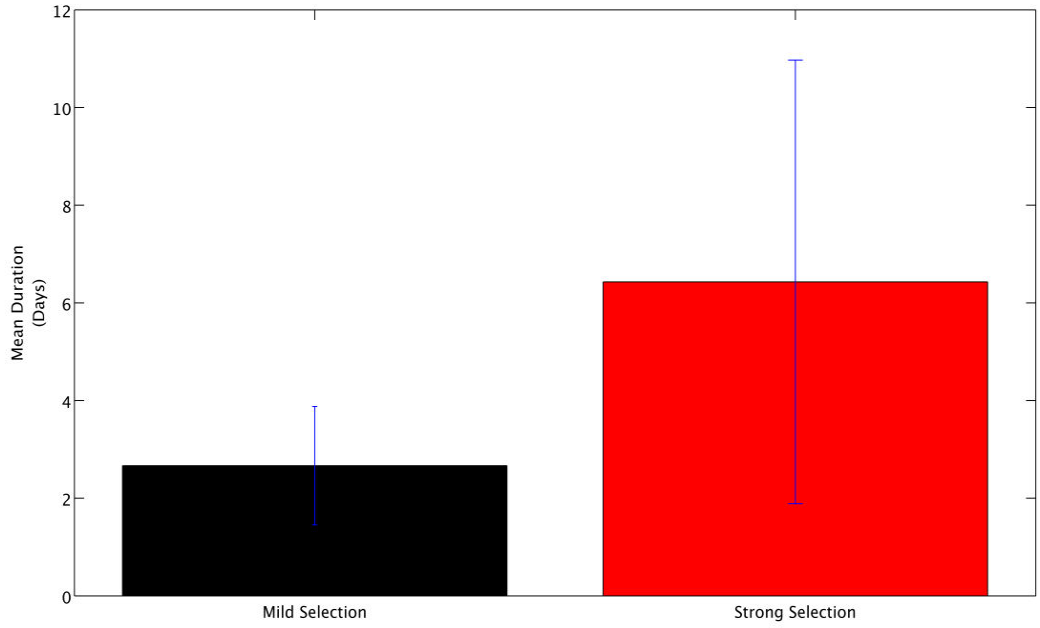


Figure 37: Comparison of mean duration of mild and strong selection. Black color represents mild selection whereas red color signifies strong selection. Navy blue shows standard deviation of calculation

In addition to F_{RS} Analysis of cultures, figure 32 also shows that mutational diversity is higher in strongly diluted population than mildly diluted population especially in first coding region mutation. Mild Selection cultures have seven paths in first coding mutation however; strong Selection cultures have ten paths for first coding region mutation. Therefore, dynamics of cultures is not same within and between dilution types.

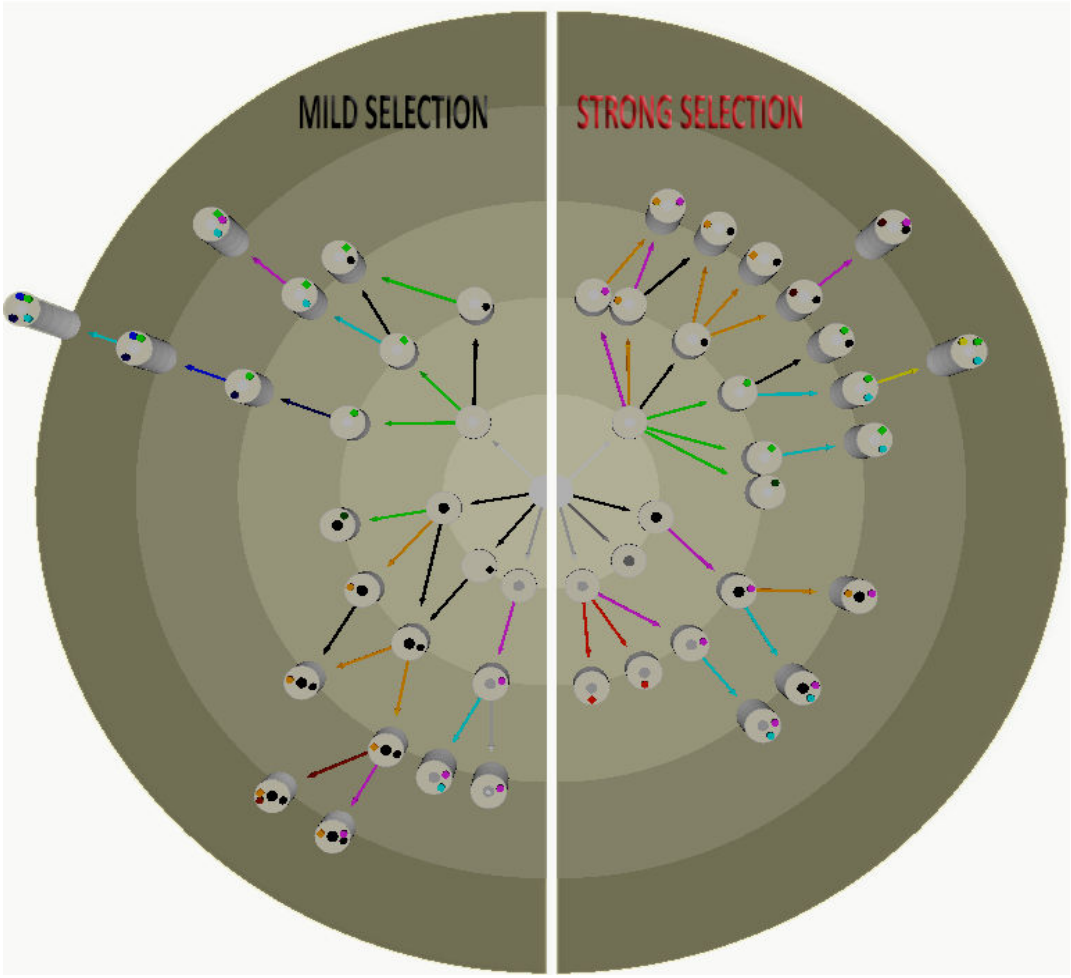


Figure 38: Mutational dynamics of strongly and mildly selected cultures. Middle line separates two selection types. Promoter mutations are located in the center of cylinders represented with colors light grey, grey and dark grey. Other colors represent different coding mutations

5. DISCUSSION

Protein motion, reorientation of domains and sub domains are all related to protein function which involves binding and release of cofactor and substrate. Such dynamics are mediated by allosteric regulation protein. In DHFR, hinge bending motions and active loop movement are primary responsible for substrate and cofactor binding and release by determination of active site cleft closure. The active site cleft separates DHFR in to two sub domains. These are adenosine binding sub domain and major sub domain. Adenosine binding domain is located between residue 38 and 88 generates binding site for cofactor. Lys 38 and Val88 provide hinge bending motions to generate movement of adenosine binding domain for cofactor binding and this movement closes the active site cleft. Major sub domain which is also called loop sub domain occupies 40-50% of DHFR in nucleotide length. Loop sub domain is also divided into 3 parts. These are Met20 loop also called Loop I found between residue 9-24, FG loop also called Loop II lying between 116 and 132nd residues and GH loop is located between residues 142 and 150. Met20 loop is directly related to enzymatic activity of DHFR protein whereas FG and GH loops are responsible from stabilizing of protein via hydrogen bond interaction with Met20 loop. Met20 loop has 2 conformations according to substrate binding or release. If substrate site is occupied, Met20 loop is found in occlude conformation. Binding of nicotamide ring of NADPH leads close conformation of Met20 loop. Transition of Met20 loop between these conformations disrupts preexisting hydrogen bond and forms new ones. [18] Upon cofactor binding, hydrogen bonds between Asn23 in Met20 loop and Ser148 in G-H loop are broken and new hydrogen bonds between Asp122 in FG loop and Ile14-Gly15-Glu17. Therefore, these residues are very significant for ligand and cofactor binding. However, we did not see any mutation on these residues and also we did not found any

mutated residues on in F-G loop or G-H loop suggesting DHFR catalytic reaction have not been disturb by any kind of change.

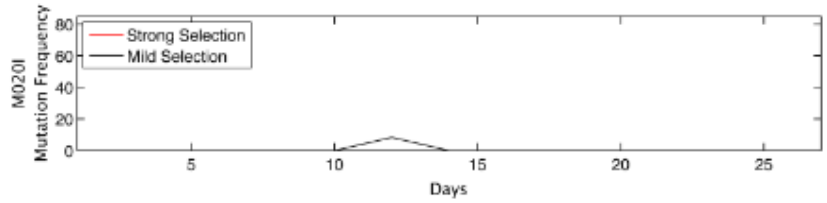


Figure 39a: Mutation Frequency of M20I versus days. It is only found in mild selection. Black color belongs to mild selection and red color corresponds to strong selection.

Only exceptions are M20 and P21 residues on Met 20 loop. If we concentrate on M20I SNPs even if this mutation have not been observed in final destination of any culture, Methionine to Isoleucine change increases residue hydrophobicity (Appendix b) and M20I mutation might enables to protect the active site cleft which is found in very deep hydophobic region. This mutation type was only observed in mild selection culture (culture 4) with mutation g-9a+D27E+F153V+M20I during day 10-14 (Figure 39a). However M20I has competed with L28R and has failed in same background. Perhaps this faillure is due to slowest catalytic activity of DHFR because more hydrophobic residue might disrupt the flexibility of Met20 loop reflecting to fitness of bacteria.

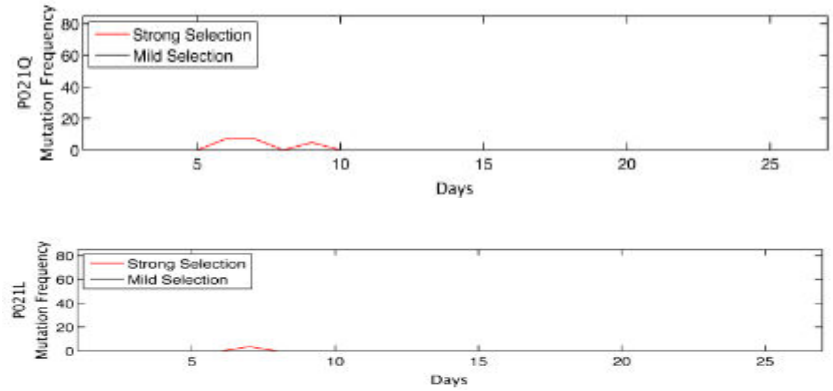


Figure 39b: Mutation Frequency of P21Q and P21L versus days. It is only found in strong selection. Black color belongs to mild selection and red color corresponds to strong selection.P21Q is more persistent than P21L

On the other hand, P21 residue is very close to M20. Similar with M20I, Both P21Q and P21L are not permanent in population (Figure 39b). Proline is non polar amino acid and in the case of P21Q, it is changed into glutamine which is polar but less hydrophobic than proline but Leucine is non polar and more hydrophobic than proline (Appendix B). P21L possesses very similar residue transition to M20I and it is also compete with L28R. Therefore, it also supports assumption about disruptions of Met20 loop flexibility. If it is more hydrophobic, it may be attracted by hydrophobic active cleft. Additionally, P21Q is more persistent than P21L and it turns into polar and less hydrophobic residue (Figure 39b – Appendix B). However, since their background mutations are not same, we cannot compare them.

