Studying Genetic and Enzymatic Constraints Driving Evolution of Antibiotic Resistance

Yusuf Talha TAMER

This MSc. Thesis is submitted to: Faculty of Engineering and Natural Sciences
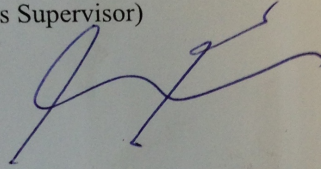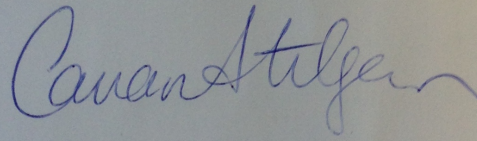
06.10.2014

Studying Genetic and Enzymatic Constraints Driving Evolution of Antibiotic Resistance
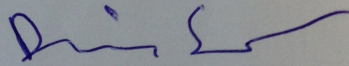
APPROVED BY:

Asst. Prof. Dr. Erdal Toprak
(Thesis Supervisor)

Prof. Dr. Canan Atılgan

Asst. Prof. Dr. Deniz Sezer

DATE OF APPROVAL: 10/06/2014

1. ABSTRACT

Studying Genetic and Enzymatic Constraints Driving Evolution of Antibiotic Resistance

YUSUF TALHA TAMER

MSc. 2014

Erdal Toprak (Thesis Supervisor)

Keywords: Antibiotic Resistance, Bacterial Evolution, Trimethoprim, Morbidostat

World is heading towards a post-antibiotic era due to emergence of antibiotic resistance. Several fatal infectious diseases caused by antibiotic resistant bacteria cannot be treated anymore using the existing antibiotic surplus. Novel antibiotics or novel strategies to use antibiotic more efficiently are therefore crucial to combat against resistance. However, both of these approaches require a clear understanding of antibiotic resistance at molecular and genetic levels. Here in this study, we studied evolutionary dynamics of trimethoprim resistance under dynamically sustained drug selection. Using a custom made continuous culture device that we call the Morbidostat; we evolved drug sensitive Escherichia coli cells against increasing levels of trimethoprim adapting strong or mild dilution rates. First, using Illumina whole genome sequencing and Sanger sequencing, we identified trimethoprim resistance conferring mutations in dihydrofolate reductase (*folA*) gene and the order that these mutations appear in the population. Our results suggest that clonal interference between different genotypes is common and longer under strong dilution where trimethoprim stress is applied in shorter and steeper pulses. Second, we cloned and purified dihydrofolate reductase (DHFR) enzymes with single mutations and carried out biochemical assays to quantify mutant enzymes' enzymatic activities. Our preliminary results showed that DHFR mutants have slightly worse substrate affinity (higher k_m values) but up to ~20 fold elevated catalysis rate (k_{cat}/k_m) compared to their wild type ancestor. We conclude that trimethoprim-resistance-conferring DHFR mutations decrease affinity to both enzyme's substrate and competing drug molecules, yet enzymatic activity, which is essential for folic acid synthesis, is still adequately efficient to maintain bacterial fitness.

2. ÖZET (IN TURKISH)

Antibiyotik Direncinin Evrilmesine Yol Açan Genetik Ve Enzimatik Etkenlerin

İncelenmesi

Yusuf Talha TAMER

Yüksek Lisans Tezi 2014

Anahtar Kelimeler: Antibiyotik Direnci Kazanılması, Trimetoprim, Folat Sentez Yolağı

Günümüz dünyası bakterilerin antimikrobiyal ilaçlara direnç kazanması nedeniyle antibiyotiklerin tamamıyla etkisiz hale geleceği güne doğru bir geçiş yaşıyor. Bir çok ölümcül bulaşıcı hastalık antibiyotiklere dirençli hale gelmiş bakteriler nedeniyle yakın gelecekte tedavisiz kalacak. Yeni antibiyotikler ve yeni tedavi yöntemlerinin geliştirilmesi ve bunlarla beraber antibiyotik direnci kazanılmasının önüne geçilmesi çok büyük önem taşıyor. Bu problemin çözülmesi adına yapılması gereken bakterilerin antibiyotik direnci kazanması işleminin genetik ve moleküler aşamalarını anlamak. Bu çalışmada trimetoprim antibiyotiğine karşı direnç kazanılmasının evrimsel temellerini farklı seçilim baskıları altında inceledik. Bu amaç doğrultusunda bakterilerin eşit bir şekilde seçilim baskılarıyla karşılaşmasına ve bakteri büyümesinin sürekli kontrol altında tutulmasına izin verebilen Morbidostat adlı makineyi kullandık. Bakteriler güçlü (Uzun süreli antibiyotik enjeksiyonuna) ve zayıf (kısa süreli antibiyotik enjeksiyonu) olmak üzere iki farklı seçilim baskısı altında antibiyotik direnci kazandılar. İllumina tüm genom sekanslama ve Sanger gen sekanslanması yöntemleriyle öncelikle deneyin sonucunda direnç kazanılmasına yol açan mutasyonları belirledik, sonrasında dihidrofolat redüktaz enzimi üzerinde görmüş olduğumuz bu mutasyonların, hangi sırayla kazanıldığını anlamak için günlük alınmış olan örnekleri sekansladık. Sonuçlarımızda güçlü seçilim baskısı altındaki popülasyonlarda genotipik çeşitlilik, zayıf seçilim gösteren popülasyonlara göre daha uzun süreli ve yaygın olarak görüldü. İkinci olarak gördüğümüz bu mutasyonları birer birer yabanıl protein üzerinde değişikliğe uğratıp deneyde gördüğümüz mutasyonların reaksiyonun biyokimyasına etkisini çalıştık. Elimizdeki ilk sonuçlar gösterdi ki; mutant proteinler yabanıl olanla karşılaştırıldığında, substrat affinitesi (K_m) adına biraz kötü olsa da reaksiyonun katalizinde (K_{cat}/K_m) 20 kata kadar daha etkili oldular. Sonuç olarak trimethoprim direnci kazanılmasında gerekli mutasyonlar, enzimin substratına karşı ilgisini azaltmış olsa da bakterinin hayatını devam ettirmesi adına çok önemli olan folat sentez yolunun çalışmasında daha etkili oldukları için bakteri popülasyonlarının Darwinsel uyumunu sağlamış oldular.

TO MY FAMILY

06.10.2014

YUSUF TALHA TAMER

3. ACKNOWLEDGEMENTS

s

Table of Contents

4. AIM OF STUDY

World is heading towards a post-antibiotic era due to emergence of antibiotic resistance. Several fatal infectious diseases caused by antibiotic resistant bacteria cannot be treated anymore using the existing antibiotic surplus. Novel antibiotics or novel strategies to use antibiotic more efficiently are therefore crucial to combat against resistance. However, both of these approaches require a clear understanding of antibiotic resistance at molecular and genetic levels. Here in this study, we studied evolutionary dynamics of trimethoprim resistance under dynamically sustained drug selection.

5. INTRODUCTION

5.1. Antibiotics

Bacterial pathogens cause severe infections and deaths over 17 million people annually.[1] Antibiotics are the substances that inhibit the growth of bacteria or kill them directly. They can be produced naturally or synthetically. From the time, Alexander Fleming first found antibiotic -Penicillin-, there are hundreds of molecules are designed as bactericidal or bacteriostatic agents but only a few of them are commercialized because of economical and safety issues.

5.2. Classification of Antibiotics

Commercial antibiotics are classified under 5-6 major classes with respect to their target mechanism. Some of these major classes are: Cell Wall Synthesis Inhibitors (e.g. β-Lactams), Protein Synthesis Inhibitors (e.g. Aminoglycosides, Macrolides), DNA Replication and Repair Inhibitors, Folic Acid Pathway Inhibitors. Beta-Lactam antibiotics hold the largest share in the antibiotic market of entire world [2]. Major targets of the β-Lactams are peptidoglycan layers and syntheses of the cell wall. This class of antibiotics has a special Lactam ring on their chemical structure. The other class, Protein synthesis inhibitors, is targeting the ribosomal small and large subunits with mimicking substances that have roles in the machinery of translation. Main targets for DNA replication and repair inhibitors are DNA and RNA synthesis precursors such

as DNA Gyrase Family proteins. Quinolones –synthetics antibiotics - and Coumarins are belonged to this group. Also there are some other targets of antibiotics directly or indirectly affecting or blocking the polymerization of nucleic acids and division of cell. Folic acid synthesis pathway inhibitors are in this group. Folic acid pathway inhibitors will be explained deeply at the second chapter of this thesis. But briefly this pathway synthesizes the precursors of nucleic acids.
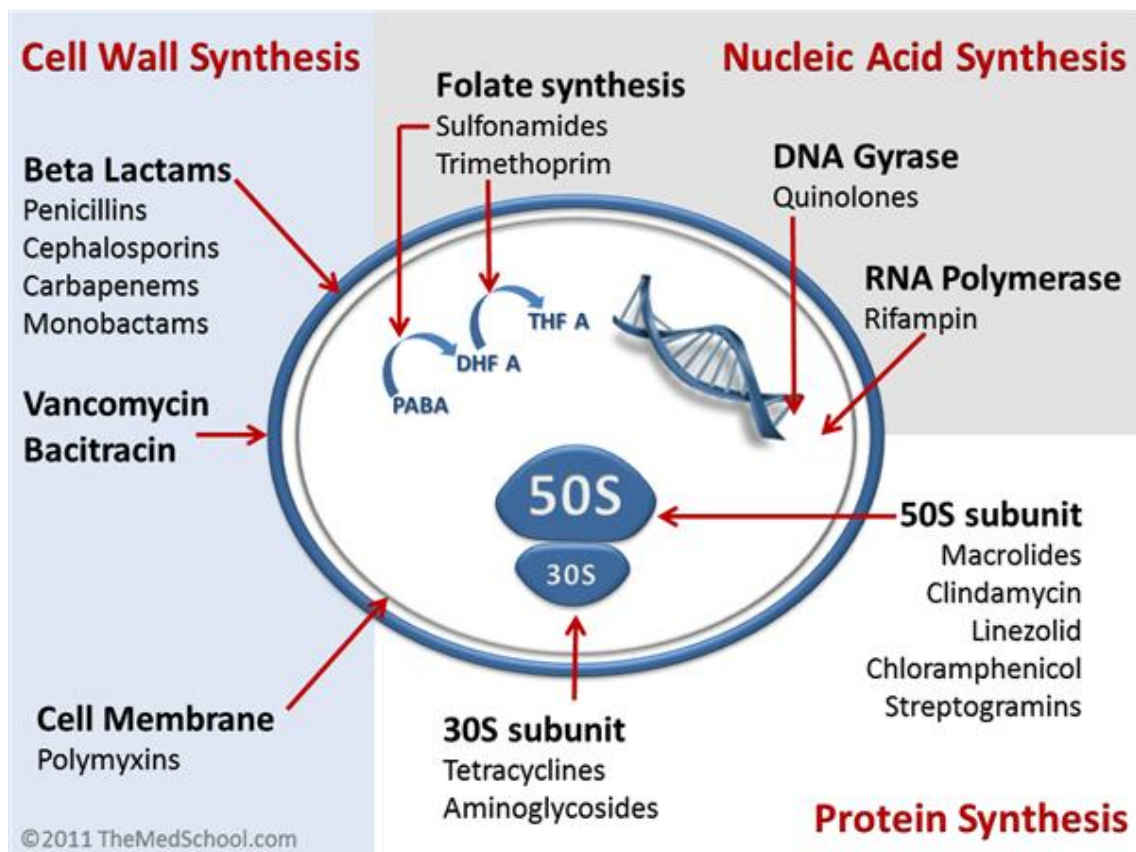
Figure 1: General Classification of Antibiotics by their targets. Figure is taken from http://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism 06/01/2014[3]

5.3. Adaptation and Genetic Diversity

Rivoire et al states that, there are three foundations that justify adaptations under the rules of natural selection:

1. Populations composed of individuals from diverse genetic backgrounds
2. These diverse characteristics associate with their fitnesses.
3. These characteristics should pass to the new generations. [4]

There are factors that facilitate adaptation process such as sexual reproduction, horizontal gene transfer, and mutation. Among these factors, Clune et al defines mutation as the ultimate source for diversification of genotypes. Thus to be able to understand the rate of evolution, the rate of mutation is an inevitable criterion [5].

Especially for prokaryotic species mutations are the major effectors that change the fitness and the surveillance of the organism.

5.4. Antibiotic Resistance
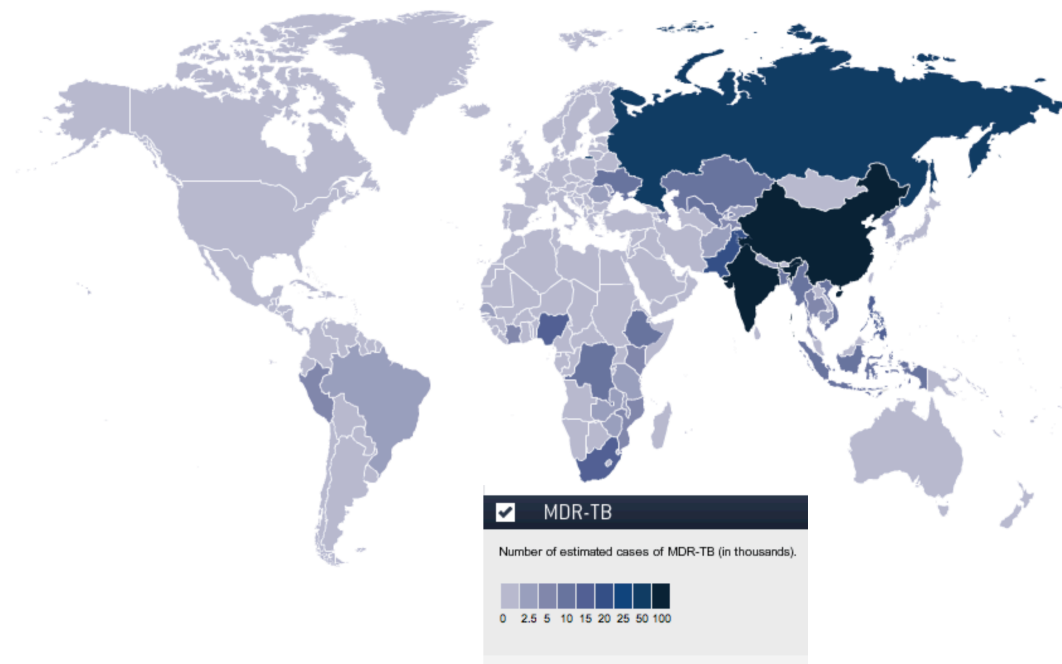
Figure 2: Number of Multi Drug Resistant Tuberculosis (TB) cases observed in world.

One of the fundamental features of living organisms is responding to an environmental or an inner signal. Right after the clinical usage of first antibiotics, bacteria started to respond this environmental stress and begin adapting this new environment. Though the resistance causing factors are differing among different species, there are 7 main factors that facilitate the tolerance of antibiotic stress.

1. Activated Specific/Non-specific efflux pumps that can control the outflow of antibiotics

2. Modifications in cell wall structures that restrict or block the influx of antibiotics. For example altered peptidoglycan structure found in Vancomycin resistant enterococcus (VRE).

3. Some species of bacteria have naturally insensitive target enzymes so they practically resistant to antibiotics. This case will be explained later in TMP resistance part.

4. Post-transcriptional or post-translational modifications may take place on target enzyme that make bacteria tolerate the antibiotic or decrease the effects of it.

5. Horizontal gene transfer of resistant protein or resistance cassette makes bacterium become resistant

6. Covering the environment with biofilm is also a big problem that makes bacterium get rid of the effects of antibiotics.

7. Last but the most important cause that makes bacterium resistant is to mutate regulatory or coding region of the target protein.

Last reason is the most problematic one between them because it makes not just one colony of bacteria resistant; this cause makes them have fitness advantage among other colonies. Thus, after certain amount of antimicrobial stress mutated bacteria become dominant among the ecosystem [6].
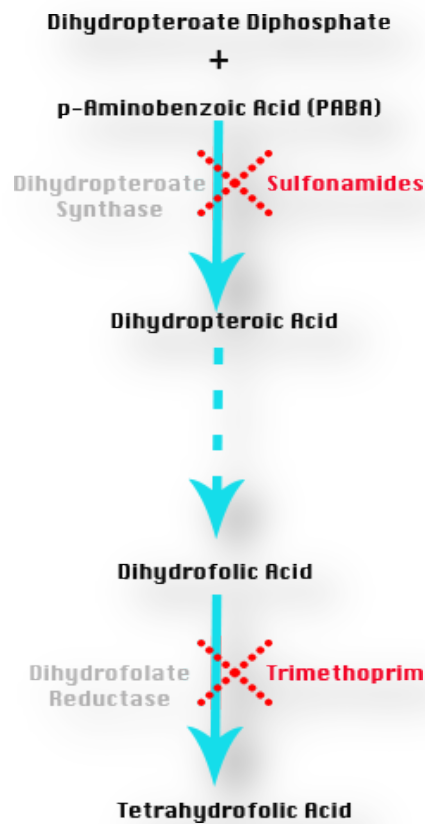
5.5. Folic Acid Pathway and DHFR

Figure 3: Folic acid pathway and targets of antibiotic found on this pathway

Studies on folic acid synthesis pathway are going back to observations of *Woods in 1940* [7]. Folic acid pathway is one of the most crucial pathways for synthesizing different kinds of cellular components in both eukaryotes and prokaryotes. For example, synthesis of purines such as thymine, and synthesis of aminoacids such as methionine, glutamic acid, and glycine are dependent to this pathway. What makes this pathway important is all the microorganisms and plant synthesize their folate through folate biosynthesis pathway but in mammals instead of just having this pathway; they also have folate pumps on their membranes. Mammals can bypass the folic acid pathway by just importing the folate from extracellular matrix through the specialized pumps. Because of its clinical and commercial importance in antibiotic market, most of the enzymes in this pathway are crystallized [8]. Folic Acid Biosynthesis Pathway has two main checkpoints controlled by two different classes of antibiotics.