Consequently, P21 and M20 residues are located on LoopI and mutations on these residues have large impacts on fitness of population

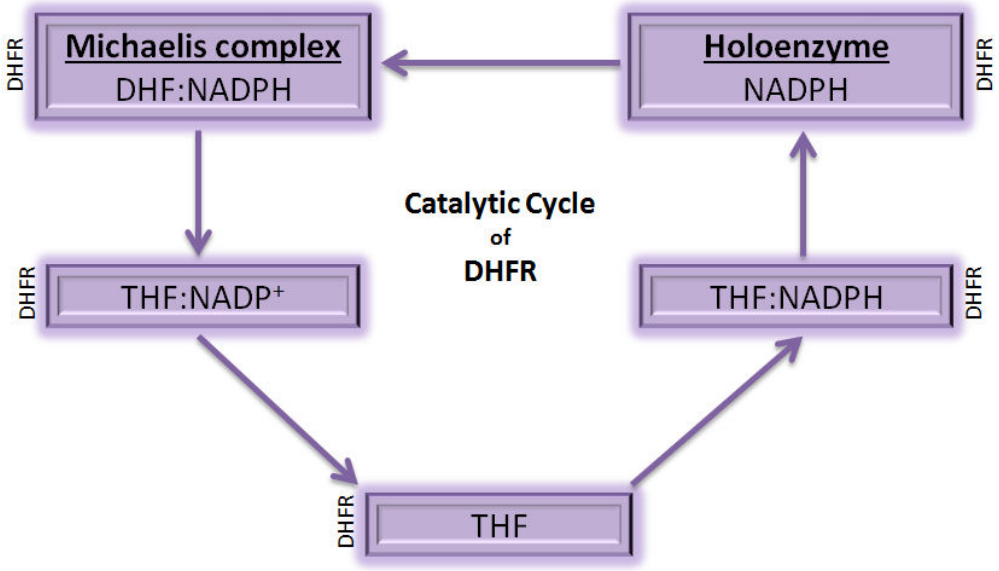


Figure 40: Catalytic cycle of DHFR composed of 5 intermediate complexes. Purple rectangles correspond to DHFR enzyme [18]

NMR studies shows that Dynamical fluctuation of DHFR is related to ligand binding cofactor binding. The kinetic cycle of DHFR consists of 5 intermediate complexes. Holoenzyme (DHFR+NADPH) intermediate enables to bind substrate

DHF and forms Michaelis Complex (DHFR+DHF+NADPH). Catalization occurs and DHFR+THF+$NADP^+$ complex is generated and $NADP^+$ is released. However, substrate cannot leave from enzyme unless another NADPH cofactor binds to DHFR, which is rate limiting step of catalytic reaction. As result other intermediate DHFR+THF+NADPH is emanated and THF is released. In physiological condition enzyme never gets free and D27 residue plays very important role in substrate release. It is responsible from hydride transfer to THF for its freedom (Figure 40) [18]. In our result we identified also D27E mutation nearly in every culture and four of them harbor this mutation in final genotype. However, if the amino acid properties of both native and mutant version of residue 27is investigated, it is inevitable to unnotice that both amino acid shares same characteristic. Both glutamic acid and aspartic acid amino acids are acidic polar and their hyrophobicity scale of site chains are same. Therefore bacteria populations may choose this conversion to decrease specificity of TMP binding without damaging to hydride transfer of 27th residue.

FolA gene is situated between 49823-50302 positions of MG1655 e.coli genome. In close proximity of FolA gene, REP5 is located between50328-50364 position. Mutation that we found in 34 nucleotide upstream of folA gene is then located on REP5. REP5 is repetitive extragenic palindromic sequence and these classes of repeats are known to have regulatory functions such as binding site for protein, cleavage site for DNA gyrase, stabilization of mRNA or transcriptional termination [25]. Arising of this mutation against TMP rises a question that this element may be related to transcription termination of FolA gene and this mutation may have stabilizing effect on mRNA of DHFR protein.

Bacterial transcriptional termination can be intrinsic or rho factor independent. About half of genes have intrinsic termination in e.coli but transcription termination of other half are rho dependent. Transcription termination of Fol A gene depends on whether Rho factor or not is unknown. In intrinsic termination, palindromic region after the stop codon of gene forms hairpin structure. Uracil rich region is found in 7-9 nucleotides after hairpin structure where RNA polymerase is stabilized and stops. Both hairpin and the sequence located in both upstream and downstream of hairpin effects the termination efficiency. [26] Therefore +34 mutation may enables more efficient termination. If transcription termination is Rho dependent, Rho protein binds its binding

sites called rut site located in upstream of gene stop codon. Rho factor tracks along the DNA until it finds RNA polymerase.[26] +34 regions may be found in rut site of Rho factor and it may help stability of Rho binding. In order to understand of +34 SNPs function, transcriptional termination type of folA gene should be determined via hairpin modelization of target sequence and if transcription termination is Rho dependent, near proximity of +34 position may be deleted or site directly mutated to find rut site.

6. CONCLUSION

Evolution occurs via continuous adaptive mutations. Selection of adaptive mutation is where the resistance of microorganism begins. In order to analyze such origin of resistance, we designed morbidostat which is computer controlled selection device. We focused especially to trimethoprim resistance. Wherefore, we evolved 13 cultures against Trimethoprim (TMP) by using morbidostat. We used mild and strong dilutions to understand evolutionary process and mutational trajectories of Mcherry Chloramphenicol resistant MG1655 E. coli strains. Chloramphenicol and Mcherry marker was used to prevent contamination risk of population. We arranged 6 cultures for mild selection and 7 cultures for strong selection by changing drug adding time as 30sec and 60sec respectively. We have maintained experiments for 504 hours = 23 days and generated at least 1000 fold resistant E. coli strains against TMP.

So as to identify the order and final destination of those spontaneous mutation acquisitions, we sequenced approximately 1300 single colony sequencing and we found 4 promoter mutation and 13 distinct coding region mutation of DHFR. Interestingly, one novel mutation was detected on the +34 nucleotide upstream of DHFR gene of e.coli. Therefore, totally 18 spontaneous mutations were identified.We concluded that mutation combinations were not specific to dilution type. In addition, we measured population dynamics with f statistics. We found out dilution types induce more mutational pathways such that the order of mutation gaining was very explicit in mild dilution whereas the strongly diluted cultures tried many mutational pathways at the same time. We also espied that the promoter mutation have come first with 12/13 ratio because bacteria prefered to change firstly expression of DHFR rather than changing protein structure.

Besides, in order to find fitness of mutations and their epistasic interaction, we restricted the growth with certain threshold and bacteria which possess lower growth rate than dilution rate have been eliminated. As a result, we compared the reproducibility of some mutation between each other. Apart from, we prolonged the experiment time to 600hours (27 days) to be sure about final destination and we found that nothing has changed.

We accomplished Statistical Coupling Analysis (SCA) and scrutinized evolutionary conserved coevolving residues (sectors) of DHFR and we noticed that sectors were highly hit after the promoter mutation except tryptophan found in 30th residue. Correspondingly, we made an interference that mutation acquisition has flown an ordered path starting with promoter mutation, following with sector region and finalizing with epistatis.

7. FUTURE WORKS

As a result of this study, we would like to suggest some future works to do. First of all, we analyzed population dynamics as a consequence of spontaneous mutation in Fol A gene but we did not performed whole genome sequencing to ensure the background of cultures. Therefore, WGS should be the first part of future experiments Secondly; replaying experiments may be designed to find out future step of population, after accumulation of predetermined mutation. Besides, pair wise interactions and fitness of mutations may be measured if strains are artificially mutated with P1 transduction. Additionally, expression level of DHFR protein might be measured for each mutation or combination of mutations. Finally, biochemical characterization DHFR enzyme may be investigated in mutational background with X-ray or NMR and catalytic efficiency of DHFR may be measured by determining k_{cat} and k_m values

REFERENCES

1. Davies, J. and D. Davies, *Origins and evolution of antibiotic resistance.* Microbiol Mol Biol Rev, 2010. 74(3): p. 417-33.

2. Bush, K., *The coming of age of antibiotics: discovery and therapeutic value.* Ann N Y Acad Sci, 2010. 1213: p. 1-4.

3. Neu, H.C. and T.D. Gootz, *Antimicrobial Chemotherapy*, in *Medical Microbiology*, S. Baron, Editor. 1996: Galveston (TX).

4. Kohanski, M.A., D.J. Dwyer, and J.J. Collins, *How antibiotics kill bacteria: from targets to networks.* Nat Rev Microbiol, 2010. 8(6): p. 423-35.

5. Agency, U.S.E.P., *Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality*. 2001

6. Walsh, C., *Molecular mechanisms that confer antibacterial drug resistance.* Nature, 2000. 406(6797): p. 775-81.

7. David E. Golan, A.H.T., Ehrin J. Armstrong, *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 2012: Lippincott Williams & Wilkins.

8. Massimo, F., et al., *Correlation of Trimethoprim and Brodimoprim Physicochemical and Lipid Membrane Interaction Properties with Their Accumulation in Human Neutrophils.* Antimicrobial Agents and Chemotherapy, 1996. 40(12): p. 2865–2873.

9. *Rang and Dale's Pharmacology* ed. M.M.D. H P Rang, J M Ritter, R J Flower, G Henderson. 2011: Elsevier.

10. Huovinen, P., *Resistance to trimethoprim-sulfamethoxazole.* Clin Infect Dis, 2001. 32(11): p. 1608-14.

11. Huovinen, P., *Trimethoprim Resistance.* Antimicrobial Agents and Chemotherapy, 1987. 31(10): p. 1451-1556.

12. Barrow, E.W., et al., *In vitro efficacy of new antifolates against trimethoprim-resistant Bacillus anthracis.* Antimicrob Agents Chemother, 2007. 51(12): p. 4447-52.

13. Leszczynska, K., et al., *Cloning and molecular analysis of the dihydrofolate reductase gene from Lactococcus lactis.* Appl Environ Microbiol, 1995. 61(2): p. 561-6.

14. Vickers, T.J. and S.M. Beverley, *Folate metabolic pathways in Leishmania.* Essays Biochem, 2011. 51: p. 63-80.

15. Alonso, H. and J.E. Gready, *Integron-sequestered dihydrofolate reductase: a recently redeployed enzyme.* Trends Microbiol, 2006. 14(5): p. 236-42.

16. Nzila, A., *The past, present and future of antifolates in the treatment of Plasmodium falciparum infection.* J Antimicrob Chemother, 2006. 57(6): p. 1043-54.

17. Rao, A.S.T., S. R., *A Study On Dihydrofolate Reductase and It's Inhibitors: A Review.* International Journal of Pharmaceutical Sciences & Research, 2013. 4(7): p. 2535-2547.

18. Schnell, J.R., H.J. Dyson, and P.E. Wright, *Structure, dynamics, and catalytic function of dihydrofolate reductase.* Annu Rev Biophys Biomol Struct, 2004. 33: p. 119-40.

19. Mireia Garcia-Viloca, D.G.T., Jiali Gao, *Reaction-Path Energetics and Kinetics of the Hydride Transfer Reaction Catalyzed by Dihydrofolate Reductase.* Biochemistry 2003. 42: p. 13558-13575.

20. Lerner, M.G., *Computational Studies of E. coli DHFR: Drug Design, Dynamics, and Method Development*. 2008.

21. Reynolds, K.A., R.N. McLaughlin, and R. Ranganathan, *Hot spots for allosteric regulation on protein surfaces.* Cell, 2011. 147(7): p. 1564-75.

22. Counago, R., S. Chen, and Y. Shamoo, *In vivo molecular evolution reveals biophysical origins of organismal fitness.* Mol Cell, 2006. 22(4): p. 441-9.

23. Toprak, E., et al., *Evolutionary paths to antibiotic resistance under dynamically sustained drug selection.* Nat Genet, 2012. 44(1): p. 101-5.

24. Reva, B., Y. Antipin, and C. Sander, *Predicting the functional impact of protein mutations: application to cancer genomics.* Nucleic Acids Res, 2011. 39(17): p. e118.

25. Messing, S.A., et al., *The processing of repetitive extragenic palindromes: the structure of a repetitive extragenic palindrome bound to its associated nuclease.* Nucleic Acids Res, 2012. 40(19): p. 9964-79.

26. Lewin, B., *Genes IX*. 2006, USA: Jones and Bartlett.

27. Kyte, J. and R.F. Doolittle, *A simple method for displaying the hydropathic character of a protein.* J Mol Biol, 1982. 157(1): p. 105-32.

APPENDIX

Appendix A

Master Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A		c1 d1	c1 d9	c1 d17	c7 d1	c7 d9	c7 d17	wt	c13 d6	c13 d14	c13 d22	
B		c1 d2	c1 d10	c1 d18	c7 d2	c7 d10	c7 d18	wt	c13 d7	c13 d15	c13 d23	
C		c1 d3	c1 d11	c1 d19	c7 d3	c7 d11	c7 d19	c13 d1	c13 d8	c13 d16	wt	
D		c1 d4	c1 d12	c1 d20	c7 d4	c7 d12	c7 d20	c13 d2	c13 d9	c13 d17		
E		c1 d5	c1 d13	c1 d21	c7 d5	c7 d13	c7 d21	c13 d3	c13 d10	c13 d18		
F		c1 d6	c1 d14	c1 d22	c7 d6	c7 d14	c7 d22	c13 d4	c13 d11	c13 d19		
G		c1 d7	c1 d15	c1 d23	c7 d7	c7 d15	c7 d23		c13 d12	c13 d20		
H		c1 d8	c1 d16		c7 d8	c7 d16		c13 d5	c13 d13	c13 d21		

Master Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A		c4 d1	c4 d9	c4 d17	c6 d1	c6 d9	c6 d17	wt	c8 d6	c8 d14	c8 d22	
B		c4 d2	c4 d10	c4 d18	c6 d2	c6 d10	c6 d18	wt	c8 d7	c8 d15	c8 d23	
C		c4 d3	c4 d11	c4 d19	c6 d3	c6 d11	c6 d19	c8 d1	c8 d8	c8 d16	wt	
D		c4 d4	c4 d12	c4 d20	c6 d4	c6 d12	c6 d20	c8 d2	c8 d9	c8 d17		
E		c4 d5	c4 d13	c4 d21	c6 d5	c6 d13	c6 d21	c8 d3	c8 d10	c8 d18		
F		c4 d6	c4 d14	c4 d22	c6 d6	c6 d14	c6 d22	c8 d4	c8 d11	c8 d19		
G		c4 d7	c4 d15	c4 d23	c6 d7	c6 d15	c6 d23		c8 d12	c8 d20		
H		c4 d8	c4 d16		c6 d8	c6 d16		c8 d5	c8 d13	c8 d21		

Master Plate 3

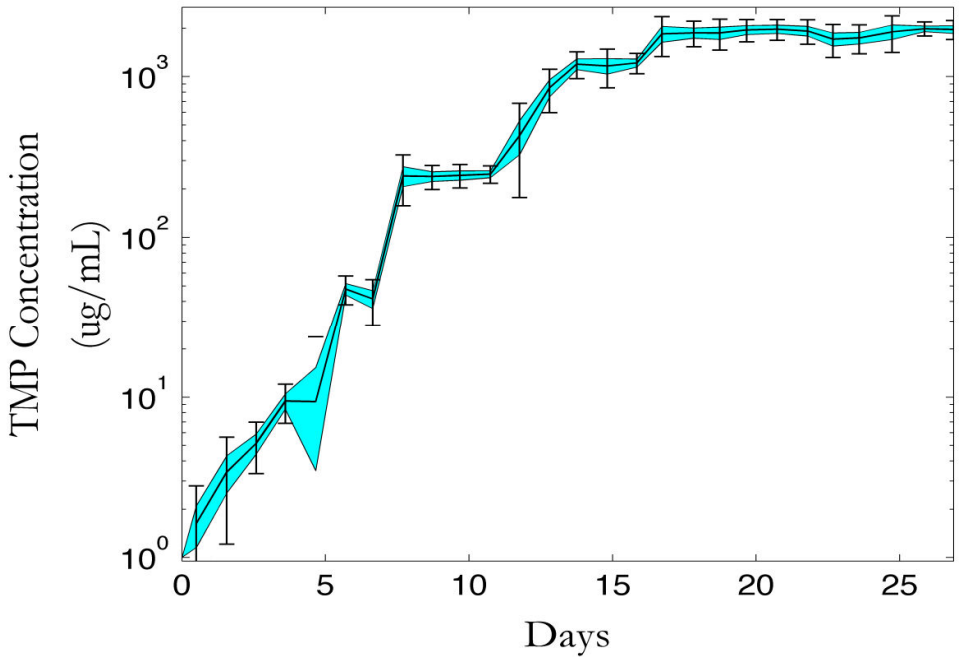
	1	2	3	4	5	6	7	8	9	10	11	12
A		c2 d1	c2 d9	c2 d17	c3 d1	c3 d9	c3 d17	c9 d1	c9 d9	wt	c15 d16	
B		c2 d2	c2 d10	c2 d18	c3 d2	c3 d10	c3 d18	c9 d2	c9 d10	c9 d17	c15 d17	
C		c2 d3	c2 d11	c2 d19	c3 d3	c3 d11	c3 d19	c9 d3	c9 d11	c9 d18	c15 d18	
D		c2 d4	c2 d12	c2 d20	c3 d4	c3 d12	c3 d20	c9 d4	c9 d12	c9 d19	c15 d19	
E		c2 d5	c2 d13		c3 d5	c3 d13	c3 d21	c9 d5	c9 d13	c9 d20	c15 d20	
F		c2 d6	c2 d14	c2 d21	c3 d6	c3 d14	c3 d22	c9 d6	c9 d14	c9 d21	c15 d21	
G		c2 d7	c2 d15	c2 d22	c3 d7	c3 d15	c3 d23	c9 d7	c9 d15	c9 d22	c15 d22	
H		c2 d8	c2 d16	c2 d23	c3 d8	c3 d16	wt	c9 d8	c9 d16	c9 d23	c15 d23	

Master Plate 4

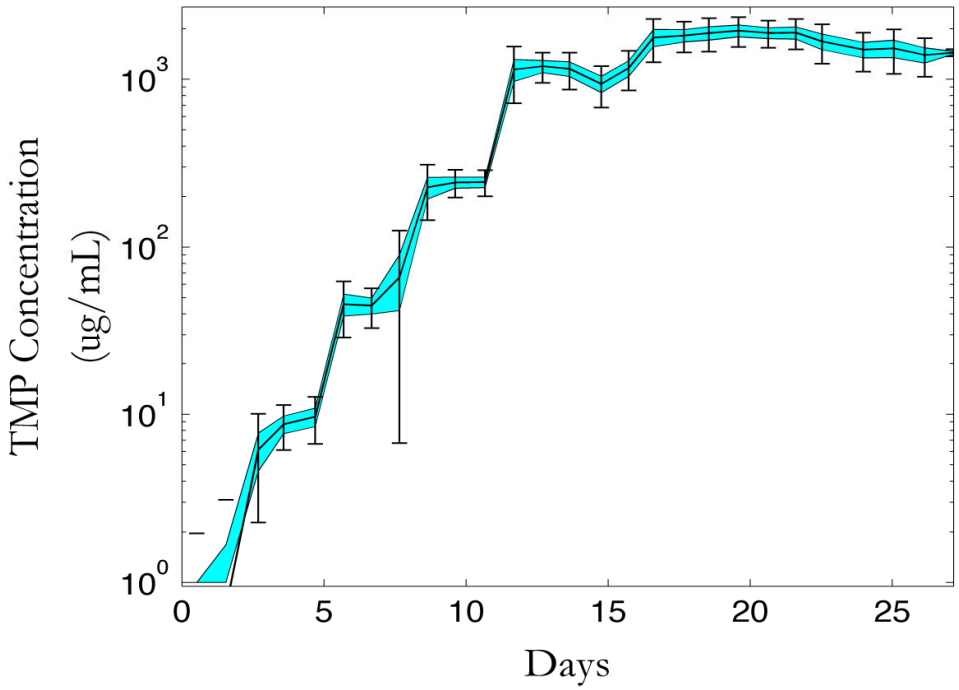
	1	2	3	4	5	6	7	8	9	10	11	12
A		c10 d7	c10 d15	c10 d23	c11 d7	c11 d15	c11 d23	c14 d7	c14 d15	c14 d23	c15 d8	
B	c10 d1	c10 d8	c10 d16		c11 d8	c11 d16	wt	c14 d8	c14 d16	c15 d1	c15 d9	
C	c10 d2	c10 d9	c10 d17	c11 d1	c11 d9	c11 d17	c14 d1	c14 d9	c14 d17	c15 d2	c15 d10	
D	c10 d3	c10 d10	c10 d18	c11 d2	c11 d10	c11 d18	c14 d2	c14 d10	c14 d18	c15 d3	c15 d11	wt
E	c10 d4	c10 d11	c10 d19	c11 d3	c11 d11	c11 d19	c14 d3	c14 d11	c14 d19	c15 d4	c15 d12	
F	c10 d5	c10 d12	c10 d20	c11 d4	c11 d12	c11 d20	c14 d4	c14 d12	c14 d20	c15 d5	c15 d13	
G	c10 d6	c10 d13	c10 d21	c11 d5	c11 d13	c11 d21	c14 d5	c14 d13	c14 d21	c15 d6	c15 d14	
H		c10 d14	c10 d22	c11 d6	c11 d14	c11 d22	c14 d6	c14 d14	c14 d22	c15 d7	c15 d15	