1. The first reaction is catalyzed by Dihydropteroate Synthase (DHPS) can be blocked by Sulfonamides class of antibiotics;

2. Trimethoprim can block the last reaction, which is catalyzed by Dihydrofolate Reductase (DHFR). This project is focused on the enzyme DHFR because of its important role on TMP resistant bacterial evolution. In *E. coli* DHFR is one chained and 159 amino acids-containing enzyme.
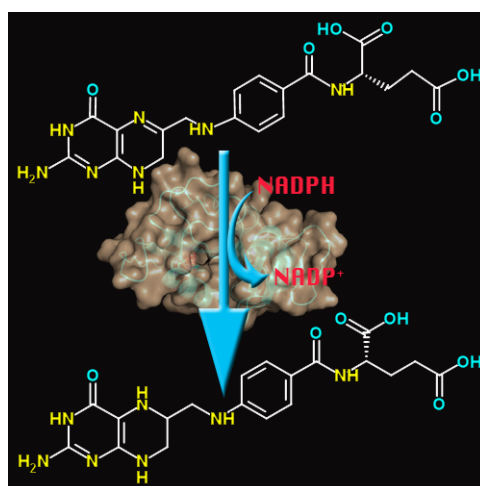
5.6. DHFR Enzyme Activity

Figure 4: Reaction catalyzed by Dihydrofolate Reductase. From Dihydrofolate to Tetrahydrofolate [9]

As shown in the pathway above, DHFR enzyme takes DHF as an input and gives THF as product. When we analyze the reaction in deep, there is a methyl group

shuttling occurs on DHF molecule (As shown in the right side of the figure). After this reaction THF can be further converted to Nucleic acids such as Thymine and certain amino acids like Methionine. In their JBC paper, *Appleman et al,* shows that on E. coli DHFR, 27th residue (Aspartic Acid) has an important role as active site [10]. D27 is interacting with DHF and helps catalysis of the reaction.

5.7. Trimethoprim: A Folic Acid Pathway inhibiting Antimicrobial Agent

Trimethoprim is a synthetic bacteriostatic antibiotic that targets on Dihydrofolate Reductase (DHFR) enzyme. This antibiotic first used successfully in a Proteus genus of bacteria in 1964 [11]. From that time to now, Trimethoprim is a commonly prescribed antibiotic either alone or combination with sulfamethoxazole (SMX) or co-methoxazole especially for the urinary tract infections. Since combination therapies with co-methoxazole later found that has side effects on bone marrows and lose its efficacy as antibiotic, this combination therapy is restricted in 1995 [12]. Unlike co-methoxazole trimethoprim (TMP-coMX) combination therapy, TMP-SMX combination therapy thought to be a better alternative and claimed that this drug combination via their synergistic effect is also decreasing the rate of evolution of resistant bacteria [13].

Trimethoprim has very high binding affinity to prokaryotic DHFR when compared to its eukaryotic ortholog[14]. When E. coli DHFR gene is blasted in non-redundant database against mammal proteins, the best alignment has the sequence identity as 30%. This affinity and sequence difference also makes trimethoprim, a good antibiotic candidate.

5.8. Resistance Mechanisms against Folic Acid Biosynthesis Pathway Inhibiting Antibiotics

Figure 5: DHFR enzyme 3D structures taken from different species. Top Line: *Bacillus anthrasis, Candida albicans, Mycobacterium tuberculosis,* Center: *Escherichia coli,* Bottom Line: *Gallus gallus, Mus musculus, Homo sapiens*

Although Trimethoprim resistance is a highly studied issue, the problem hasn't been completely solved yet. General pathways that are mentioned for becoming resistant to the antibiotics are also applicable for TMP. For example, *S. aereus* and *S. pneumoniae* have insensitive DHFRs in their metabolisms. Another defense mechanism found in P. aeroginosa, these gram-positive bacteria, has cell wall structure that doesn't let TMP enter the cell. The highest level of resistance is acquired by mutating DHFR gene and also in literature, *E. coli* cells which bears mutant folA gene, have resistance level up to solubility limit of TMP in media. In their paper, *Toprak et al,* explains how mutations occurring on regulatory and/or coding region of folA gene make insensitive *E. coli* cell against Trimethoprim stress [15]. Their paper is mainly focused on the genome. Though, TMP resistance issue has genomics causes, but because of competitive inhibition of DHFR, the main reason why cell become resistant is because

TMP can't stop the kinetics of the enzyme. In this project, main aim is to understand the resistance issue as protein stability and an enzyme kinetics problem.

5.9. Morbidostat

Morbidostat is a continuous bacterial culturing device to understand the evolutionary constraints of different stress conditions [16]. This device automatically allows us to monitor the growth of cultured species under different continuously changing antibiotic concentration with respect to resistance levels. Thus, besides the growth rate and drug concentration at a certain time period, resistance level at certain time can also be measured. How morbidostat works is simply illustrated in the figure taken from the *Nature Protocols paper of Dr. Toprak and his colleagues* [15, 16]. Bacterial growth is measured with detectors located in tube holders periodically such as in every second. If growth curve in a period exceeds the limit OD or slope of the growth curve in that period exceeds certain limits given by the user, machine adds stock antibiotic solution to the culture tube. Volume of culture is kept under control by taking excess amount of culture out of the tube regularly. Thus, by adjusting the type of antibiotic added, amount of antibiotic added, and upper-lower limits of growth; different stress conditions can be studied in morbidostat easily. There are also other potential applications for morbidostat, such as host-pathogen interactions, long-term adaptation experiments, or drug resistance in cancer cells.
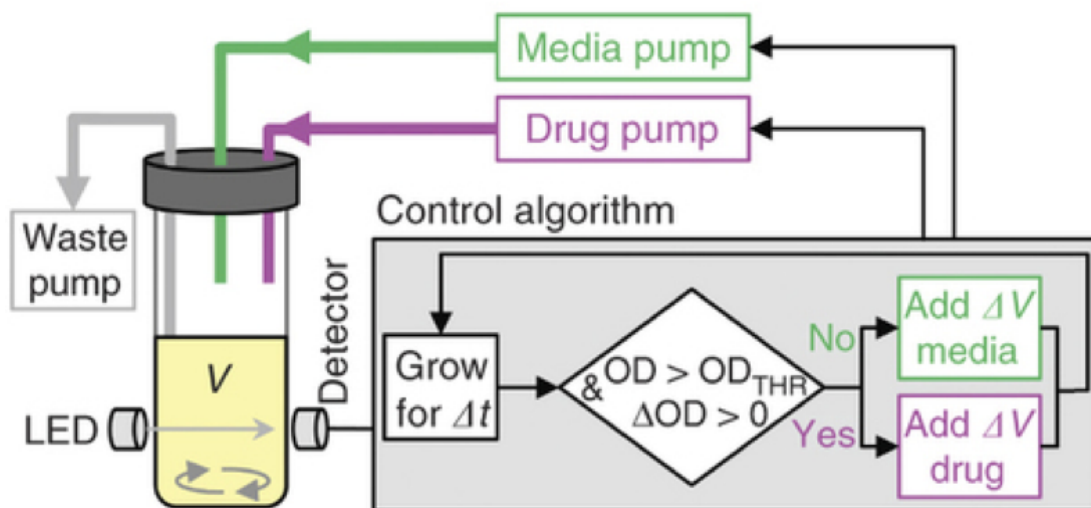

Figure 6: Controlling algorithm schema showed in *Toprak et al* paper. [15, 16]

5.10. Statistical Coupling Analysis (SCA)

Statistical Coupling Analysis (SCA) is a tool for showing the sparsely and contiguously spaced and interacted groups of aminoacids found as a blueprint of natural

proteins and these groups of residues are called sector [17-20]. This tool provides co-evolved residues of proteins that are important for function and proper folding of them [17-20]. Sector residues are spanning through the 20% of the protein and they are physically linked with each other. Recent studies on SCA showed that sector regions spanning through the protein makes a communicative network between allosteric and active sites of the protein so that mutations on one site of the proteins can be compensated with other mutations on the other site of the proteins. Thus folding and function can be regained appropriately [18]. *McLaughlin et al,* in their Nature paper explained analysis and the construction of the SCA matrices in detail. As a brief explanation of how SCA find evolutionarily driven residues is that SCA needs sufficient amount of multiple sequences from different sources of organisms and size of the alignment can vary by the question of interest. Then to construct the SCA matrices the conservations of each pairs are used with the normalization of randomized shuffling of these pairs on their multiple sequence alignment columns. After this normalization eigenvectors of each columns are calculated and graphed as color-coded matrix. This matrix is *n* by *n* and the *n* is the number of sequences aligned. There are some hotspots found on the SCA matrices that highly red areas showing the groups of residues coevolved with each other (sector sites). With changing the *n* and the sources of these *n* sequences, sector regions allow us to see insights of evolutionary architecture of proteins. For instance, it can be seen that some functionalities are conserved in some of the branches of organisms, also some structural patterns can be seen all the related proteins found in literature.

6. MATERIALS & METHODS

6.1. Buffers, Media Solutions and Preparations

M9 Media

M9 defined media is used for morbidostat experiments to decrease the artifacts coming from environment. Media is prepared with M9 media salts, 0.4% Glucose, 0.2% Protein Hydrolysate Amicase, 2mM $MgSO_4$, and 100uM $CaCl_2$.

TB Media

This media is used to grow the cells for protein purification and

protein synthesis induction. 900mL of this medium is 12g of Tryptone, 24g of Yeast Extract, 4mL of 99% Glycerol is mixed with 100mL of TB salts - 0.17M KH2PO4, and 0.72M K_2HPO_4- [21].

Ni-NTA Agarose Beads

For DHFR protein purification, Ni-NTA Agarose Beads were used (purchased from QIAGEN Firm).

Ni-NTA Binding Buffer

This buffer is used to bind the His6-tagged DHFR to the Nickel beads designed for protein purification. Buffer includes 50mM Tris-HCl, 10mM Imidazole, 0.5M NaCl. pH is adjusted to the 8.0.

Ni-NTA Elution Buffer

To elute the bound proteins from the Nickel Beads, this buffer is used and it composed of 100mM Tris-HCl, 400mM Imidazole, 1M NaCl. pH is adjusted to 8.0.

Dialysis Buffers for Kinetics Experiments

Dialysis process is important for DHFR enzyme kinetics because Imidazole in Binding and Elution buffers absorbs the light at 340nm like NADPH does [22]. Thus imidazole in protein solution has to be minimized before Kinetics measurements. Buffer designed for Kinetics experiments includes 50mM Tris-HCl, 300mM NaCl and 1% Glycerol. pH for this buffer is also 8.0.

Dialysis Buffer for Differential Scanning Calorimetry (DSC) Measurements

Tris-HCl buffer is very sensitive to the temperature changes. Thus, the dialysis buffer used for Kinetics experiments is not suitable for DSC experiments. Therefore, another dialysis buffer is used for stability measurements that composed of 10mM Potassium-Phosphate Buffer,

0.2mM EDTA, and 1mM β-Mercaptoethanol solutions with pH 8.2.

MTEN Buffer

For kinetics measurements MTEN buffer is used for Kinetics measurements of DHFR such as K_m, K_{cat}, V_{max}, K_I. Buffer includes 50mM MES, 25mM Tris-Base, 25mM Ethanolamine, 100mM NaCl. 5mM fresh DTT is added at the beginning of the assays. pH is 7.0.

Dihydrofolic acid (DHF) and NADPH solutions

25 mg of DHF is mixed with 10 mL of MTEN buffer (pH 7.0) and 35μL of β-Mercaptoethanol. Quantitation of DHF is done in A_{282}.

NADPH solutions are prepared with adding 8mg of NADPH powder into 1.5mL of pH7.0 MTEN Buffer. Concentrations are measured in NanoDrop© machine at 340nm. Both solutions are stored in -80 °C for further use.

6.2. Morbidostat Experiment Setup for TMP resistance

To be able understand the affects coming from the selection strength; two different selection environment is used in this study. First group has 7 different cultures and has the dilution rate of $0.6h^{-1}$ –a.k.a. strong selection- and the second group has 6 different cultures and their selection rate $0.3h^{-1}$ – a.k.a. mild selection-. In other words, for strong selection ~60% of the culture is changed with whether stock antibiotic solution or media and for the mild selection this rate is about 30%. To reach this dilution rates, strong selection has 60 seconds of drug or media injection; moreover, in mild selection injections last 30 seconds.

6.3. Morbidostat Replay Experiment Setup

To verify the results, morbidostat experiment is repeated with a single mutant as new parental strain. This regulatory site mutation is commonly popped up at the beginning of the first experiment (c-35t). In literature, this mutation is known to increase the expression of DHFR [23]. Setup for the experiment is the same as first one i.e. there are two different selection conditions called strong and mild and the experiments lengths are 5 days and after experiment single colonies are picked and sent

to sanger gene sequencing.

6.4. Sanger Sequencing and SNP analyses

Sanger sequencing is done with the help of Genewiz®. For this experiment more than a thousand colonies are picked and sequenced for their folA coding and its cis-regulatory region. To analyze the results, CLC Biology MainWorkbench and MacVector softwares are used. In these programs, one can easily align the query sequence to the reference WT folA sequence. After analyzing the alignment with respect to reference, SNP positions are determined and included in results section.

6.5. Heterogeneity or Diversity Calculations

After each day of the morbidostat experiment bacterial cultures are taken for further usage and analyzed to reveal the daily changes on the folA gene. These daily changes are shown as trajectories and diversity of the daily trajectories are calculated with the formula below:

$$Diversity\ Score\ (Entropy\ (H)) = \sum_{i=1}^{n} f_i \ln(f_i)$$

In this formula n represents the number of different genotypes; p,q,r are the ratios of the each genotype found after analyses of multiple sequences for each days. To make this more understandable lets make two different examples.

1. If there is only one mutant found after sequencing:

$$H = |(1 * \ln(1))| = 0$$

So there is no diversity in the environment.

2. There are three different mutants in one day and ratio for one of them is 50% and the other two are 25% each:

$$H = \left| \left(\frac{1}{2} * \ln(.5) + \frac{1}{4} * \ln(.25) + \frac{1}{4} * \ln(.25) \right) \right| = 1.03$$

Thus the diversity increased from 0 to 1.03.

6.6. Site Directed Mutagenesis and Colony Screening Protocol

To make new single mutant colonies, QuickChange® site directed mutagenesis protocol have been used. In this protocol, complete homologous primers are used and they just have one nucleotide changed from wild type to be able to make targeted

mutation. For this purpose, the mutated primers are designed and ordered from IDT DNA Technologies®. In this protocol, plasmids including the target gene are amplified with using mutated primers (forward and reverse). Then plasmids, which are already methylated, cleaved by an enzyme called Dpn1. What is specific for this enzyme is that this enzyme only recognizes a palindromic region that has methylated residues on it. Thus, newly synthesized plasmids cannot affect from incubation with enzyme. All the end products of this amplification and incubation processes are transformed in a plasmid compatible cell line and plated on a selective media plate. This protocol and reagents are found as a kit from Agilent Genomics firm but two of the Ranganathan lab members optimized the protocol for non-kit users as described above. Detailed protocols found in this reference [24].

Although incubation with enzyme cuts out all the WT plasmids, to make sure that plasmids have the intended mutation, colonies found on the selective plates are screened, and Sanger sequenced.

6.7. Plasmid Isolation Protocols (Boiling Mini Preparation Protocol)

To isolate mutant plasmids produced in QuickChange step, traditional phenol-chloroform plasmid isolation protocol is used. Detailed protocol for this part is found in the AddGene webpage. [25]

6.8. Homologous Recombination Protocol

Recombineering protocols are used in this step. In recombineering protocols, query gene is designed with having homologous arms in two ends so that when bacteria started to polymerize the DNA query gene is also amplified and added into the genome of interest. Detailed protocols are found in the references [26-30].