Appendix B

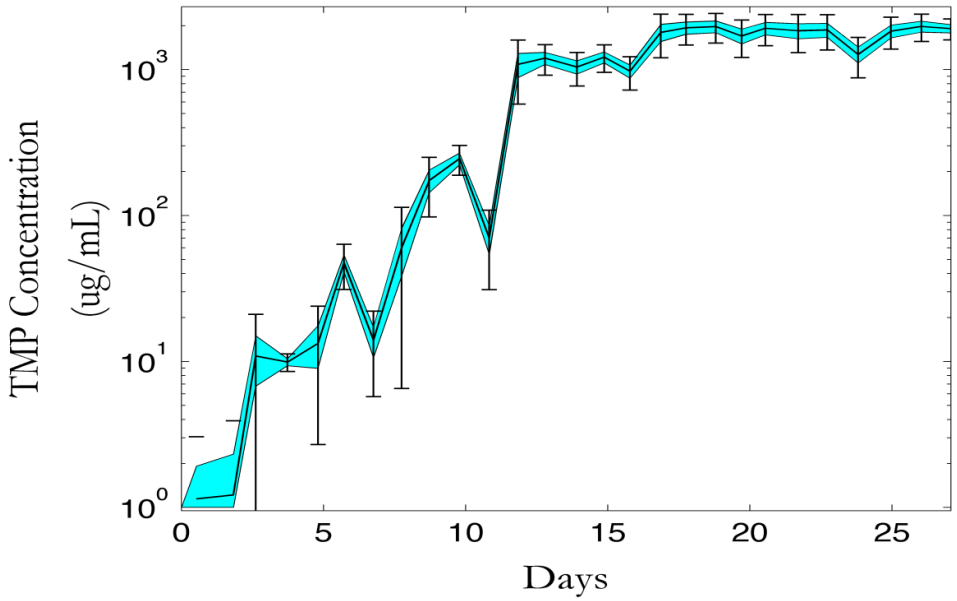
Culture 1



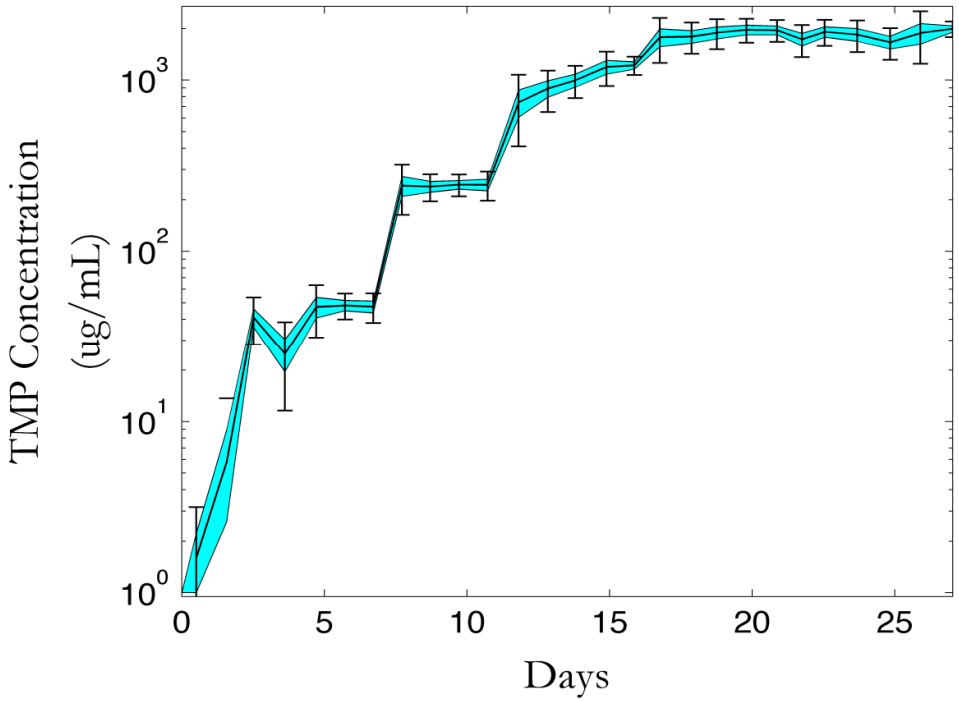
Culture 2



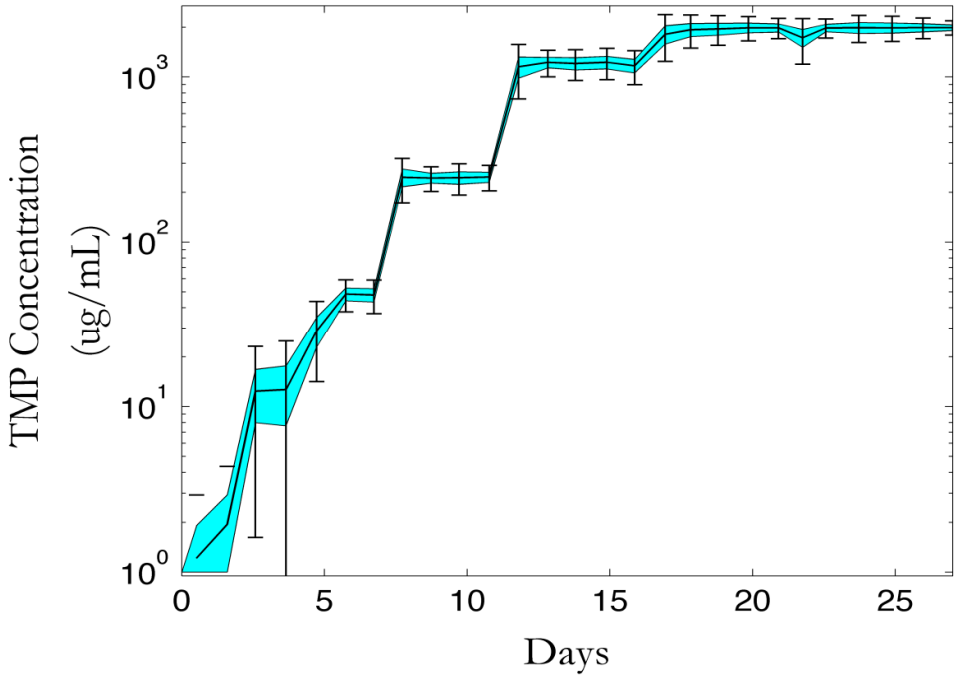
Culture 3



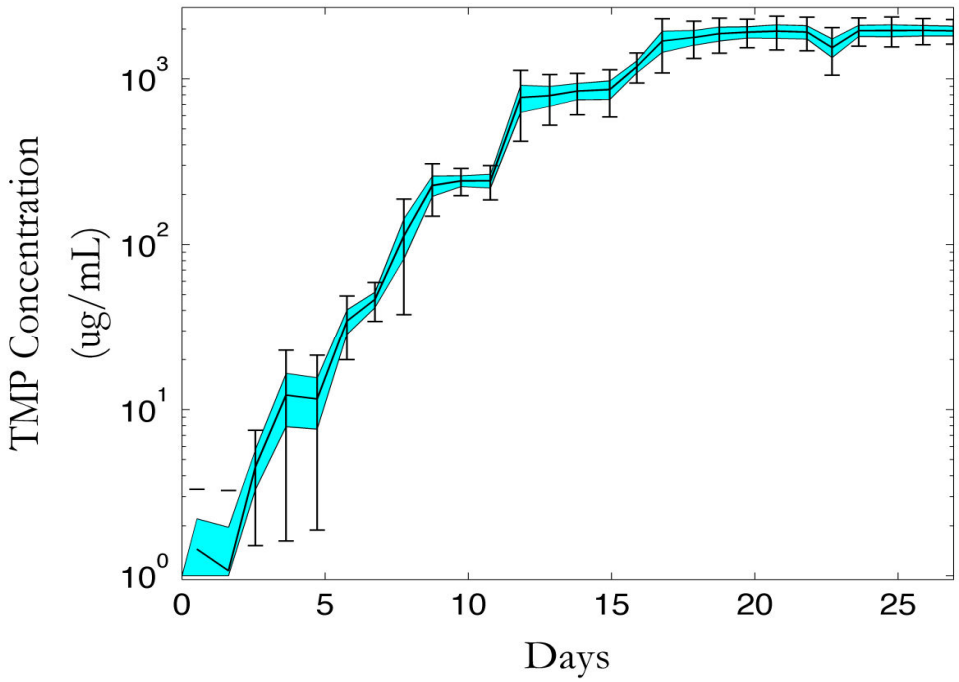
Culture 4



Culture 6



Culture 7



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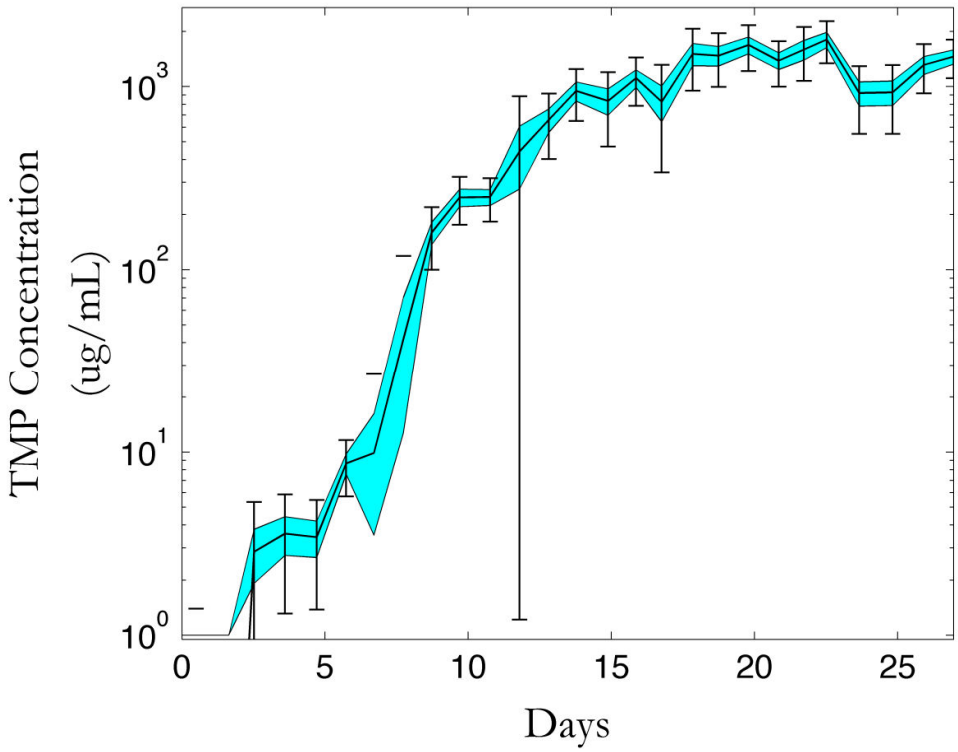

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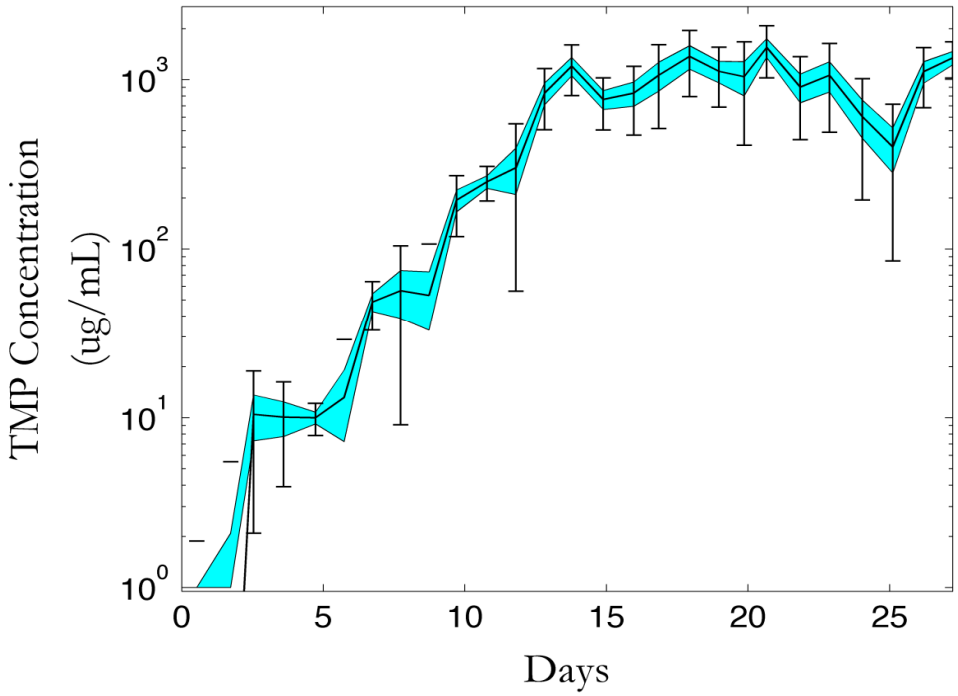
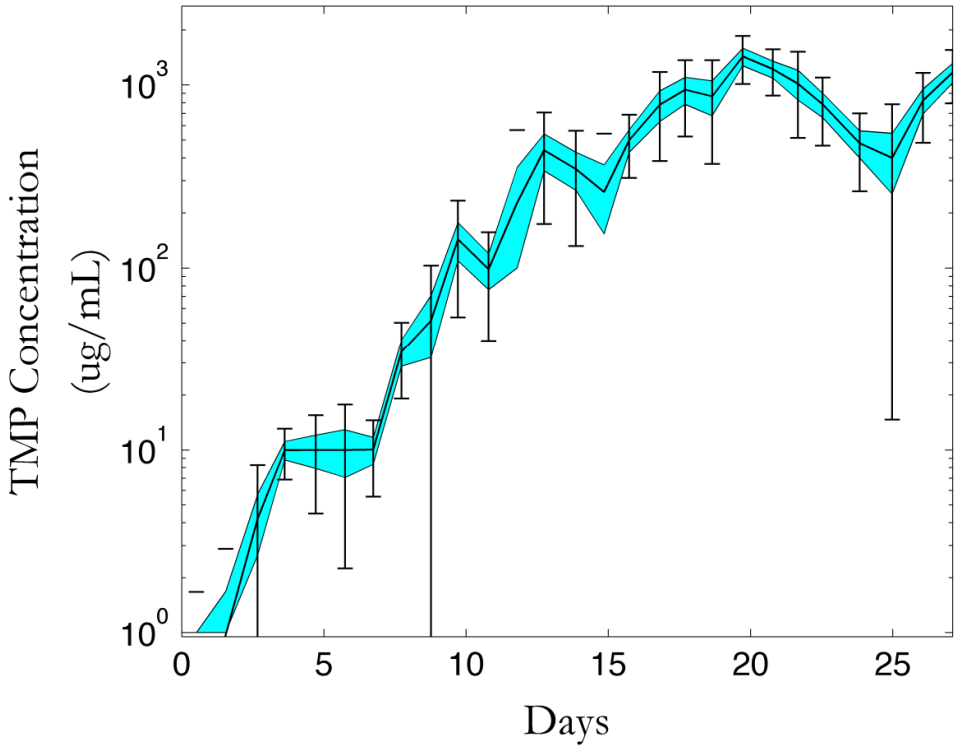
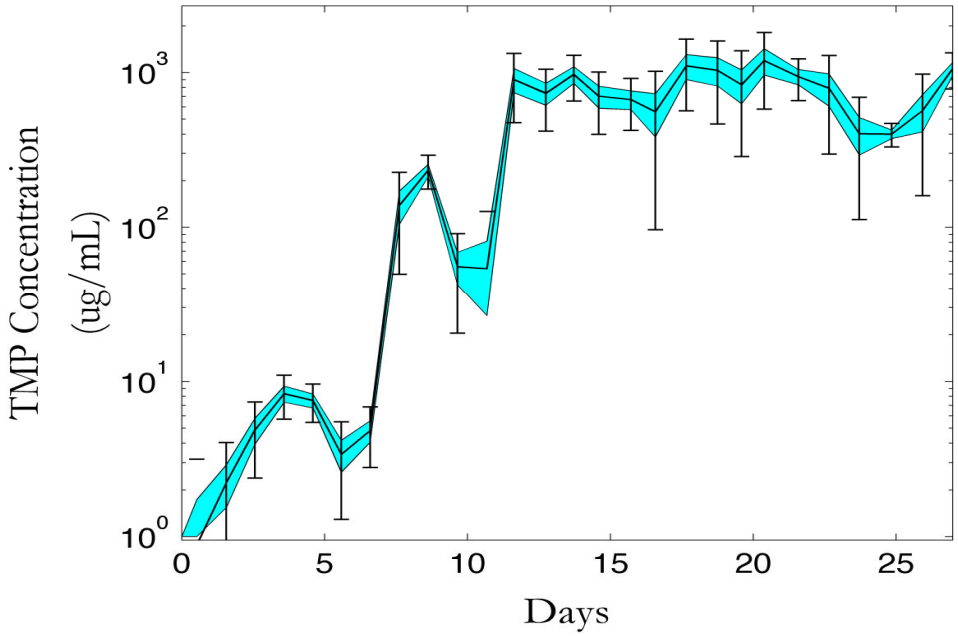