6.9. Protein Purification Protocol

Purification of the protein is necessary for making biochemical assay and the purer the protein, the better the results. To make this happen *pet24a* plasmid is used. This plasmid has T7 promoter, lac operator and his tags on it. Thus when induced with IPTG (inducer of lac operator), folA gene found between his tag and T7 promoter is expressed in high amount at lower temperatures of incubation. To purify the proteins

Ni-NTA Agarose beads are used (Ordered from QIAGEN). These beads have high affinity to bind His-tags; hence high purity can be achieved with these steps.

6.10. Enzyme Affinity Assay and $K_{m,}$ V_{max}, K_{cat} calculations

For these measurements GraphPad Prism© software is used. In this software, first one-fourth of the A340 vs. Time data coming from Spectrophotometry instrument is nonlinearly regressed and slope values are used for Michelis Menten Kinetics calculations. This software has the feature for the data needs for nonlinear fitting such as Michelis-Menten curves. Kcat values are calculated by following formula:

$$\frac{Vmax}{\epsilon_{NADPH}} = Kcat * [Enzyme]$$

$$Kcat = \frac{V_{max}}{[Enzyme] * \epsilon_{NADPH}}$$

$$\epsilon_{NADPH} = 12.300M \ cm^{-1}$$

6.11. Enzyme Stability Assay

For this assay Differential Scanning Calorimetry is used. Main logic in this assay is to cover a wide range of temperature interval to monitor the required enthalpy to stabilize the temperature of the cell. In this assay no inhibitor used. Stabilities of empty proteins (WT and mutant counterparts) are measured.

7. RESULTS

7.1. Morbidostat Experiment Results

After 28 days of evolution experiments, all the OD growth graphs are collected and linked end to end. To briefly explain the working mechanism of morbidostat, figure below added. Every 18 mins, controlling algorithm runs and decides which pump to open separately for each culture and adds media, antibiotics or neither. Green circles show where media pump opened; red circles show where low concentrations antibiotic injection started, and purple circles show where high concentration antibiotic injection started. Respectively triangles showing the closing of each pumps. All opening and closing are controlled by algorithm explained in deep in *Toprak et al.* Nature Genetics and Protocols papers [15, 16]. Red and Purple Line shows the OD point where

respective antibiotic addition starts. After certain amount of time bacteria start to become resistant to antibiotic concentration added and growth start not to influence from that concentration of antibiotic addition. Continuous average OD increase between 120th and 130th hours coming from this resistance level increase. But after highly concentrated TMP addition –shown as purple pump opening-, population size is highly shrunk. Next figure series are showing the whole experiments done for all 7 strong and 6 mild selection replicates.
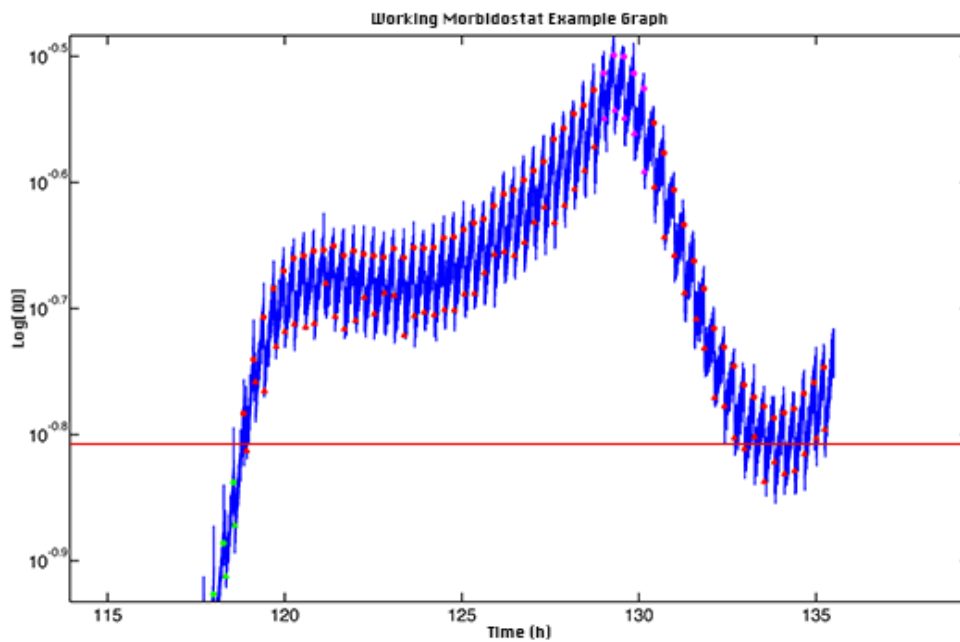
Figure 7: An example time interval of working Morbidostat experiment. Circles are showing the pump openings and triangles underneath each circle show when respective pump is closed.
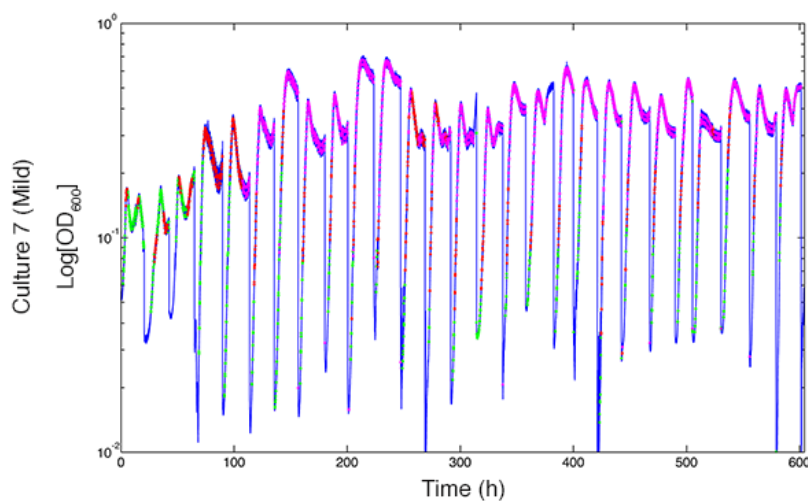
Figure 8: Whole experiment shown for one culture of Mild Selection
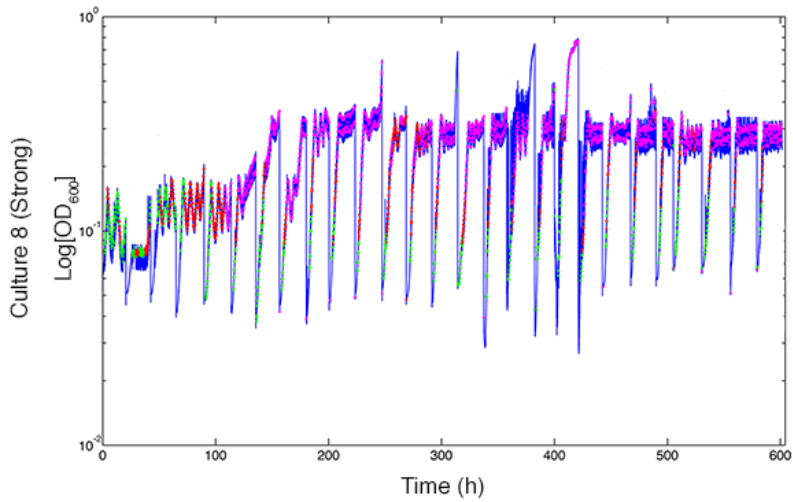
Figure 9: Whole experiment shown for one of the strongly selected cultures.

Whole experiment graphs for other 11 cultures are put in Appendix. By analyzing the fluctuations in trimethoprim concentrations in the culture tubes, bacterial resistance levels are monitored. Graphs below show changes of trimethoprim in time course for one mild and one strongly selected culture.
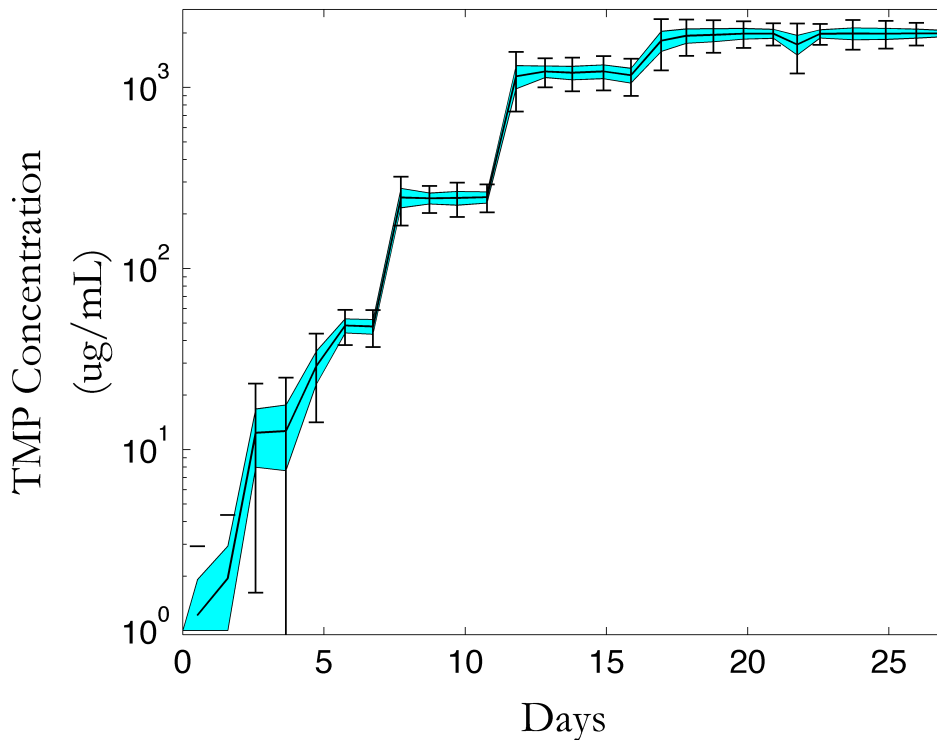
Figure 10: Mildly selected colonies generally show this stepwise increasing pattern.
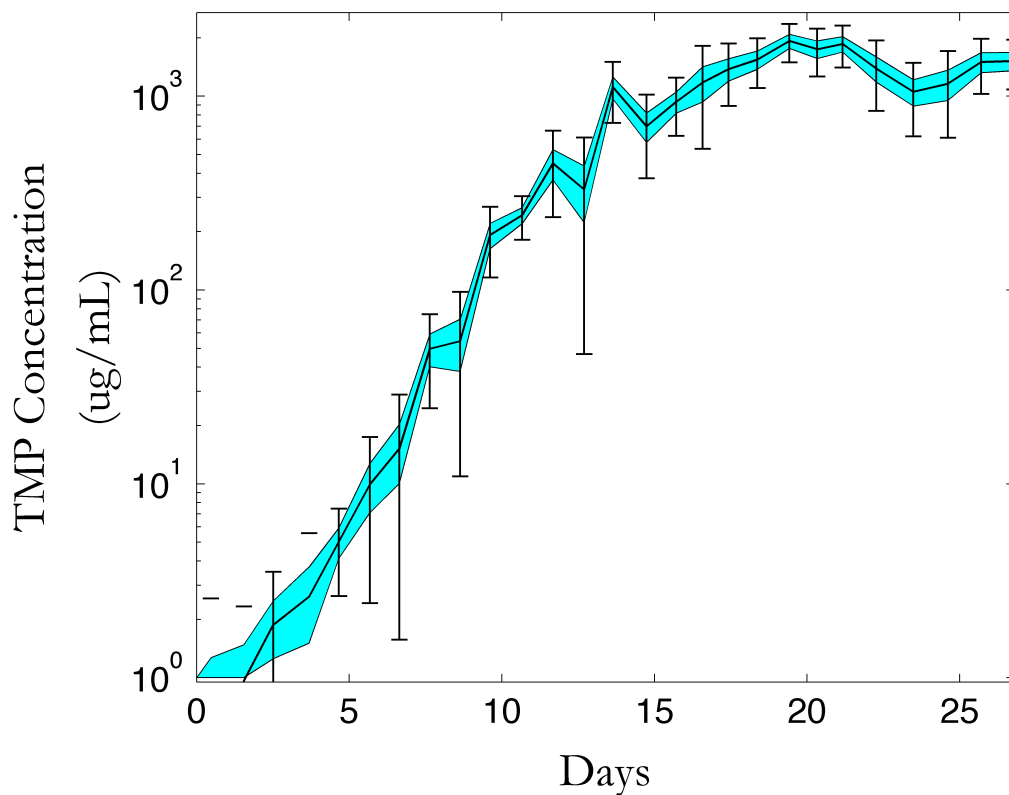
Figure 11: Clonal interferences revealed in strong selection make stepwise pattern disappear. Thus, resistance levels are increasing sharper in strong selections.

Change in TMP concentrations for other colonies are put in Appendix. Also to see the general picture for the TMP concentrations next graph is plotted. This graph shows change in TMP concentrations by selections. It is clearer to see that in mild selection populations have been increased their resistance levels in more than one-step. Unlike mild selection, in strong selection conditions daily fluctuations of trimethoprim is sharper.
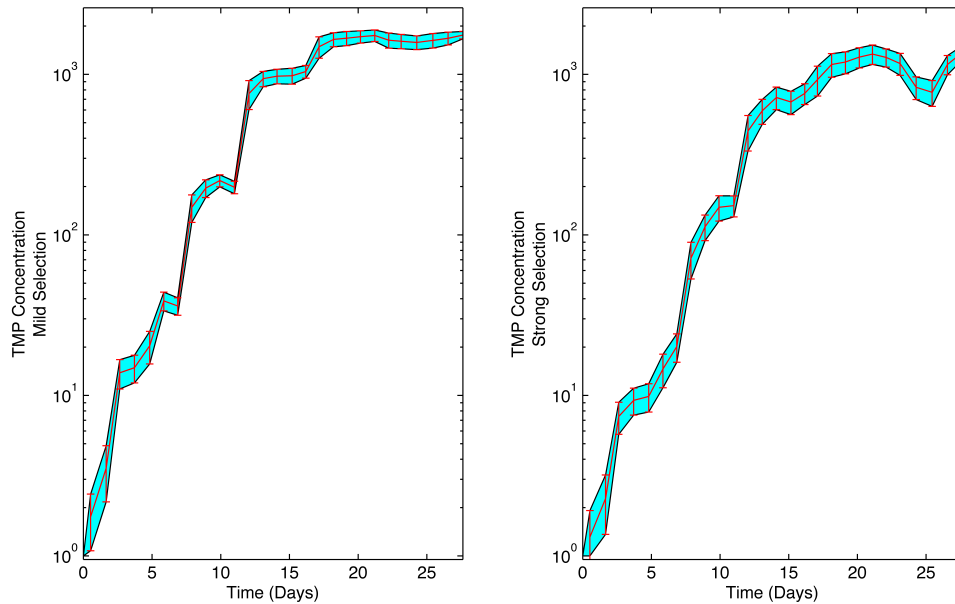
Figure 12: Left graph shows the TMP change in mild selection and the right graph shows the strongly selected colonies.

Growth rates and changes in drug concentrations are calculated with using adjacent pump closings and openings. All figures for growth rate changes shown for both selections and error bars show standard deviations between the cultures.

7.2. Growth Rate Measurements of Daily Samples

Growth rates for daily samples are also measured with TECAN® that is a specialized instrument for 96 well plate growth readings. Graphs are plotted with respect to selections. Red Circles indicates the growth rate at a certain day for strong selection and the blue circles are showing the mild selection. Results show that in both selections growth rates of mixed populations daily taken from the experiment is not affected and the values are similar for both selection conditions. It is important to note that fluctuations seen as error bars in strong selection is bigger than mild selection. Reason for these bigger fluctuations is tried to explain further in discussion section.

26
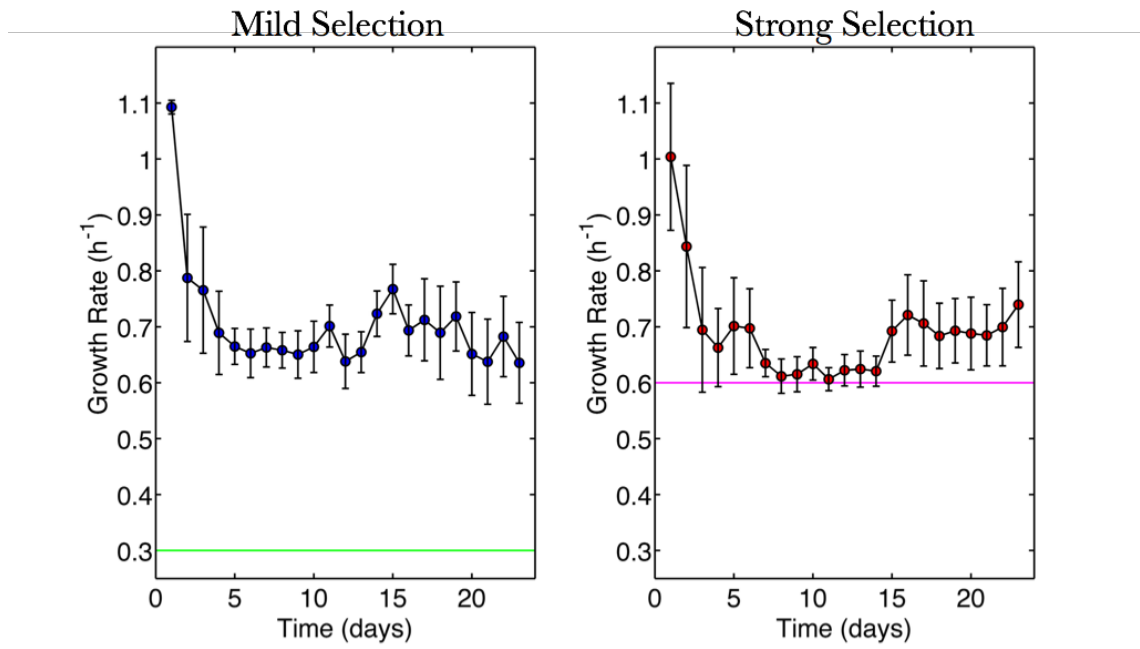
Figure 13: Growth Rates for daily mixed populations measured in 96 well plate reader instrument. This experiments are done in TMP free conditions.