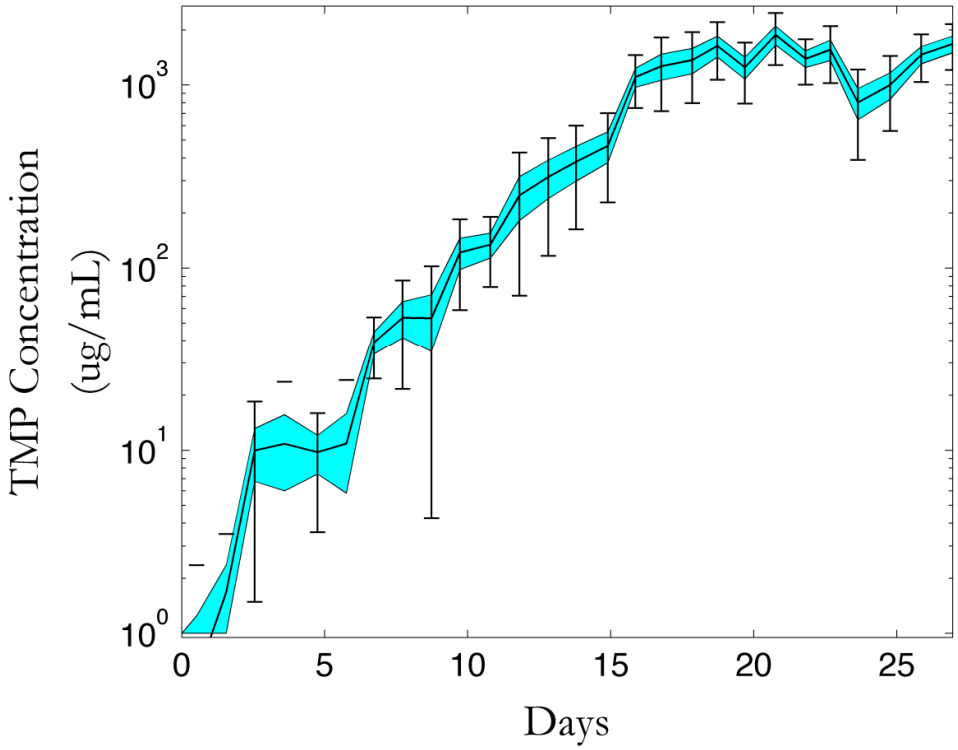
Culture 10



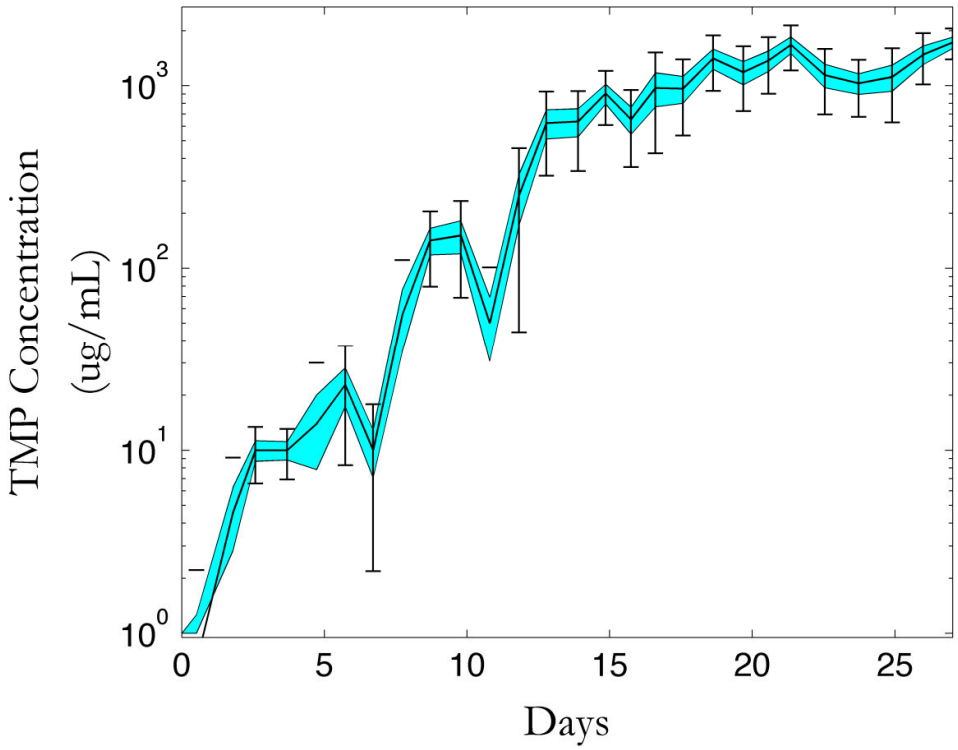
Culture 11



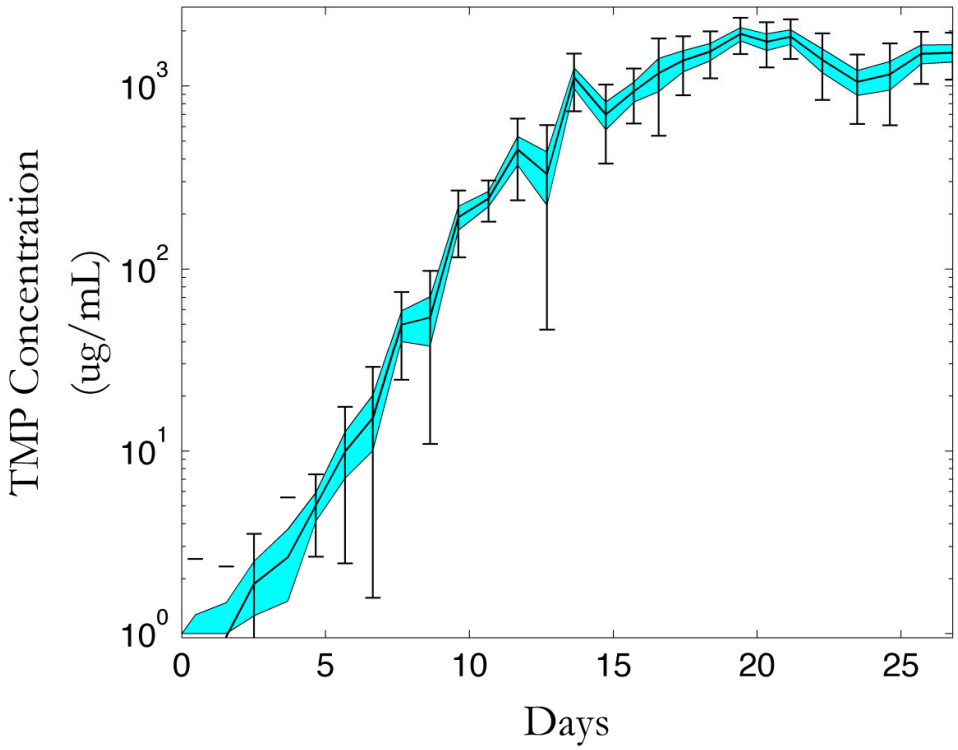
Culture 13



Culture 14

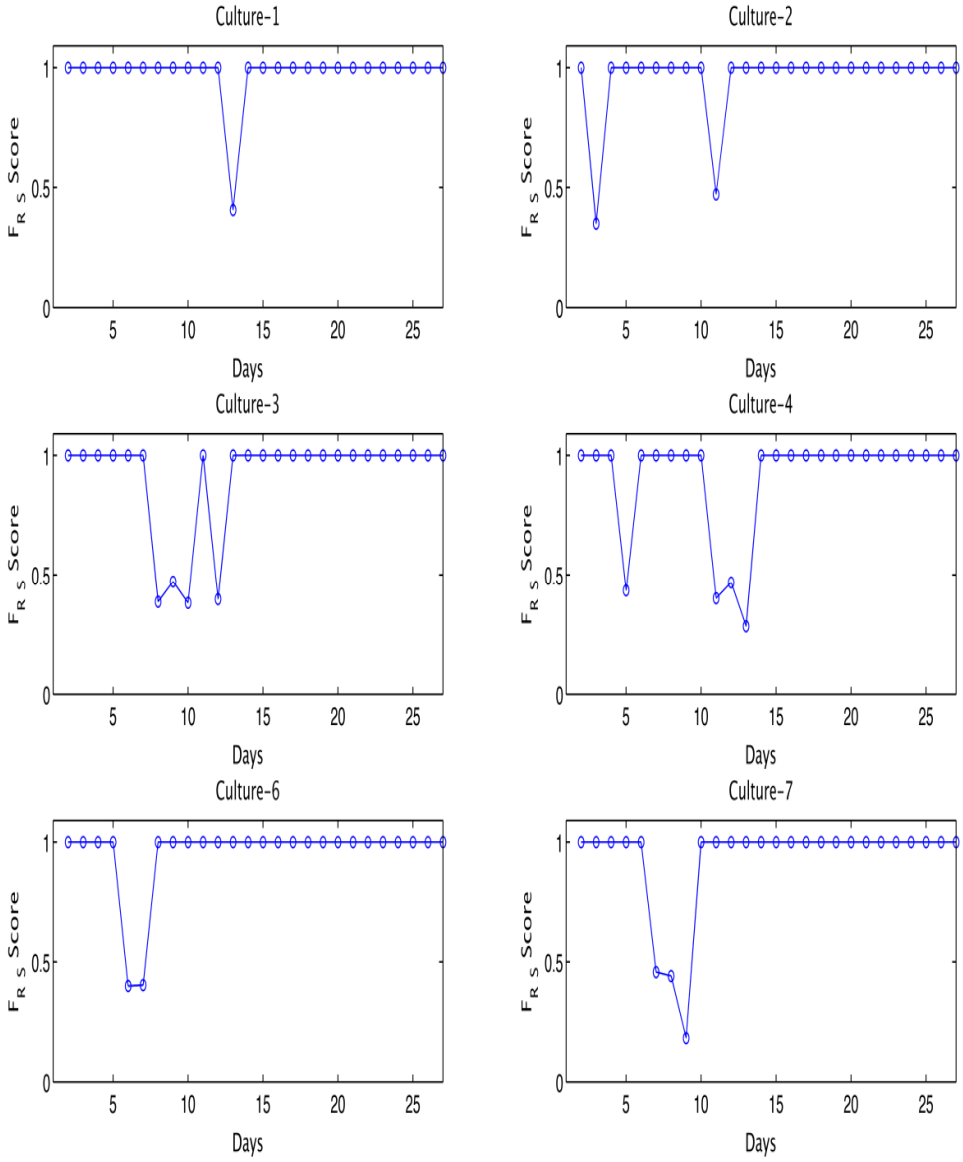


Culture 15

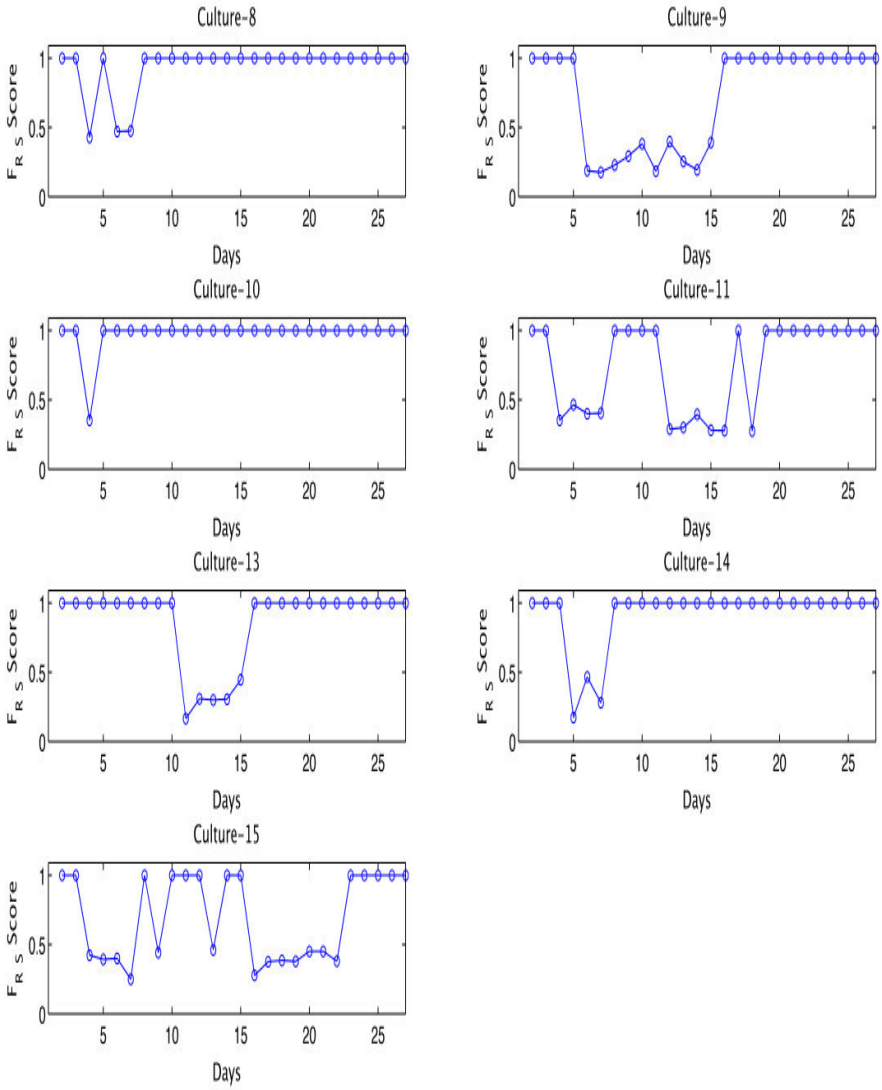


Appendix C

F_{RS} analysis of Mildly Diluted Cultures



F_{RS} analysis of Strongly Diluted Cultures



Appendix D

	M20I		
	native residue	position	mutant residue
	Methionine	20	Isoleucine
side chain polarity	nonpolar		nonpolar
hydrophobicity	1,9		4,5
	P21L		
	native residue	position	mutant residue
	Proline	21	Leucine
side chain polarity	nonpolar		nonpolar
hydrophobicity	-1,6		3,8
	P21Q		
	native residue	position	mutant residue
	Proline	21	Glutamine
side chain polarity	nonpolar		polar
hydrophobicity	-1,6		-3,5
	A26T		
	native residue	position	mutant residue
	Alaline	26	Threonine
side chain polarity	nonpolar		polar
hydrophobicity	1,8		-0,7
	D27E		
	native residue	position	mutant residue
	Aspatic Acid	27	Glutamic Acid
side chain polarity	acidic polar		acidic polar
hydrophobicity	-3,5		-3,5
	L28R		
	native residue	position	mutant residue
	Leucine	28	Arginine
side chain polarity	nonpolar		basic polar
hydrophobicity	3,8		-4,5
	W30R		
	native residue	position	mutant residue
	Tyrptophan	30	Arginine
side chain polarity	nonpolar		basicpolar
hydrophobicity	-0,9		-4,5

Adapted from a reference [27]

	W30G		
	native residue	position	mutant residue
	Tyrptophan	27	Glycine
side chain polarity	nonpolar		nonpolar
hydrophobicity	-0,9		-0,4
	W30C		
	native residue	position	mutant residue
	Tyrptophan	30	Cystein
side chain polarity	nonpolar		nonpolar
hydrophobicity	-0,9		2,5
	I94L		
	native residue	position	mutant residue
	Isoleucine	94	Leucine
side chain polarity	nonpolar		nonpolar
hydrophobicity	4,5		3,8
	R98P		
	native residue	position	mutant residue
	Arginine	98	Proline
side chain polarity	basic polar		nopolar
hydrophobicity	4,5		-1,6
	F153S		
	native residue	position	mutant residue
	Phenylalaline	153	Serine
side chain polarity	nonpolar		polar
hydrophobicity	2,8		-0,8
	F153V		
	native residue	position	mutant residue
	Phenylalaline	153	Valine
side chain polarity	nonpolar		nonpolar
hydrophobicity	2,8		4,2
	F153L		
	native residue	position	mutant residue
	Phenylalaline	153	Leucine
side chain polarity	nonpolar		nonpolar
hydrophobicity	2,8		3,8

Adapted from a reference [27]

Appendix E

Sequencing Results

Culture 1 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	g-31a
Colony 2	g-31a
Colony 3	g-31a
Colony 4	g-31a

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 11 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R

Day 12 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/c-35t
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R

Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T
Colony 5	g-31a/L28R/A26T
Colony 6	g-31a/L28R/A26T
Colony 7	g-31a/L28R/A26T

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Culture 2 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation

Colony 4	No mutation

Day 3 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	D27E
Colony 2	D27E
Colony 3	D27E

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	D27E
Colony 2	D27E
Colony 3	D27E
Colony 4	D27E

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a
Colony 2	D27E/g-9a
Colony 3	D27E/g-9a
Colony 4	D27E/g-9a

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153V
Colony 2	D27E/g-9a/F153V
Colony 3	D27E/g-9a/F153V
Colony 4	D27E/g-9a/F153V

Day 11 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153V
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153V

Day 12 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S

Day 19 (8 different single colonies were sent to sequencing but one did not work well)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S
Colony 5	D27E/g-9a/F153S
Colony 6	D27E/g-9a/F153S
Colony 7	D27E/g-9a/F153S

Day 20 (8 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S
Colony 5	D27E/g-9a/F153S
Colony 6	D27E/g-9a/F153S
Colony 7	D27E/g-9a/F153S
Colony 8	D27E/g-9a/F153S

Day 21 (8 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S
Colony 5	D27E/g-9a/F153S
Colony 6	D27E/g-9a/F153S
Colony 7	D27E/g-9a/F153S
Colony 8	D27E/g-9a/F153S

Day 22 (8 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S
Colony 5	D27E/g-9a/F153S
Colony 6	D27E/g-9a/F153S
Colony 7	D27E/g-9a/F153S
Colony 8	D27E/g-9a/F153S

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	D27E/g-9a/F153S
Colony 2	D27E/g-9a/F153S
Colony 3	D27E/g-9a/F153S
Colony 4	D27E/g-9a/F153S
Colony 5	D27E/g-9a/F153S
Colony 6	D27E/g-9a/F153S
Colony 7	D27E/g-9a/F153S

Culture 3 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-9a

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-9a

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a/W30C
Colony 4	g-9a

Day 9 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S
Colony 2	g-9a/F153S
Colony 3	g-9a/W30C
Colony 4	g-9a/W30C

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S
Colony 2	g-9a/F153S
Colony 3	g-9a/W30S
Colony 4	g-9a/W30C

Day 11 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S
Colony 2	g-9a/F153S
Colony 3	g-9a/F153S
Colony 4	g-9a/F153S

Day 12 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E
Colony 5	g-9a/F153S/D27E
Colony 6	g-9a/F153S/D27E
Colony 7	g-9a/F153S/D27E

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E

Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/F153S/D27E
Colony 2	g-9a/F153S/D27E
Colony 3	g-9a/F153S/D27E
Colony 4	g-9a/F153S/D27E

Culture 4 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-9a

Day 5 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	c-35t
Colony 2	g-9a/D27E
Colony 3	g-9a/D27E

Day 6 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E
Colony 2	g-9a/D27E
Colony 3	g-9a/D27E
Colony 4	g-9a/D27E

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V
Colony 2	g-9a/D27E/F153V
Colony 3	g-9a/D27E/F153V
Colony 4	g-9a/D27E/F153V

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V
Colony 2	g-9a/D27E/F153V
Colony 3	g-9a/D27E/F153V
Colony 4	g-9a/D27E/F153V

Day 11 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/M20I
Colony 2	g-9a/D27E/F153V

Colony 3	g-9a/D27E/F153V
Colony 4	g-9a/D27E/F153V

Day 11 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/M20I
Colony 2	g-9a/D27E/F153V
Colony 3	g-9a/D27E/F153V/M20I
Colony 4	g-9a/D27E/F153V

Day 12 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/M20I
Colony 2	g-9a/D27E/F153V
Colony 3	g-9a/D27E/F153V/M20I
Colony 4	g-9a/D27E/F153V

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/M20I
Colony 2	g-9a/D27E/F153V
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V

Day 14 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 15 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R

Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R
Colony 5	g-9a/D27E/F153V/L28R
Colony 6	g-9a/D27E/F153V/L28R
Colony 7	g-9a/D27E/F153V/L28R

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/D27E/F153V/L28R
Colony 2	g-9a/D27E/F153V/L28R
Colony 3	g-9a/D27E/F153V/L28R
Colony 4	g-9a/D27E/F153V/L28R

Culture 6 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R

Colony 4	c-35t/W30R

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L
Colony 2	c-35t/W30R/I94L
Colony 3	c-35t/W30R/I94L
Colony 4	c-35t/W30R/I94L

Day 9 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L
Colony 2	c-35t/W30R/I94L
Colony 3	c-35t/W30R/I94L
Colony 4	c-35t/W30R/I94L

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L
Colony 2	c-35t/W30R/I94L
Colony 3	c-35t/W30R/I94L
Colony 4	c-35t/W30R/I94L

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L
Colony 2	c-35t/W30R/I94L
Colony 3	c-35t/W30R/I94L
Colony 4	c-35t/W30R/I94L

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T
Colony 5	c-35t/W30R/I94L/A26T
Colony 6	c-35t/W30R/I94L/A26T
Colony 7	c-35t/W30R/I94L/A26T

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/I94L/A26T
Colony 2	c-35t/W30R/I94L/A26T
Colony 3	c-35t/W30R/I94L/A26T
Colony 4	c-35t/W30R/I94L/A26T

Culture 7 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 5 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 6 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G

Colony 4	c-35t/W30G

Day 7 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G
Colony 4	c-35t/W30G
Colony 5	c-35t/W30G
Colony 6	c-35t/D27E
Colony 7	c-35t/D27E
Colony 8	c-35t/D27E

Day 8 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G
Colony 4	c-35t/W30G
Colony 5	c-35t/D27E
Colony 6	c-35t/D27E
Colony 7	c-35t/D27E
Colony 8	c-35t/D27E

Day 9 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/W30G/A26T
Colony 4	c-35t/W30G/A26T
Colony 5	c-35t/W30G/A26T
Colony 6	c-35t/W30G/A26T
Colony 7	c-35t/W30G/D27E
Colony 8	c-35t/D27E

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/W30G/A26T
Colony 4	c-35t/W30G/A26T

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/W30G/A26T
Colony 4	c-35t/W30G/A26T

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R

Colony 4	c-35t/W30G/A26T/L28R

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R
Colony 5	c-35t/W30G/A26T/L28R
Colony 6	c-35t/W30G/A26T/L28R
Colony 7	c-35t/W30G/A26T/L28R

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T/L28R
Colony 2	c-35t/W30G/A26T/L28R
Colony 3	c-35t/W30G/A26T/L28R
Colony 4	c-35t/W30G/A26T/L28R

Culture 8 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	g-31a
Colony 4	g-31a

Day 5 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	g-31a
Colony 2	g-31a
Colony 3	g-31a

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	g-31a
Colony 2	g-31a
Colony 3	g-31a/P21Q
Colony 4	g-31a/P21Q

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 9 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T
Colony 5	g-31a/L28R/A26T
Colony 6	g-31a/L28R/A26T
Colony 7	g-31a/L28R/A26T

Day 24 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T

Day 25 (4 different single colonies were sent to sequencing but one did not work well)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Culture 9 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 5 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 6 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G
Colony 4	c-35t/D27E
Colony 5	c-35t/D27E
Colony 6	c-35t/F153S
Colony 7	c-35t
Colony 8	c-35t

Day 7 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S
Colony 6	c-35t/F153S
Colony 7	c-35t/D27E
Colony 8	c-35t

Day 8 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/W30G
Colony 3	c-35t/W30G
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S
Colony 6	c-35t/F153S
Colony 7	c-35t/F153S
Colony 8	c-35t/D27E

Day 9 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G
Colony 2	c-35t/ F153S
Colony 3	c-35t/ F153S
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S

Colony 6	c-35t/F153S
Colony 7	c-35t/F153S
Colony 8	c-35t/ F153S

Day 10 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/W30G/A26T
Colony 4	c-35t/W30G/A26T
Colony 5	c-35t/W30G/A26T
Colony 6	c-35t/W30G/A26T
Colony 7	c-35t/F153S
Colony 8	c-35t/ F153S

Day 11 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/W30G/A26T
Colony 4	c-35t/W30G/A26T
Colony 5	c-35t/W30G/A26T
Colony 6	c-35t/W30G/A26T
Colony 7	c-35t/F153S/D27E
Colony 8	c-35t/ F153S

Day 12 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/W30G/A26T
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/D27E
Colony 7	c-35t/F153S/D27E
Colony 8	c-35t/F153S/D27E

Day 13 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30G/A26T
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/L28R
Colony 6	c-35t/F153S/L28R
Colony 7	c-35t/F153S/L28R
Colony 8	c-35t/F153S/L28R

Day 14 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E

Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/L28R
Colony 7	c-35t/F153S

Day 15 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R
Colony 5	c-35t/F153S/L28R
Colony 6	c-35t/F153S/L28R
Colony 7	c-35t/F153S/L28R
Colony 8	c-35t/F153S/L28R

Day 15 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R
Colony 5	c-35t/F153S/L28R
Colony 6	c-35t/F153S/L28R
Colony 7	c-35t/F153S/L28R
Colony 8	c-35t/F153S/L28R

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R
Colony 5	c-35t/F153S/L28R
Colony 6	c-35t/F153S/L28R
Colony 7	c-35t/F153S/L28R

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R

Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/L28R
Colony 2	c-35t/F153S/L28R
Colony 3	c-35t/F153S/L28R
Colony 4	c-35t/F153S/L28R

Culture 10 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 3 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-9a

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E
Colony 2	c-35t/D27E
Colony 3	c-35t/D27E
Colony 4	c-35t/D27E

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/F153V
Colony 2	c-35t/D27E/F153V
Colony 3	c-35t/D27E/F153V
Colony 4	c-35t/D27E/F153V

Day 14 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/F153V
Colony 2	c-35t/D27E/F153V
Colony 3	c-35t/D27E/F153V
Colony 4	c-35t/D27E/F153V

Day 15 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/F153V
Colony 2	c-35t/D27E/F153V
Colony 3	c-35t/D27E/L28R
Colony 4	c-35t/D27E/L28R/F153L

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 22 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L
Colony 5	c-35t/D27E/L28R/F153L
Colony 6	c-35t/D27E/L28R/F153L
Colony 7	c-35t/D27E/L28R/F153L

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L

Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/D27E/L28R/F153L
Colony 2	c-35t/D27E/L28R/F153L
Colony 3	c-35t/D27E/L28R/F153L
Colony 4	c-35t/D27E/L28R/F153L

Culture 11 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-31a

Day 5 (5 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-9a
Colony 3	g-9a
Colony 4	g-9a
Colony 5	g-31a

Day 6 (4 different single colonies were sent to sequencing)

Colony 1	g-9a
Colony 2	g-31a
Colony 3	g-31a
Colony 4	g-31a

Day 7 (8 different single colonies were sent to sequencing)

Colony 1	g-31a/P21L
Colony 2	g-31a/P21L
Colony 3	g-31a
Colony 4	g-31a
Colony 5	g-31a
Colony 6	g-31a
Colony 7	g-31a
Colony 8	g-31a

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R
Colony 2	g-9a/L28R
Colony 3	g-9a/L28R
Colony 4	g-9a/L28R

Day 9 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R
Colony 2	g-9a/L28R
Colony 3	g-9a/L28R
Colony 4	g-9a/L28R

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R
Colony 2	g-9a/L28R
Colony 3	g-9a/L28R
Colony 4	g-9a/L28R