7.3. Sequencing Results and Mutation Trajectories
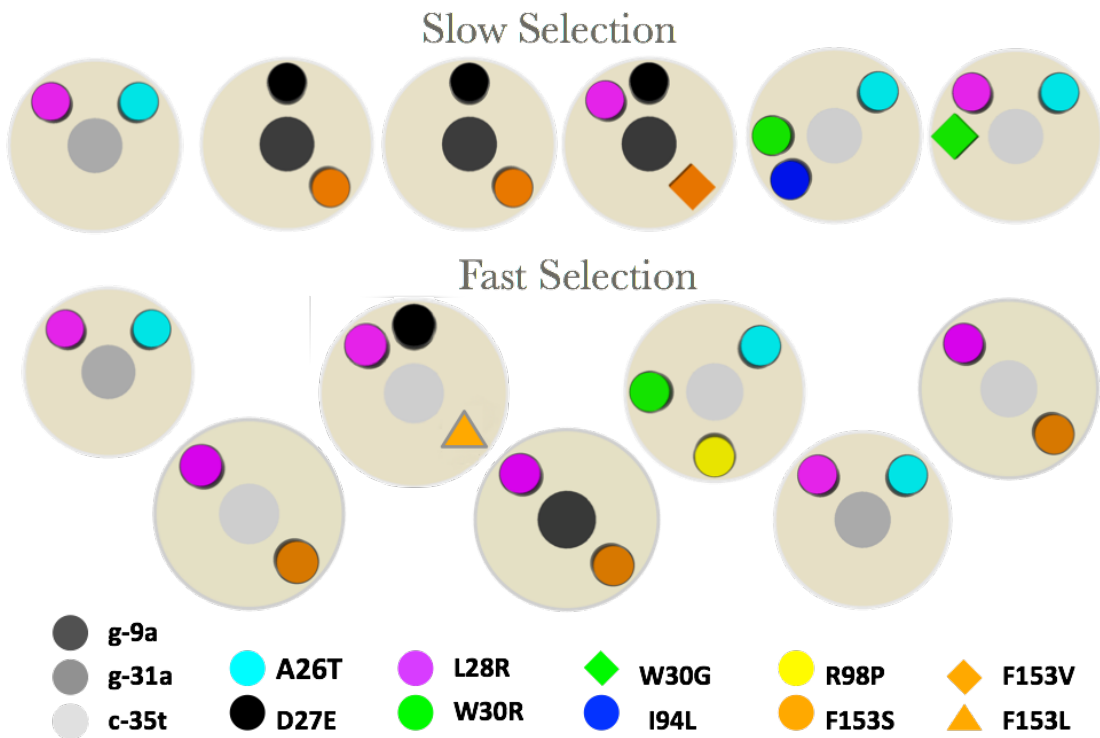
Figure 14: Mutations found on cultures on final day of experiment.

Throughout the Morbidostat experiment, to avoid biofilm formation at the walls of the culture tubes, after each day small amount of culture (~100 µL) taken and diluted in fresh media and experiment continued for next day. Also mixed populations are taken and stocked in 50% glycerol for further use. After the experiment, these daily mixed populations are plated and 4-12 single colony sequenced for each day and each culture. Totally ~1500 single colony is Sanger sequenced for their *folA* promoter and coding region in this study. To show all the sequencing results special cylinder graphs are used and these trajectories shows the mutations gained in time course. Starting from the left first rectangle days are shown on the base part of the graphs. Each big cylinder shows a genotype shown on that day. Top of the each cylinder there are circles on the center and cylinders at the periphery. Central circle shows the promoter mutations and peripheral cylinders shows the coding region mutations. Each radial angle is specific for a mutation. Also these peripheral cylinders are shown like pie charts i.e. colored part of the pie chart shows the percentage of the single colonies seen as that genotype. Also one of the cultures acquired two promoter mutations at the same time and this genotype is plotted as diamond on top of the central circle.
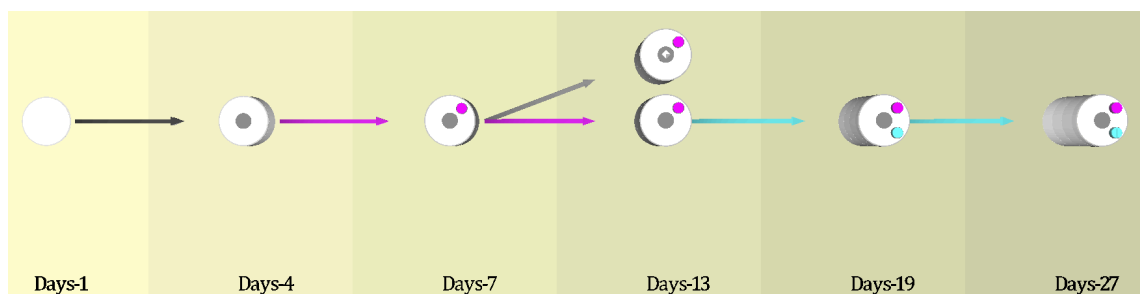
Figure 15: Genotypic Diversity found in first culture in mild selection

When we look at the Figure 13, diversity seen only on day 13 and it lasts just 1 day after the day 13, all the colonies seen has the same genotype till the end of the experiment.

Figure 16: Genotypic Diversity found in second mildly selected culture
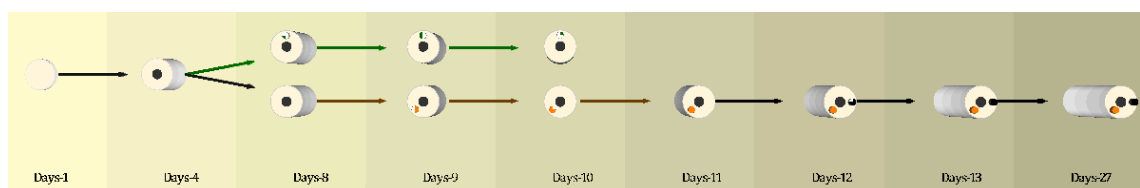
Also for the other mildly selected culture diversified state continues only for three days.
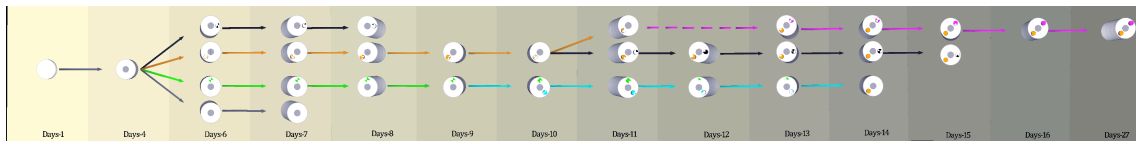
Figure 17: Genotypic Diversity seen in of the Strong Culture
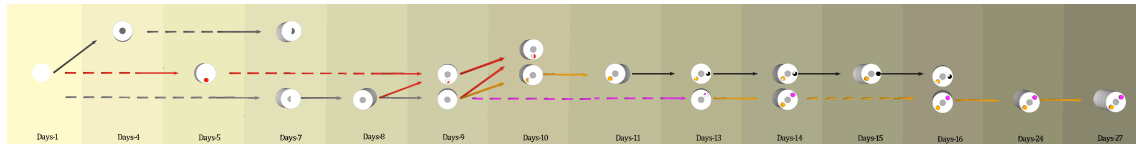
Figure 18: Clonal Genotypic Interference found in other strongly selected culture
Cultures of the both selections there are significant difference in diversity of genotypes and the duration of these diversities are more common in strongly selected cultures than mildly selected cultures. These graphs are plotted in VPython module of Python language. Scripts for these plots can be given with request. Genotypic Diversity Graphs for all other cultures are found in appendix part.

7.4. Diversity Plots

To quantitate the diversity found on genotypes of cultures, F statistics are optimized for haploidic genotype structure of bacteria. Detailed explanations on Diversity scores can be found in Materials & Methods section. Diversity scores for each culture are plotted and added below.
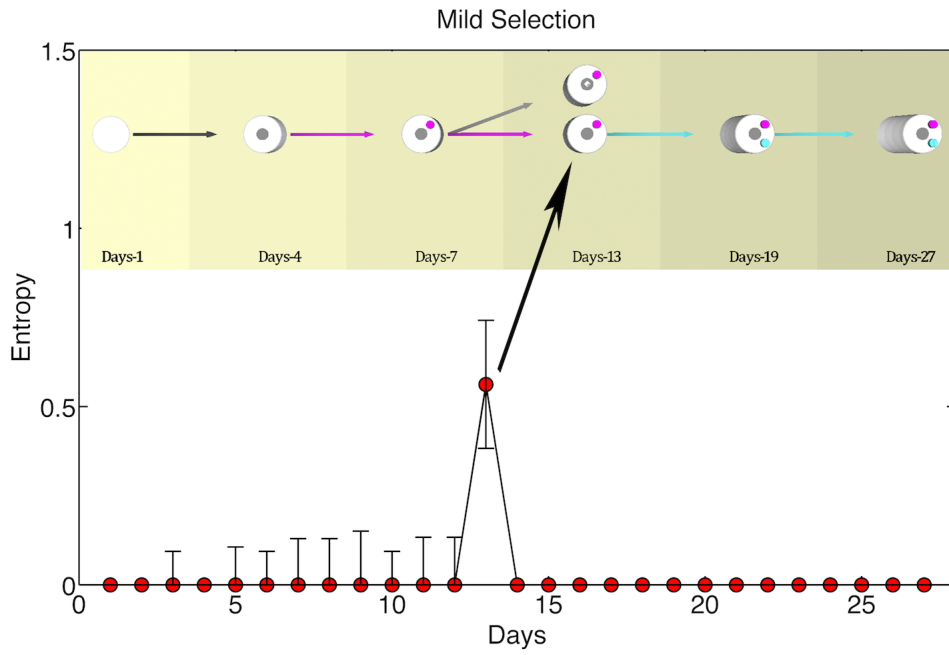
Figure 19: Plot shows the Diversity score for each day of the experiment. Arrow shows the day of diversity found in mild selection culture.
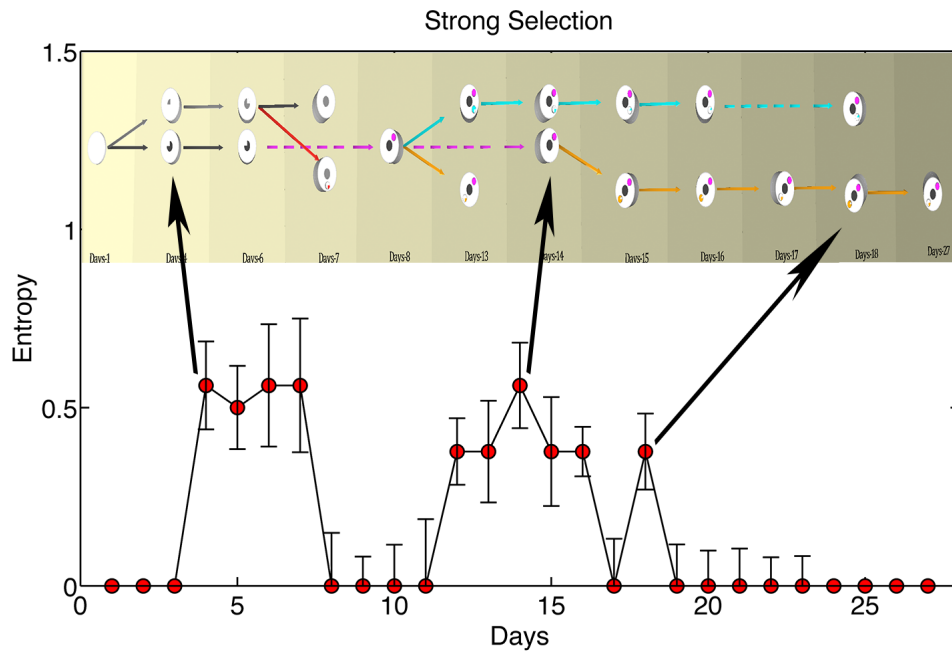
Figure 20: Plot shows the diversity and cylinder graph shows the genotypes found on that day.
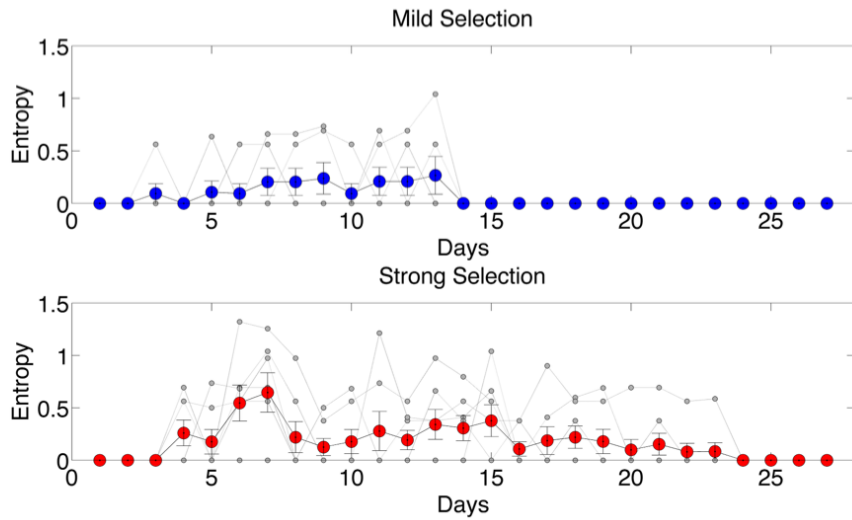
Figure 21: Diversity scores by selection strength

These two figures show the diversities as groups of selections and the error bars show the standard errors of diversity scores in days. Straight line in both figures, show the mean diversity score for that selection. This graph apparently shows the score for diversity is really high in strong selection. Also diversity continues to day 14 in strong selection but in strong selection it lasts one more week to genotypically stabilize the population.
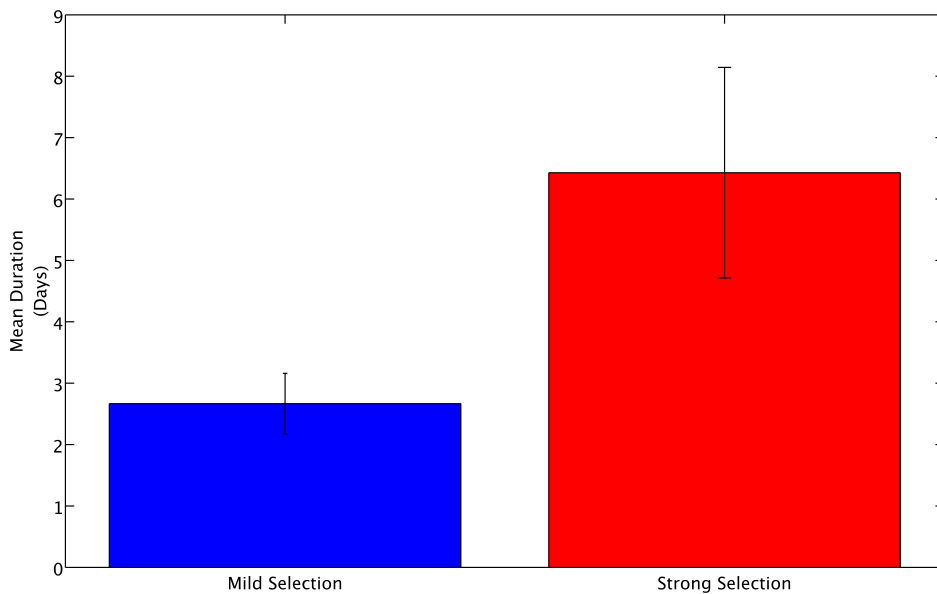
Figure 22: Durations for diversified populations

Lastly, the durations of genotypic diversities found on selections are compared and this bar graph is plotted for this purpose. Error bars on these histograms show the standard errors in replicates in selections. As a result, cultural diversity in strong selection surprisingly more common and lasts longer than mild selection conditions.

7.5. Whole Genome Sequencing (WGS) Results

Each culture final days are sent WGS and results revealed that main cause for TMP resistance is coming from the mutations on folA gene regulatory or coding site. Detailed table for WGS results added as Appendix.