Day 13 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/A26T
Colony 2	g-9a/L28R/A26T
Colony 3	g-9a/L28R/A26T
Colony 4	g-9a/L28R/A26T
Colony 5	g-9a/L28R/A26T
Colony 6	g-9a/L28R/A26T
Colony 7	g-9a/L28R/A26T
Colony 8	g-9a/L28R/F153S

Day 14 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/A26T
Colony 2	g-9a/L28R/A26T
Colony 3	g-9a/L28R/A26T
Colony 4	g-9a/L28R/A26T
Colony 5	g-9a/L28R/A26T
Colony 6	g-9a/L28R/A26T
Colony 7	g-9a/L28R

Colony 8	g-9a/L28R

Day 15 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S
Colony 5	g-9a/L28R/F153S
Colony 6	g-9a/L28R/F153S
Colony 7	g-9a/L28R/F153S
Colony 8	g-9a/L28R/A26T

Day 16 (7different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/A26T
Colony 2	g-9a/L28R/A26T
Colony 3	g-9a/L28R/A26T
Colony 4	g-9a/L28R/A26T
Colony 5	g-9a/L28R/A26T
Colony 6	g-9a/L28R/A26T
Colony 7	g-9a/L28R/F153S

Day 17 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S
Colony 5	g-9a/L28R/F153S
Colony 6	g-9a/L28R/F153S
Colony 7	g-9a/L28R/F153S
Colony 8	g-9a/L28R/F153S

Day 18 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S
Colony 5	g-9a/L28R/F153S
Colony 6	g-9a/L28R/F153S
Colony 7	g-9a/L28R/F153S
Colony 8	g-9a/L28R/A26T

Day 19 (8 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S
Colony 5	g-9a/L28R/F153S
Colony 6	g-9a/L28R/F153S

Colony 7	g-9a/L28R/F153S
Colony 8	g-9a/L28R/F153S

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S
Colony 5	g-9a/L28R/F153S
Colony 6	g-9a/L28R/F153S
Colony 7	g-9a/L28R/F153S

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-9a/L28R/F153S
Colony 2	g-9a/L28R/F153S
Colony 3	g-9a/L28R/F153S
Colony 4	g-9a/L28R/F153S

Culture 13 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (4 different single colonies were sent to sequencing)

Colony 1	c-35t

Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 7 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R
Colony 4	c-35t/W30R

Day 8 (3 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R

Day 9 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R
Colony 4	c-35t/W30R
Colony 5	c-35t/W30R
Colony 6	c-35t/W30R
Colony 7	c-35t/W30R
Colony 8	c-35t/W30R

Day 10 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R
Colony 4	c-35t/W30R
Colony 5	c-35t/W30R
Colony 6	c-35t/W30R
Colony 7	c-35t/W30R
Colony 8	c-35t/W30R

Day 11 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T
Colony 2	c-35t/W30R/A26T
Colony 3	c-35t/W30R/A26T
Colony 4	c-35t/W30R/A26T
Colony 5	c-35t/W30R/D27E
Colony 6	c-35t/W30R
Colony 7	c-35t/W30R
Colony 8	g-31a/L28R

Day 12 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T
Colony 2	c-35t/W30R/A26T

Colony 3	c-35t/W30R/A26T
Colony 4	c-35t/W30R/A26T
Colony 5	c-35t/W30R/A26T
Colony 6	c-35t/W30R/A26T
Colony 7	g-31a/L28R

Day 13 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T
Colony 2	c-35t/W30R/A26T
Colony 3	c-35t/W30R/A26T
Colony 4	c-35t/W30R/A26T
Colony 5	c-35t/W30R/A26T
Colony 6	c-35t/W30R/A26T
Colony 7	c-35t/W30R/A26T
Colony 8	g-31a/L28R

Day 14 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T
Colony 2	c-35t/W30R/A26T
Colony 3	c-35t/W30R/A26T
Colony 4	c-35t/W30R/A26T
Colony 5	c-35t/W30R/A26T
Colony 6	c-35t/W30R/A26T
Colony 7	g-31a/L28R

Day 15 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T
Colony 5	c-35t/W30R/A26T
Colony 6	c-35t/W30R/A26T
Colony 7	c-35t/W30R/A26T
Colony 8	c-35t/W30R/A26T

Day 16 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/A26T/R98P
Colony 8	c-35t/W30R/F153S

Day 17 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P

Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/F153S

Day 18 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/F153S
Colony 7	c-35t/W30R/F153S

Day 19 (11 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/F153S
Colony 8	c-35t/W30R/F153S
Colony 9	c-35t/W30R/F153S
Colony 10	c-35t/W30R/F153S
Colony 11	c-35t/W30R/F153S

Day 20 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/A26T/R98P
Colony 8	c-35t/W30R/A26T/R98P

Day 21 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/A26T/R98P
Colony 8	c-35t/W30R/F153S

Day 22 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/A26T/R98P
Colony 8	c-35t/W30R/A26T/R98P

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P
Colony 5	c-35t/W30R/A26T/R98P
Colony 6	c-35t/W30R/A26T/R98P
Colony 7	c-35t/W30R/A26T/R98P

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R/A26T/R98P
Colony 2	c-35t/W30R/A26T/R98P
Colony 3	c-35t/W30R/A26T/R98P
Colony 4	c-35t/W30R/A26T/R98P

Culture 14 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation

Colony 3	No mutation
Colony 4	No mutation

Day 2 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 3 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t

Day 4 (8 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t
Colony 5	c-35t
Colony 6	c-35t
Colony 7	c-35t
Colony 8	c-35t

Day 5 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/W30C
Colony 2	c-35t/D27E
Colony 3	c-35t
Colony 4	c-35t
Colony 5	c-35t
Colony 6	c-35t
Colony 7	c-35t
Colony 8	c-35t

Day 6 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/W30C
Colony 2	c-35t/W30C
Colony 3	c-35t/W30C
Colony 4	c-35t/W30C
Colony 5	c-35t/W30R
Colony 6	c-35t/W30R
Colony 7	c-35t/W30R

Day 7 (8 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R

Colony 4	g-31a/L28R
Colony 5	c-35t/W30C
Colony 6	c-35t/W30C
Colony 7	c-35t/W30R
Colony 8	c-35t/W30R

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R
Colony 4	c-35t/W30R

Day 8 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/W30R
Colony 2	c-35t/W30R
Colony 3	c-35t/W30R
Colony 4	c-35t/W30R

Day 10 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R
Colony 2	g-31a/L28R
Colony 3	g-31a/L28R
Colony 4	g-31a/L28R

Day 13 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 16 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 19 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 21 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 22 (7 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T
Colony 5	g-31a/L28R/A26T
Colony 6	g-31a/L28R/A26T
Colony 7	g-31a/L28R/A26T

Day 23 (13 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T
Colony 5	g-31a/L28R/A26T
Colony 6	g-31a/L28R/A26T
Colony 7	g-31a/L28R/A26T
Colony 8	g-31a/L28R/A26T
Colony 9	g-31a/L28R/A26T
Colony 10	g-31a/L28R/A26T
Colony 11	g-31a/L28R/A26T
Colony 12	g-31a/L28R/A26T
Colony 13	g-31a/L28R/A26T

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	g-31a/L28R/A26T
Colony 2	g-31a/L28R/A26T
Colony 3	g-31a/L28R/A26T
Colony 4	g-31a/L28R/A26T

Culture 15 Sequencing Results

Day 1 (4 different single colonies were sent to sequencing)

Colony 1	No mutation
Colony 2	No mutation
Colony 3	No mutation
Colony 4	No mutation

Day 4 (5 different single colonies were sent to sequencing)

Colony 1	c-15g
Colony 2	c-15g
Colony 3	c-15g
Colony 4	c-15g
Colony 5	c-15g

Day 5 (4 different single colonies were sent to sequencing)

Colony 1	P21Q
Colony 2	P21Q
Colony 3	P21Q
Colony 4	P21Q

Day 7 (8 different single colonies were sent to sequencing)

Colony 1	c-15g
Colony 2	c-15g
Colony 3	c-15g
Colony 4	c-35t
Colony 5	c-35t
Colony 6	c-35t
Colony 7	c-35t
Colony 8	No mutation

Day 8 (7 different single colonies were sent to sequencing)

Colony 1	c-35t
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t
Colony 5	c-35t
Colony 6	c-35t
Colony 7	c-35t

Day 9 (5different single colonies were sent to sequencing)

Colony 1	c-35t/P21Q
Colony 2	c-35t
Colony 3	c-35t
Colony 4	c-35t
Colony 5	c-35t

Day 10 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/P21Q
Colony 2	c-35t/P21Q
Colony 3	c-35t/P21Q
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S
Colony 6	c-35t/F153S
Colony 7	c-35t/F153S

Day 11 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S
Colony 2	c-35t/F153S
Colony 3	c-35t/F153S
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S
Colony 6	c-35t/F153S
Colony 7	c-35t/F153S
Colony 8	c-35t/F153S

Day 12 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S
Colony 2	c-35t/F153S
Colony 3	c-35t/F153S
Colony 4	c-35t/F153S
Colony 5	c-35t/F153S
Colony 6	c-35t/F153S
Colony 7	c-35t/F153S
Colony 8	c-35t/F153S

Day 13 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/L28R
Colony 7	c-35t/L28R
Colony 8	c-35t/L28R

Day 14 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/D27E
Colony 7	c-35t/F153S/D27E

Colony 8	c-35t/L28R/F153S

Day 15 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/D27E
Colony 7	c-35t/F153S/D27E
Colony 8	c-35t/F153S/D27E

Day 16 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/D27E
Colony 7	c-35t/F153S/D27E
Colony 8	c-35t/L28R/F153S

Day 17 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/L28R/F153S
Colony 7	c-35t/L28R/F153S
Colony 8	c-35t/ F153S

Day 18 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/F153S/D27E
Colony 4	c-35t/F153S/D27E
Colony 5	c-35t/F153S/D27E
Colony 6	c-35t/F153S/D27E
Colony 7	c-35t/L28R/F153S
Colony 8	c-35t/ L28R/F153S

Day 19 (8 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S
Colony 5	c-35t/L28R/F153S

Colony 6	c-35t/L28R/F153S
Colony 7	c-35t/L28R/F153S
Colony 8	c-35t/ L28R/F153S

Day 21 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S

Day 22 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/L28R/F153S
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S

Day 23 (7 different single colonies were sent to sequencing)

Colony 1	c-35t/F153S/D27E
Colony 2	c-35t/F153S/D27E
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S
Colony 5	c-35t/L28R/F153S
Colony 6	c-35t/L28R/F153S
Colony 7	c-35t/L28R/F153S

Day 24 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/L28R/F153S
Colony 2	c-35t/L28R/F153S
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S

Day 25 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/L28R/F153S
Colony 2	c-35t/L28R/F153S
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S

Day 26 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/L28R/F153S
Colony 2	c-35t/L28R/F153S
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S

Day 27 (4 different single colonies were sent to sequencing)

Colony 1	c-35t/L28R/F153S
Colony 2	c-35t/L28R/F153S
Colony 3	c-35t/L28R/F153S
Colony 4	c-35t/L28R/F153S