7.6. Biochemical Assays on Single Mutant DHFRs

Results of these genomic studies showed that amount of DHFR and whether the change of stability or the change on catalysis rate of enzyme is important to become TMP resistant. Hence, to understand the biochemical changes found on DHFR enzyme, single mutant genes are made and expressed in *E. coli* cells. After purification of the single mutant enzymes, biochemical assays below are done.

Mutant Name	K_m (nM)	K_{cat} (1/sec)	K_{cat}/K_m (1/sec.mM)	Fold Change
WT	1.233	0.589	477.697	1.000
A26T	3.880	2.359	607.990	1.273
P21L	3.994	3.567	893.090	1.870
W30R	8.731	10.496	1202.153	2.517
I94L	2.736	4.870	1779.971	3.726
W30G	1.986	4.095	2061.934	4.316
W30C	1.847	5.564	3012.453	6.306
L28R	0.163	1.415	8702.337	18.217

Table 1: Binding affinity and Catalysis Rate values for DHFR enzyme single mutants

Details about measurements are explained in Materials & Methods section. Reaction catalyzed by DHFR enzyme occurs really fast and all mutants are analyzed by their Km values for substrate of the enzyme (Dihydrofolate-DHF). Figures showing the catalysis rates are put in the appendix. Here is the table showing the results of Binding affinity and catalysis measurements.

Binding affinity assays also gives the V_{max} for the enzyme. Thus, turn over numbers for the enzymes can be calculated. But to assess the differences of catalysis K_{cat}/K_m is a better measure used in literature.

As listed on the table above, catalysis of DHF molecule can be increased up to ~20 fold. These results reveal that increase in catalysis rates is one of the reasons that help to become resistant.

7.6.2. Effects of TMP on Catalysis
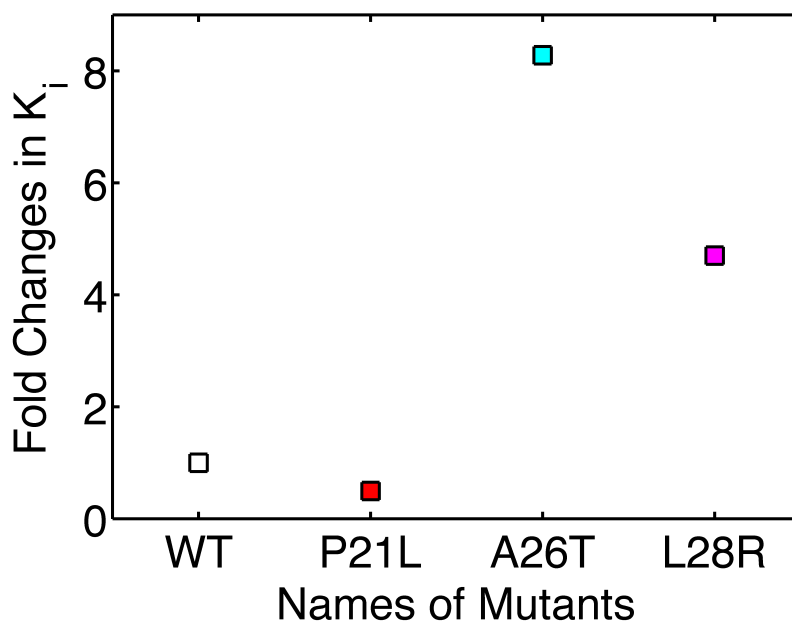
Figure 23: This figure shows the fold change in affinities of DHFR single mutants to antibiotic TMP.

Figure above indicates that A26T and L28R mutations decrease the affinity for TMP so that these proteins are more insensitive to TMP when compared to wild type protein. Interestingly, P21L mutation slightly more sensitive to TMP; although, this situation explains why there are no P21L mutations seen at the end of the experiments, why this mutation is repeatedly acquired in different cultures and different days of experiment is still unknown.

7.6.3. Protein Stability Results

As explained before, stability of the proteins are measured in Differential Scanning Calorimetry instrument. T_m values of the mutant proteins generally have similar values except W30R and W30G. These results give a brief intuition why W30R and W30C mutation bearing cultures are eliminated by L28R mutation bearing clones in culture 14 (See appendix for this result). Also when they compete with the other mutations generally they acquire other mutation to make conditions even. Thus they can survive.
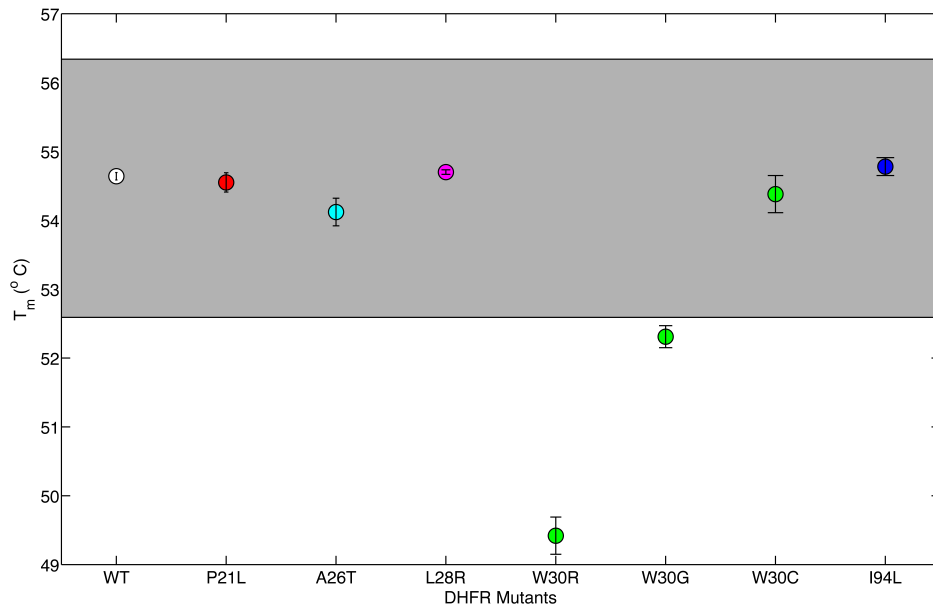
Figure 24: Stabilities of Mutant Proteins are compared with *wild type.* Gray area shows all standard deviation of database of mutations. Error bars are coming from the individual fits.

8. DISCUSSION

8.1. What have been learned from Morbidostat results?

Morbidostat result graphs for each culture shows that in mild selection conditions there are stepwise increase in resistance levels unlike the sharp increase in strong selection conditions. Also when drug concentration changes are analyzed in both selections, there is a distinct pattern. For strong selection, drug concentration increase occurs immediately after pumps open and population size is shrunk and later injections decrease the concentration of drug in culture immediately. On the other hand, in mild cultures, drug concentration changes slowly. When the scope is turned to molecular picture, Trimethoprim is specifically affects the regulation of folA gene. When all the final genotypes are investigated only three-four folA mutations are enough for bacterium to become completely insensitive to antibiotic. To understand the mechanisms against the selection strengths further experiments are needed. Since in both selection conditions, growth rates are not completely affected (see figure 12). It is

hard to completely compare and contrast the big picture with this two dilution factors. In fact, to completely understand the affect of short but dense pulses of antibiotic injections at least dilution factor' of 0.8 is needed to be done and analyzed. Also for the mild selection conditions to be able to compare and see complete difference of long sparse pulses i.e. lower dilution factors such as .2 or .15 have to be done and analyzed.

8.2. Why the mutation trajectories are changing in different selection conditions?

Although, final day results are not showing any difference in different selection strengths, progression for becoming resistant is highly different between selection strengths. As shown with the cylinder graphs, diversities among the strong selection cultures are more common than mild selection cultures. These results are kind of unexpected because when the dilution factors are thought, in each hour, strongly selected populations lose their ~60% of their population size unlike this number is ~30% in mild selection conditions. Thus, an expected result for this experiment was the exactly the opposite; however, drug concentration changes in strong selection conditions are highly dynamic that the concentration increases sharply and decreases in an instant. Dynamic changes found in strong selection environment doesn't let the different genotypes to compete and stabilize in one genotype. On the contrary, mild selection conditions are changing slower and genotypic interference for different bacteria are diminished because of the difference in fitness of these different genotypes. Also in mild selection conditions, bacteria that have higher fitness have time to dominate the environment and sequencing results just shows that one genotype in most cases. Moreover, to prove this concept, single DHFR mutants are necessary and their growth rates' had to be measured. This part of the project is work in progress.

8.3. Orders of Mutations on DHFR related to TMP resistance

To understand the response of the bacteria chronologically under TMP stress, it is needed to generalize the mutations acquired by bacteria. 12 out of 13 cultures first mutation acquired is on the promoter mutation. That mutation is required for overcoming the affects of TMP. The simplest strategy for overcoming the competitive inhibition effect, bacteria increase the number of DHFR protein with promoter mutation. There are many both clinical and basic science studies showing the c-35t mutations that we also seen is increasing the amount of DHFR expressed in the cell [23,

31]. This first mutation doesn't have a direct effect on protein structure and function. Only the amount of mRNA and protein is changing. Since environment is overwhelmed with high concentrations of TMP, bacterium has no choice to mutate the coding part of the gene. Albeit, these whole processes occurs randomly, fitnesses of these promoter mutants are not enough to dominate the environment or the inhibitory effects of high concentrations of TMP starting to become enough to kill the whole population. Hence, bacteria repeatedly chose to mutate some residues that they are more related to SCA or sector positions on DHFR. 4 out of 5 first popped up mutation is on the sector position. But after first mutation, it is very hard to predict the location of next mutation. When sector regions are extended with the secton positions on the DHFR protein, almost all the antibiotic resistance related mutations are occurring on these regions. This hypothesis is tested with Two Tailed Fisher's Exact Test, albeit, p value is slightly higher to .05, if we can achieve to increase the database of both sector regions of resistance related proteins and the mutations acquired to become resistant this value will be lowered and become significant. To further understand the nature of the resistance related mutations biochemical properties of these single mutant proteins are examined.

8.4. What are the effects of single mutations on DHFR activity?

DHFR is an essential enzyme for *E. coli*. After examining 7 single mutant by their K_m and K_{cat} values, results show that other than L28R mutations, mutant proteins decrease their affinity to bind DHF. Despite their increased K_m values when compared to wild type protein, all mutations have higher catalysis rate (K_{cat}/K_m) than wild type. These results explain why these mutations are acquired and stayed. Since their catalysis rates are higher than wild type, they can endure the reaction catalyzed by DHFR at higher concentrations of TMP. In this part also there are missing experiments, for example, biochemical assays for newly found mutations in our Morbidostat experiment has to be done. What we have now in our hand is also briefly says that mutations like A26T is not good at both protein stability and binding affinity but this mutation decrease the affinity of DHFR to TMP up to 8 fold. Thus, this explains why bacteria generally acquire A26T mutations through the end of the experiment because desensitization of DHFR is more important because TMP concentration is gone really high. This observation is also valid and seen in *Toprak et al Nature Genetics* paper. L28R mutation was a very strong mutation found in 9 out of 13 cultures, is also biochemically-desired mutation. Since the catalysis rate of L28R mutation is about 20 fold higher than the wild type protein and

the affinity of this mutation to TMP is about 5 fold lower than wild type. Thus having L28R mutation is highly possible and highly advantageous for bacterium. Also protein stability data showed why W30R mutation found in culture 14 is dominated with L28R is the stability issue. Though, W30R mutant has higher catalysis rate about 2.5 fold, it has lower stability values than the other mutants that are analyzed, thus this mutation is replaced with other mutations in mixed populations. When biochemical assays for the other single and some interesting double mutants are analyzed our understanding of resistance related mutations would be increase.

9. CONCLUSIONS

9.1. Morbidostat Results and Implications

Morbidostat experiment shows us that wild type bacterium is not really far away from being completely insensitive to TMP. One promoter and two-three coding region mutations on DHFR are making bacterium resistant to trimethoprim up to its solubility level. This study is important to understand the effects of environmental changes such as dilution rates cause differences in diversity of populations genotypes. Pulse rate, length and the concentrations are important in population genetics of the bacterial culture. For instance, short but high concentration pulses of antibiotic injections preserve diversity more than long and sparse pulses. Main logic behind this observation is long and sparse pulses makes high fitness bacteria to dominate the environment and stabilize the genotypic diversity. To understand the diversity, an optimization of F statistics is used.

9.2. Orders of Mutation and Further Understandings

Other than general Morbidostat results, genomic studies and biochemical studies revealed that main cause for TMP resistance is mutations acquired on folA gene regulatory and coding region. Generalizations on these mutations and their locations showed us that first rule for overcoming the competitive TMP resistance is acquiring promoter mutations. Second rule is to mutate the sector regions so that function and the folding of the protein can change and makes TMP an undesired mimicking molecule. After second rule, epistasis of genes have important role that needs to be understood further. Since biochemical observations are preliminary we can only say that bacteria

wants to overcome the stress with changing the biochemical parameters of DHFR such as K_m, K_{cat}, Stability.

10. FURTHER WORKS

10.1. Mutant genes and their impacts on cell

To understand the fitness effects of each mutation on DHFR, mutations are homologous recombined and put into the genome. Thus all the effects of mutant proteins can be monitored by the means of fitness and growth rate. Also their resistance levels against TMP and cross-resistance levels against other antibiotics would be assessed. These allow us to understand whether there are some cross talks between the folate pathway and the other antibiotic resistance related pathways. Also is there any other missed cause for TMP resistance other than mutations on folA.

10.2. Single and Double mutant proteins and their activities

Single mutant proteins that are novel literature, we couldn't have time to express and measure their activity. Also some of the double mutant proteins would be interesting to study and understand the evolution of antibiotic resistance. For example, after characterization of each single mutant we may choose one highly desirable and one highly defective mutation and make double mutant protein. These allow us to find out the rules of epistasis DHFR have and what makes natural selection to acquire these mutations on top of each other.

11. BIBLIOGRAPHY

1. Arnvig, K.B. and F. Werner, *A new spanner in the works of bacterial transcription.* Elife, 2014. **3**: p. e02840.
2. Hamad, B., *The antibiotics market.* Nat Rev Drug Discov, 2010. **9**(9): p. 675-6.
3. Orthobullets. *Antibiotic Classification and Mechanism.* 2013 10.23.2013 [cited 2014 06.01.2014]; Available from: http://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism.
4. Rivoire, O. and S. Leibler, *A model for the generation and transmission of variations in evolution.* Proc Natl Acad Sci U S A, 2014.
5. Clune, J., et al., *Natural selection fails to optimize mutation rates for long-term adaptation on rugged fitness landscapes.* PLoS Comput Biol, 2008. **4**(9): p. e1000187.
6. Huovinen, P., *Resistance to trimethoprim-sulfamethoxazole.* Clin Infect Dis, 2001. **32**(11): p. 1608-14.
7. Woods, D.D., *The relationship of p-aminobenzic acid to the mechanism of the action of sulphanilamide.* Br J Exp Pathol, 1940. **21**: p. 74-90.
8. Bermingham, A. and J.P. Derrick, *The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery.* BioEssays, 2002. **24**(7): p. 637-648.
9. Sawaya, M.R. and J. Kraut, *Loop and Subdomain Movements in the Mechanism of Escherichia coli Dihydrofolate Reductase: Crystallographic Evidence†,‡.* Biochemistry, 1997. **36**(3): p. 586-603.
10. Appleman, J.R., et al., *Role of aspartate 27 of dihydrofolate reductase from Escherichia coli in interconversion of active and inactive enzyme conformers and binding of NADPH.* Journal of Biological Chemistry, 1990. **265**(10): p. 5579-5584.
11. Cooper RG, W.M., *SUCCESSFUL TREATMENT OF PROTEUS SEPTICAEMIA WITH A NEW DRUG TRIMETHOPRIM.* Med J Aust., 1964. **2**: p. 93-96.
12. *Co-trimoxazole use restricted.* Drug and Therapeutics Bulletin, 1995. **33**(12): p. 92-93.
13. Brogden, R.N., et al., *Trimethoprim: A Review of its Antibacterial Activity, Pharmacokinetics and Therapeutic Use in Urinary Tract Infections.* Drugs, 1982. **23**(6): p. 405-430.
14. T.D., N.H.C.G., *Antimicrobial Chemotherapy in Medical Microbiology.* 1996, Galveston TX.
15. Toprak, E., et al., *Evolutionary paths to antibiotic resistance under dynamically sustained drug selection.* Nat Genet, 2012. **44**(1): p. 101-5.
16. Toprak, E., et al., *Building a morbidostat: an automated continuous-culture device for studying bacterial drug resistance under dynamically sustained drug inhibition.* Nat Protoc, 2013. **8**(3): p. 555-67.
17. Reynolds, K.A., et al., *Chapter Ten - Evolution-Based Design of Proteins*, in *Methods in Enzymology*, E.K. Amy, Editor. 2013, Academic Press. p. 213-235.
18. Reynolds, K.A., R.N. McLaughlin, and R. Ranganathan, *Hot spots for allosteric regulation on protein surfaces.* Cell, 2011. **147**(7): p. 1564-75.
19. McLaughlin, R.N., Jr., et al., *The spatial architecture of protein function and*

adaptation. Nature, 2012. **491**(7422): p. 138-42.

20. Halabi, N., et al., *Protein sectors: evolutionary units of three-dimensional structure.* Cell, 2009. **138**(4): p. 774-86.

21. *Terrific Broth.* Cold Spring Harbor Protocols, 2006. **2006**(1): p. pdb.rec8620.

22. Johnson, A.R. and E.E. Dekker, *Woodward's reagent K inactivation of Escherichia coli L-threonine dehydrogenase: increased absorbance at 340-350 nm is due to modification of cysteine and histidine residues, not aspartate or glutamate carboxyl groups.* Protein Sci, 1996. **5**(2): p. 382-90.

23. Al-Shakfa, F., et al., *DNA Variants in Region for Noncoding Interfering Transcript of Dihydrofolate Reductase Gene and Outcome in Childhood Acute Lymphoblastic Leukemia.* Clinical Cancer Research, 2009. **15**(22): p. 6931-6938.

24. Technologies, A. *QuickChange II XL Site-Directed Mutagenesis Kit.* 2013 [cited 2013 05/10/2013]; Instruction Manual]. Available from: http://www.chem.agilent.com/library/usermanuals/Public/200521.pdf.

25. Addgene. *Recovering Plasmid DNA from Bacterial Culture.* 2014 [cited 2014; Available from: http://www.addgene.org/plasmid_protocols/purify_plasmid_DNA/ - phenol.

26. Thomason, L.C., N. Costantino, and D.L. Court, *E. coli genome manipulation by P1 transduction.* Curr Protoc Mol Biol, 2007. **Chapter 1**: p. Unit 1 17.

27. Thomason, L., et al., *Recombineering: genetic engineering in bacteria using homologous recombination.* Curr Protoc Mol Biol, 2007. **Chapter 1**: p. Unit 1 16.

28. Thomason, L., et al., *Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination*, in *Current Protocols in Molecular Biology*. 2001, John Wiley & Sons, Inc.

29. Sharan, S.K., et al., *Recombineering: a homologous recombination-based method of genetic engineering.* Nat Protoc, 2009. **4**(2): p. 206-23.

30. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.

31. Flensburg, J. and O. Skold, *Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim.* Eur J Biochem, 1987. **162**(3): p. 473-6.

12. APPENDIX

12.1. Whole Morbidostat Results for all Cultures

Culture 14 (Strong) — Log[OD$_{600}$] vs Time (h)

Culture 15 (Strong) — Log[OD$_{600}$] vs Time (h)

46
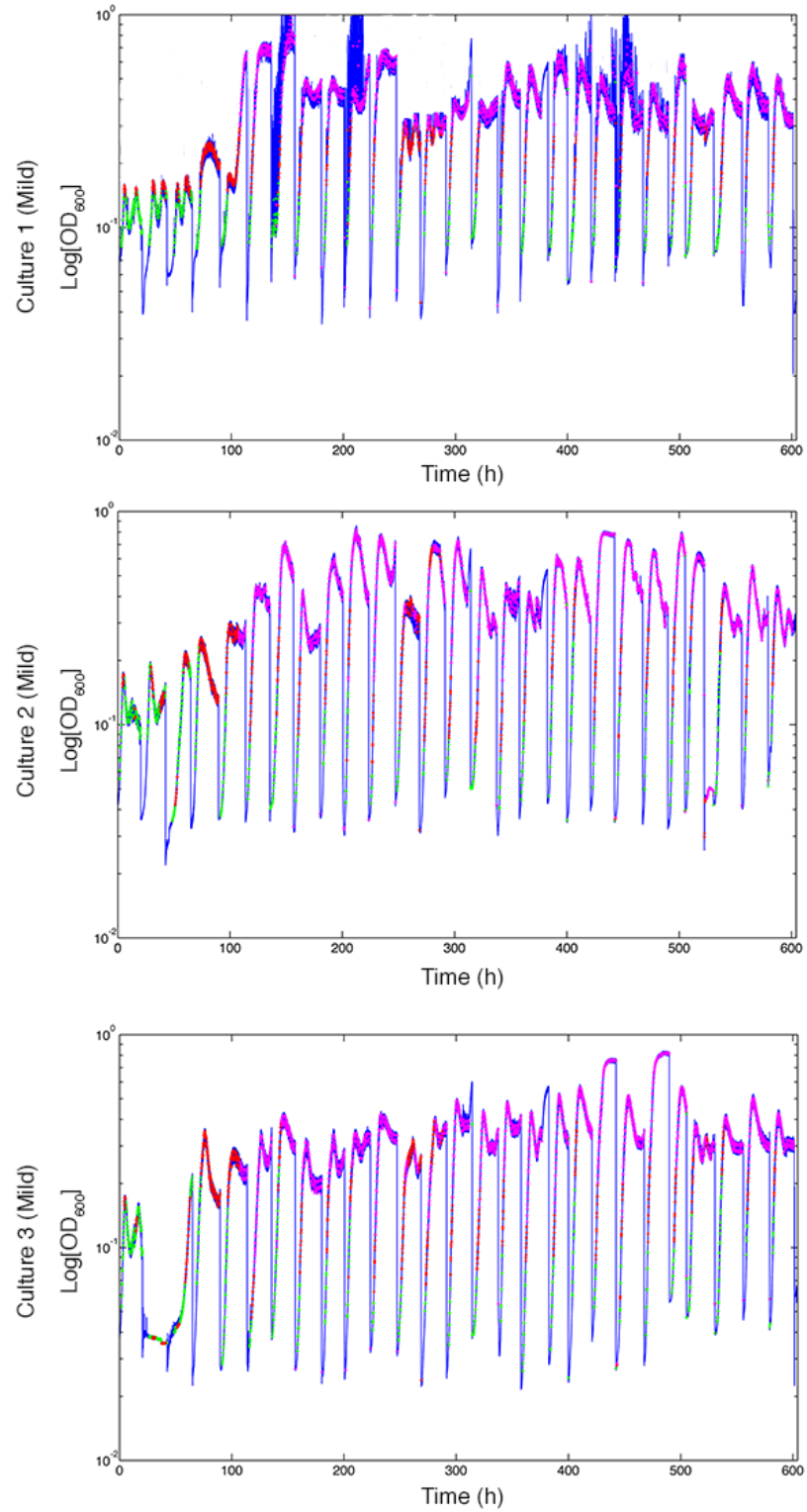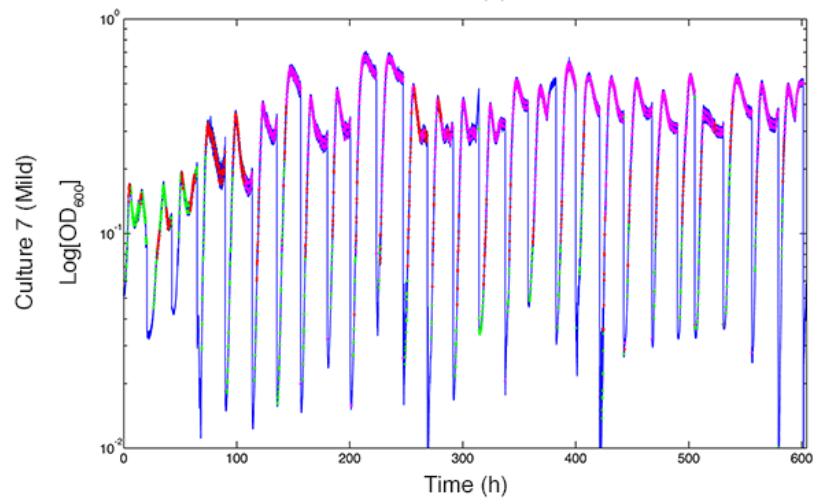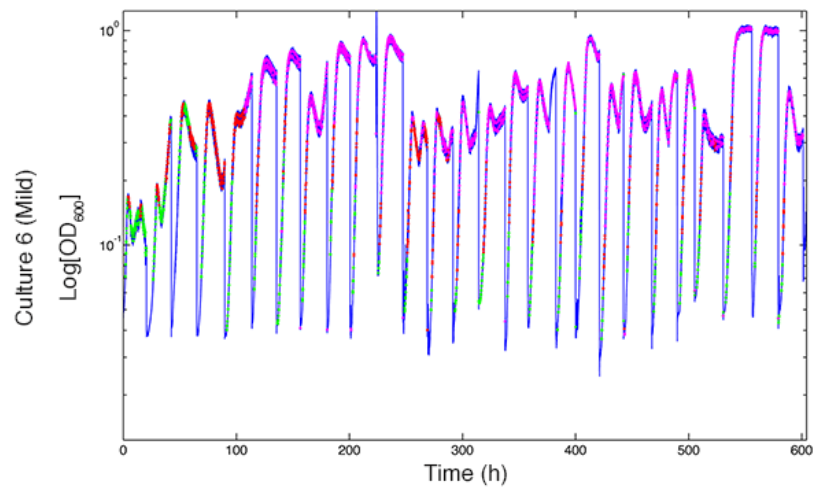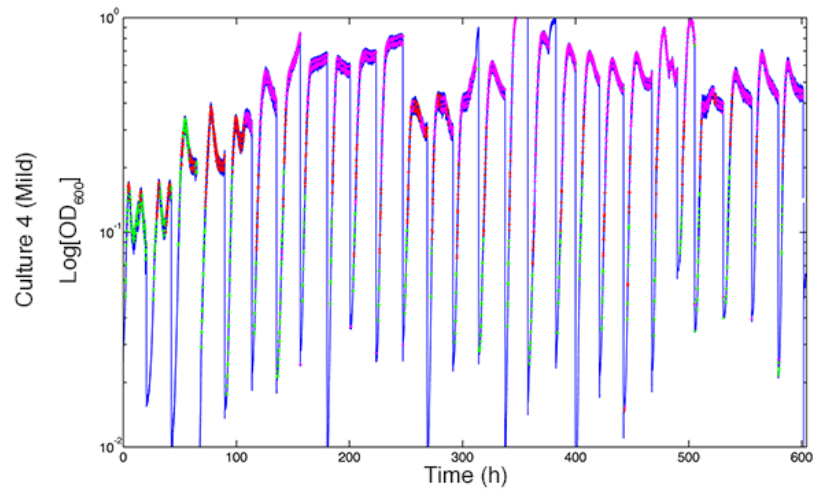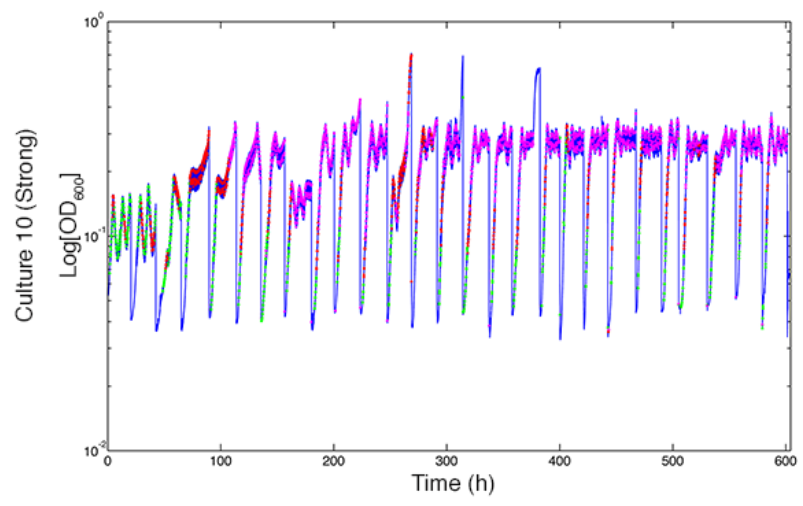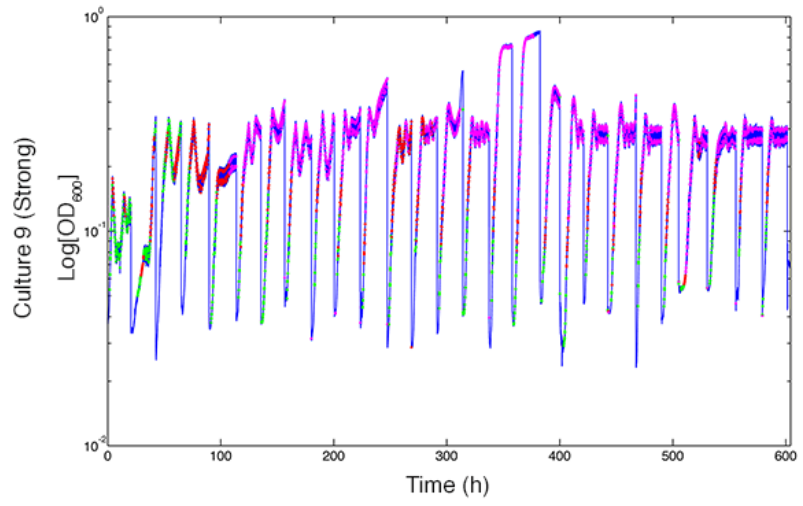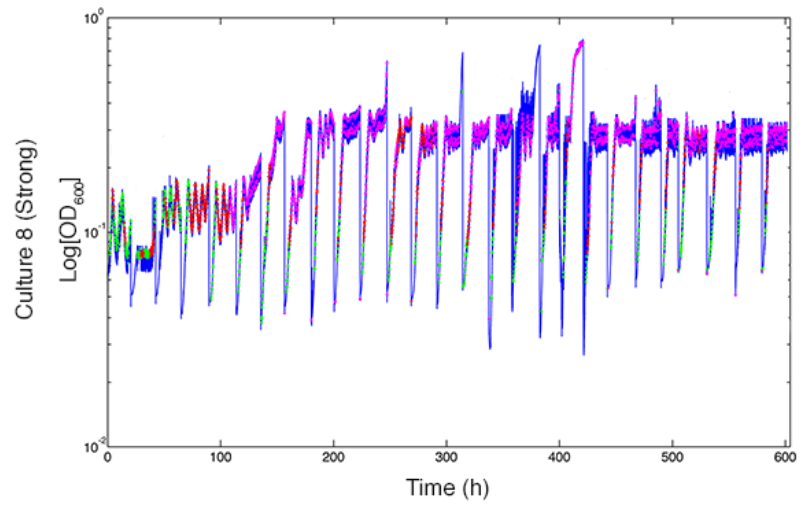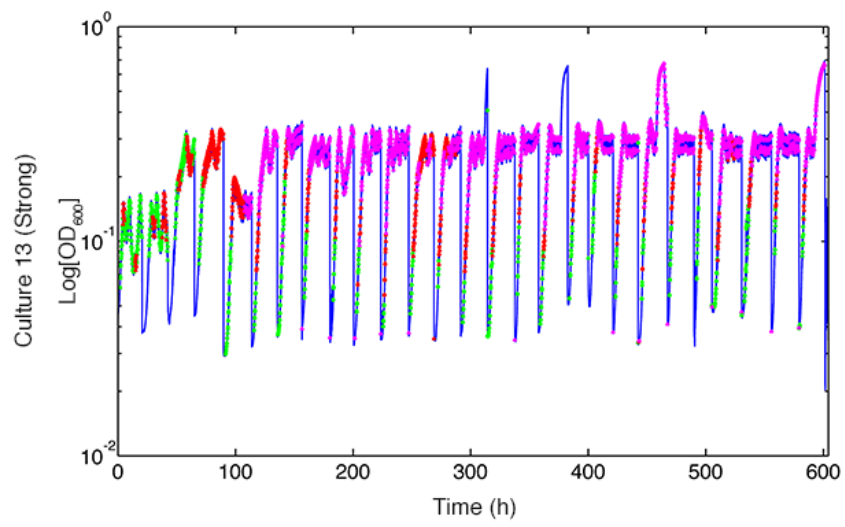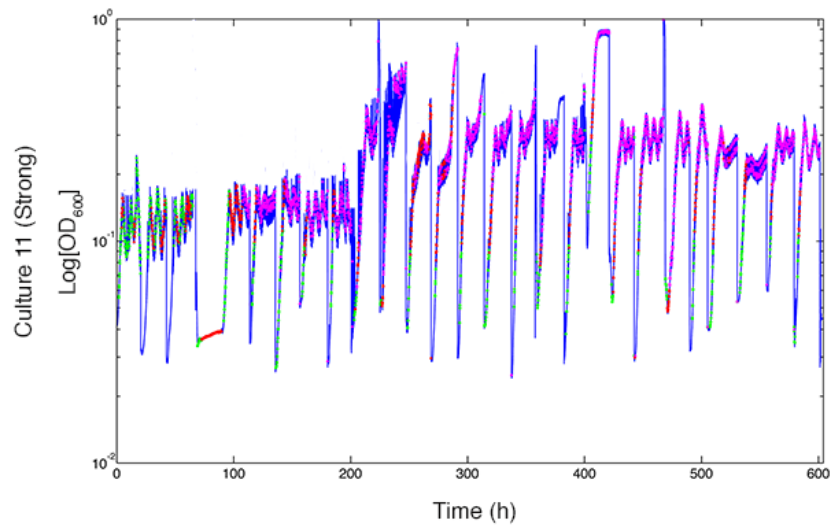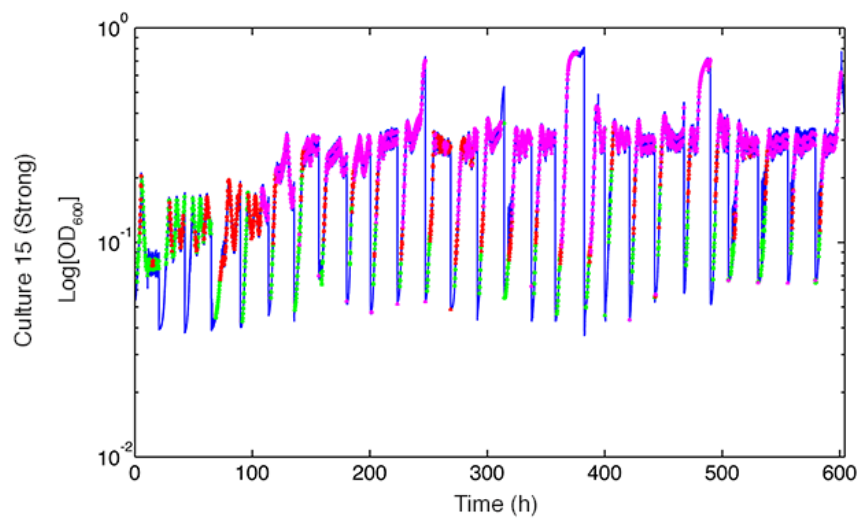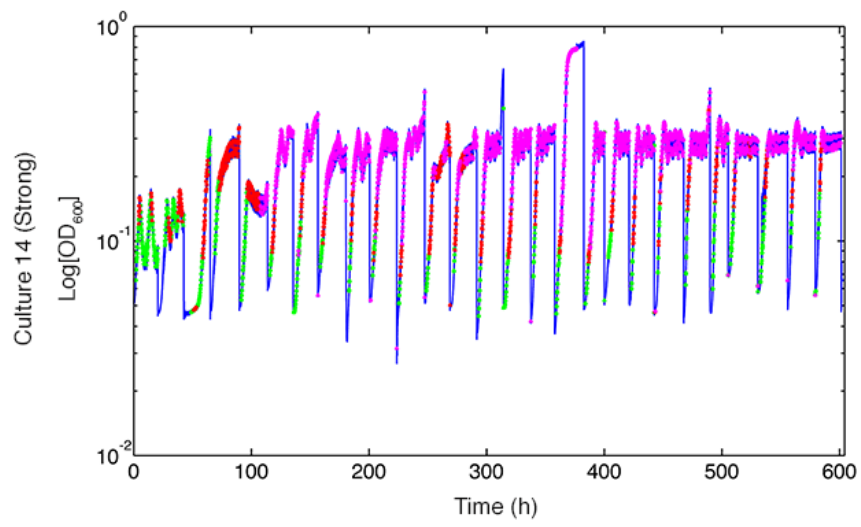
12.2. TMP concentration change with respect to time

Culture 1:
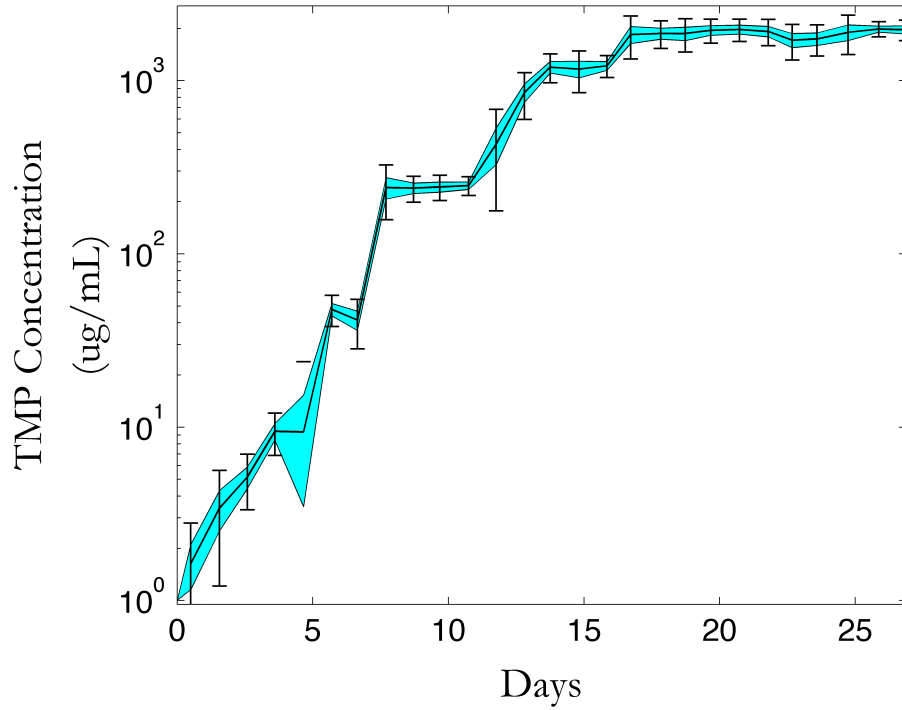
Culture 2:
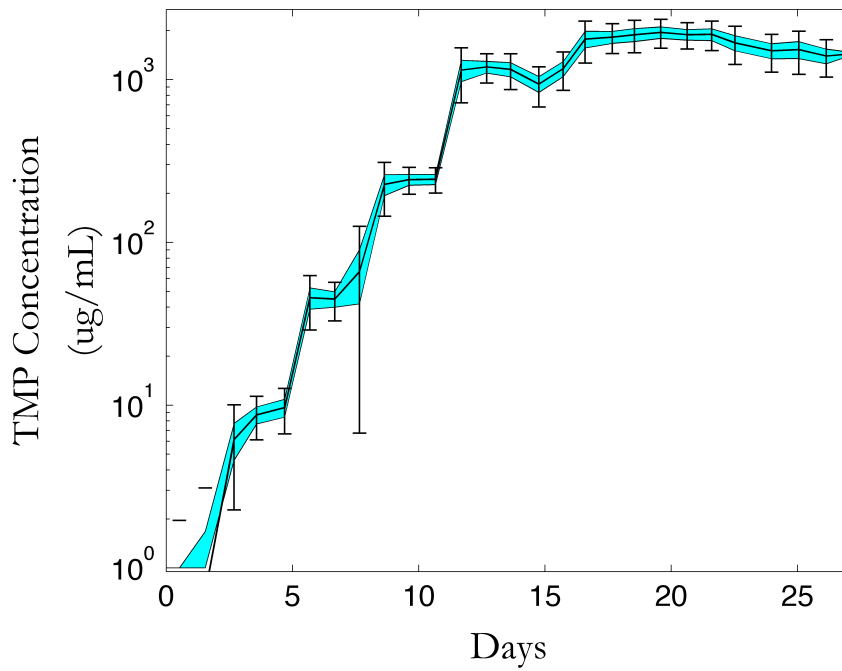
Culture 3:
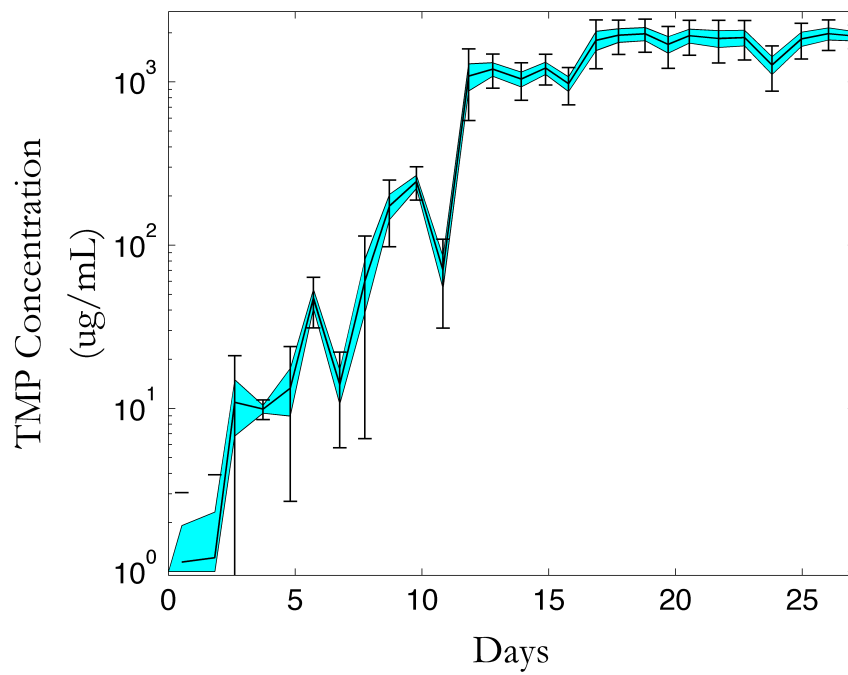
Culture 4:
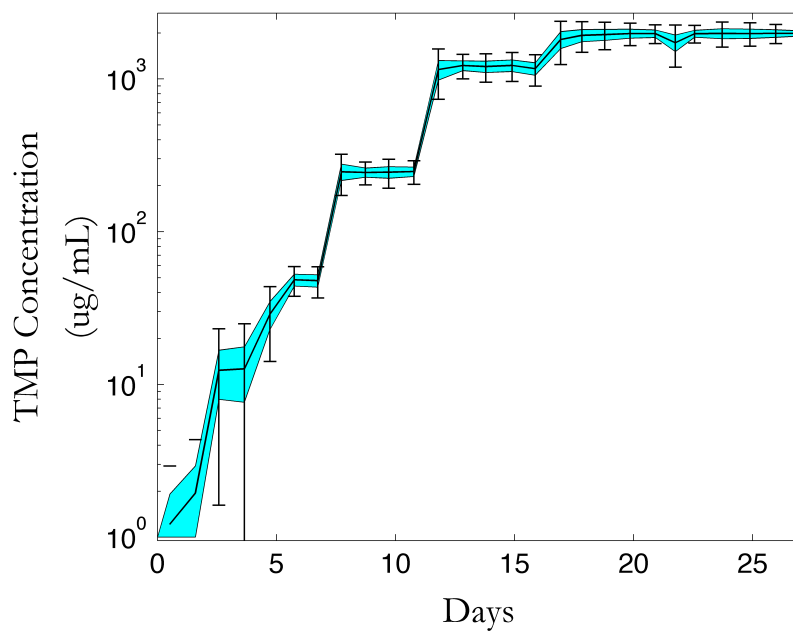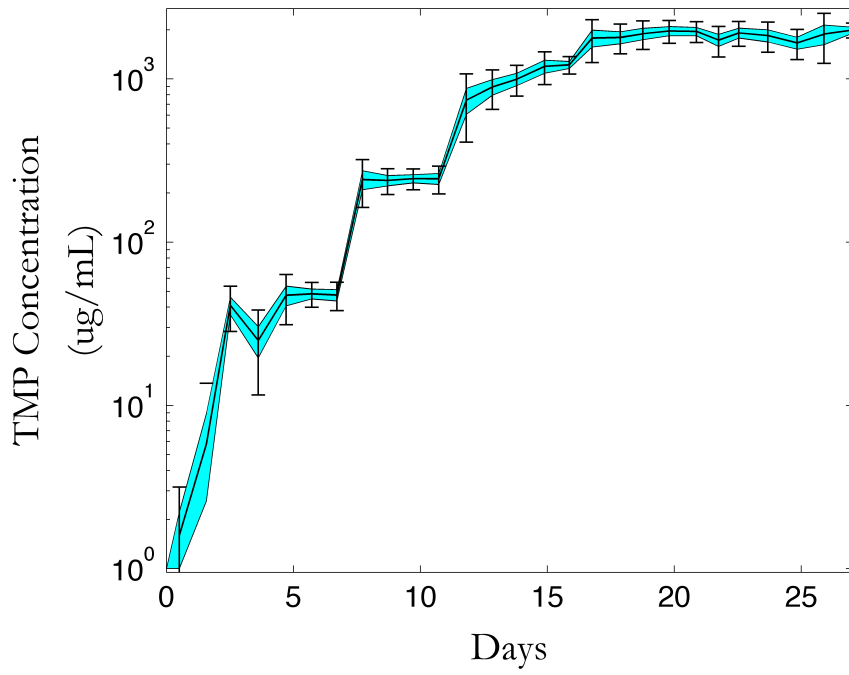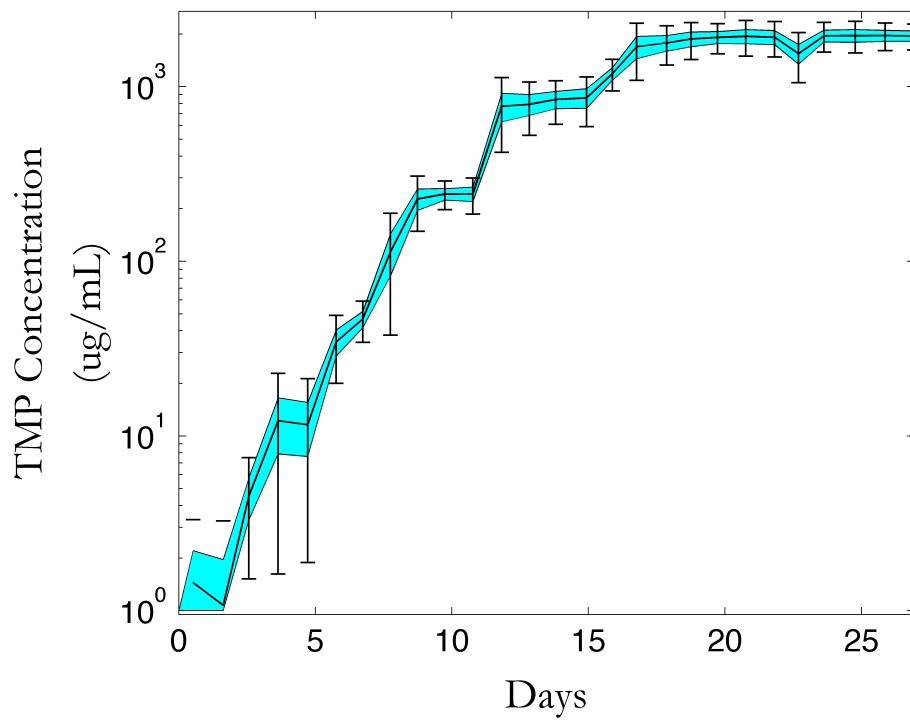
Culture 6:
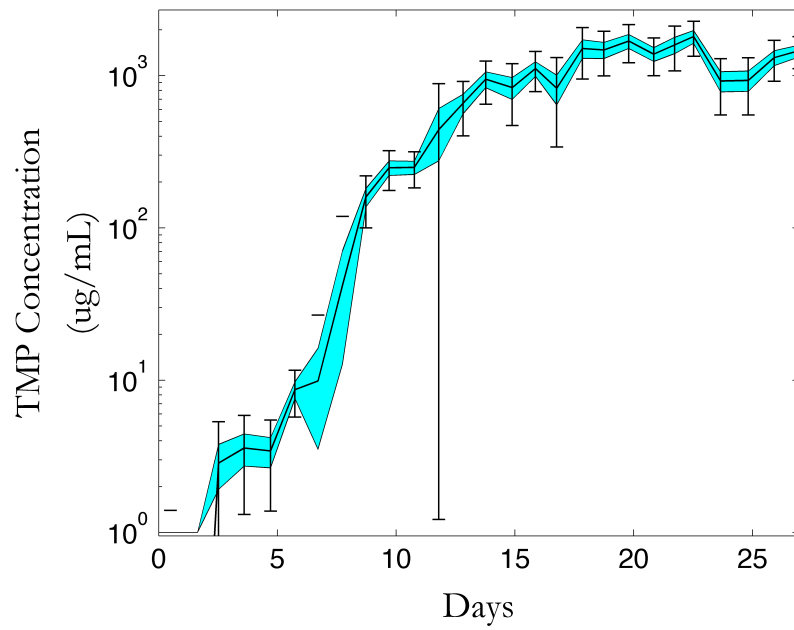
Culture 7:
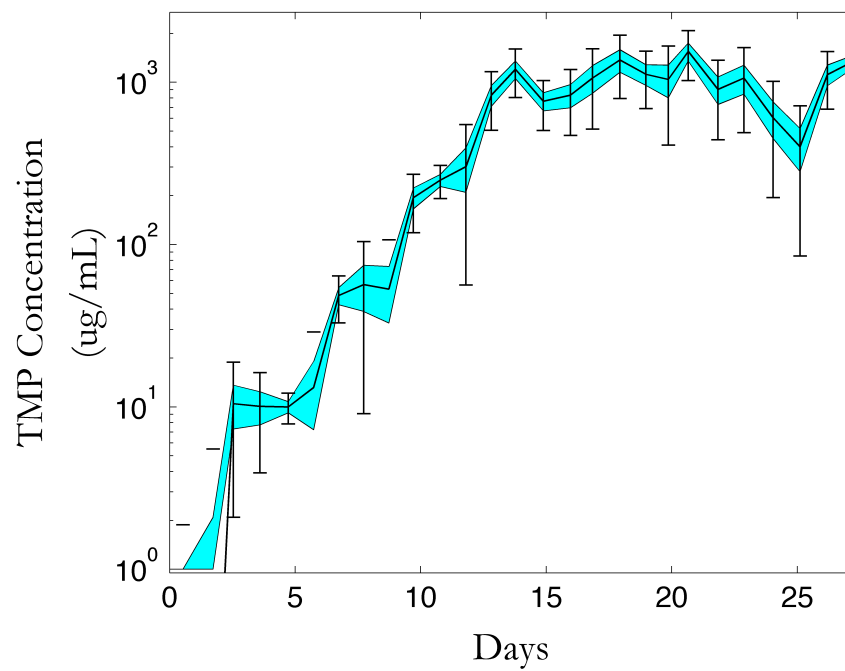
Culture 8:

Culture 9:
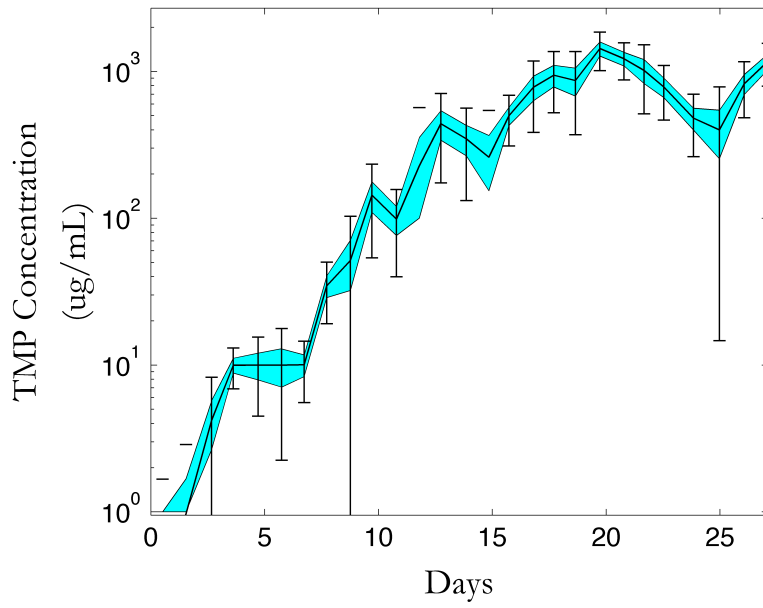
Culture 10:
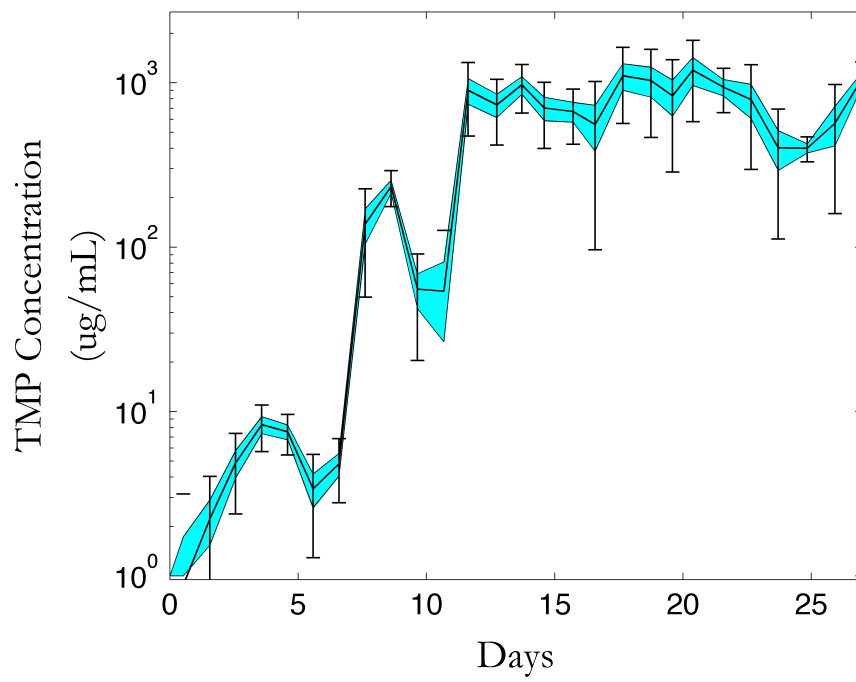
Culture 11:
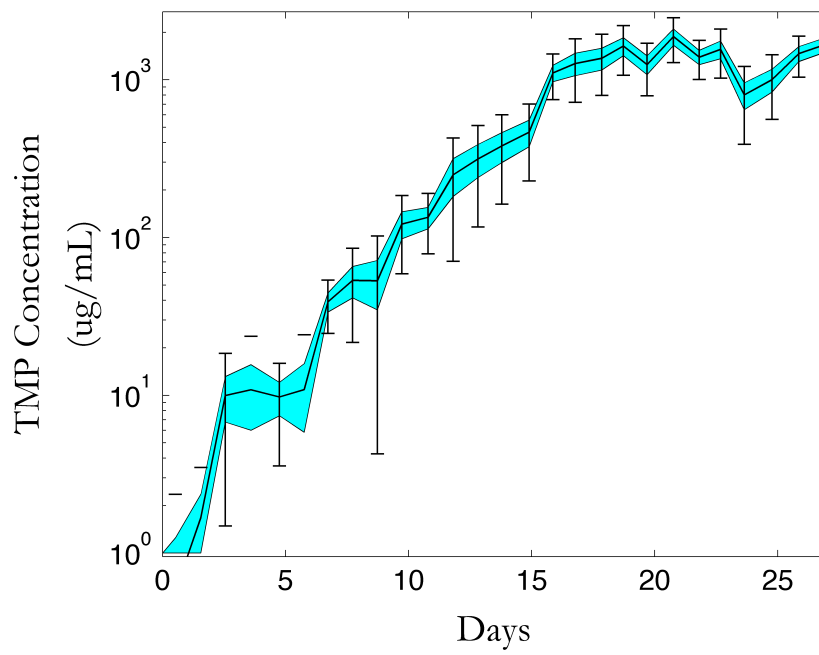
Culture 13:
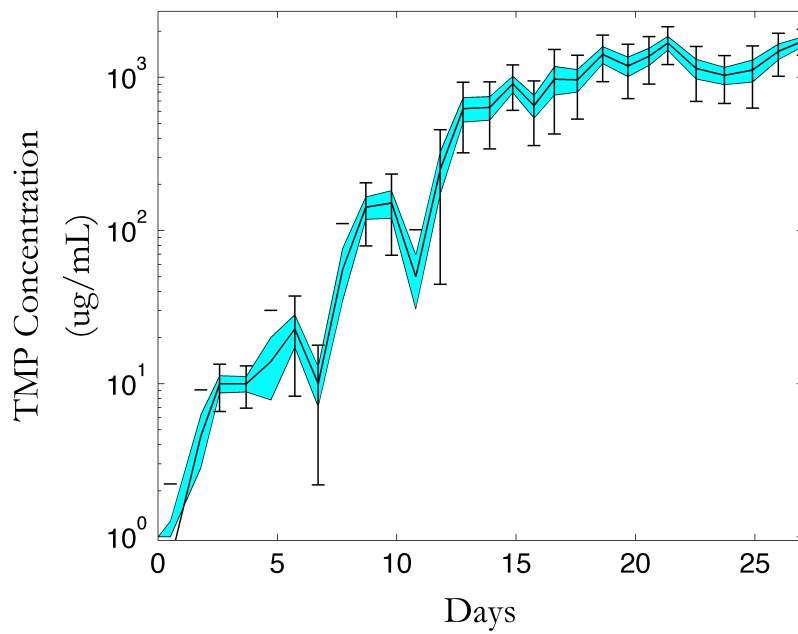
Culture 14:
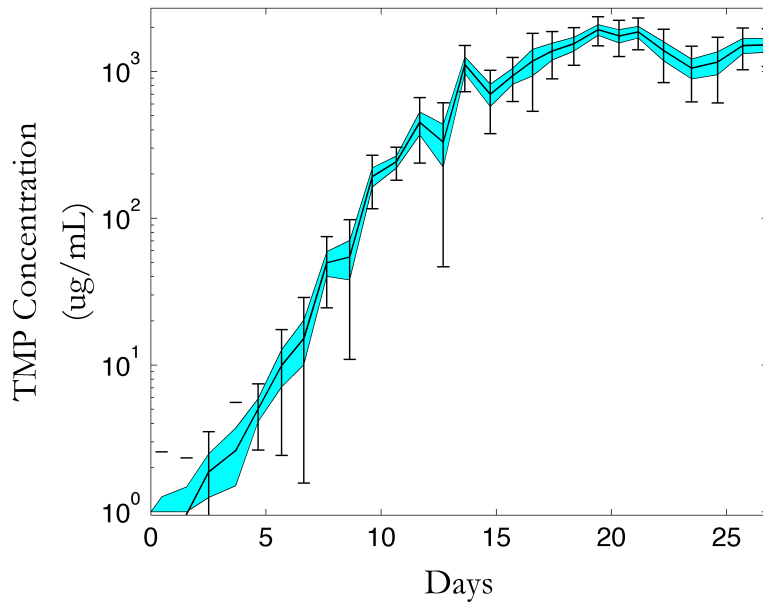
Culture 15:

12.3. Cylinder Graphs

Culture 1:
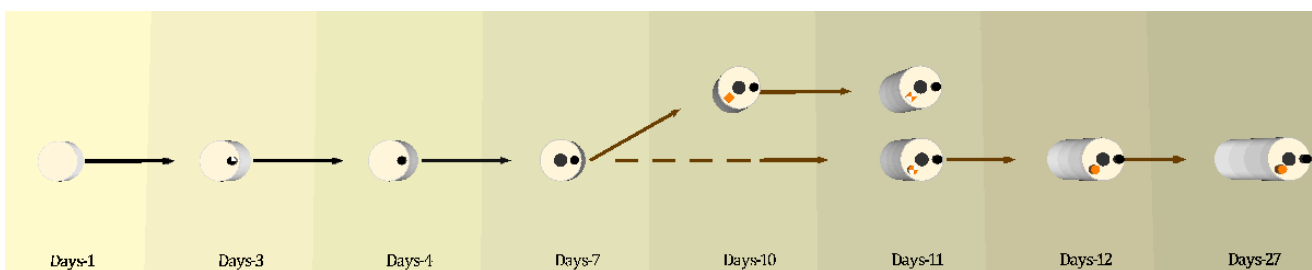
| Days-1 | Days-4 | Days-7 | Days-13 | Days-19 | Days-27 |

Culture 2:

| Days-1 | Days-3 | Days-4 | Days-7 | Days-10 | Days-11 | Days-12 | Days-27 |

Culture 3:

Culture 4:

Culture 6:

Culture 7:

Culture 8:

Culture 9:

Culture 10:

Culture 11:

Culture 13:

Culture 14:

Culture 15:

56
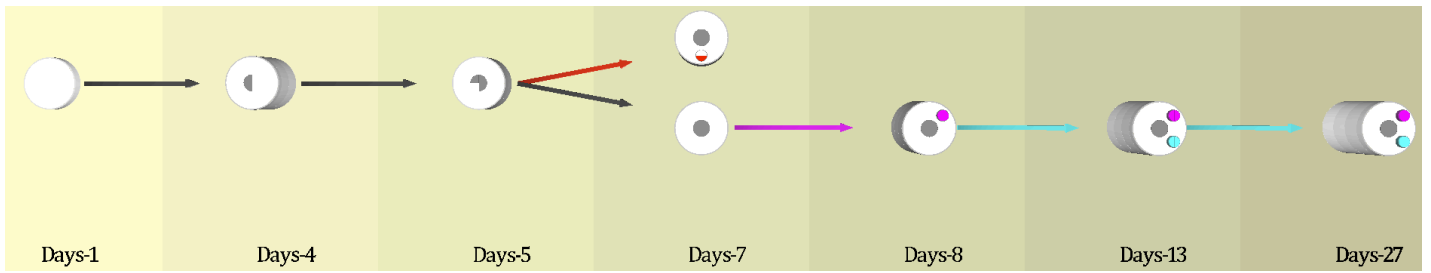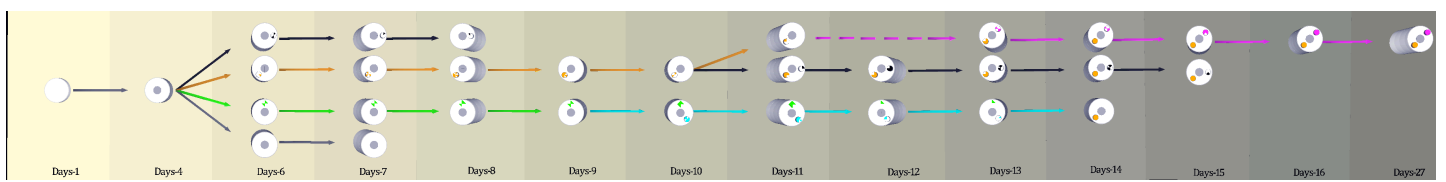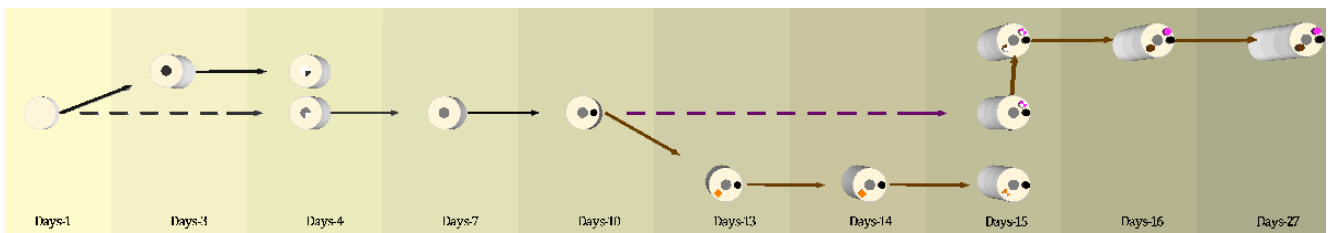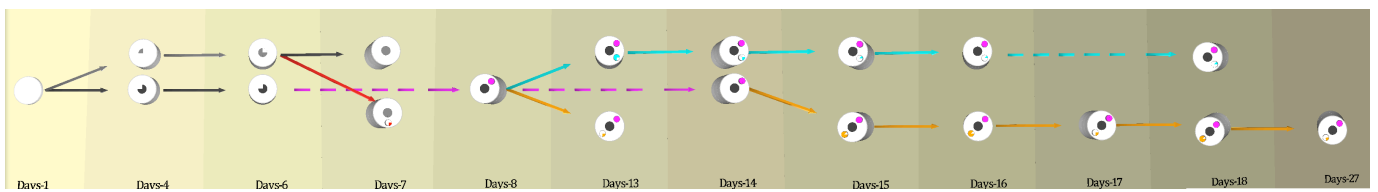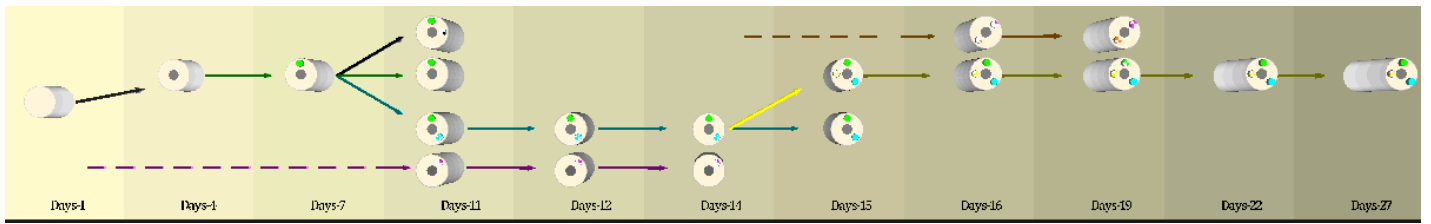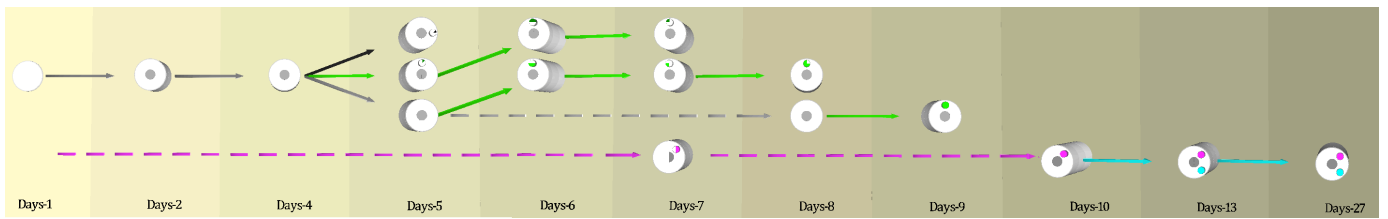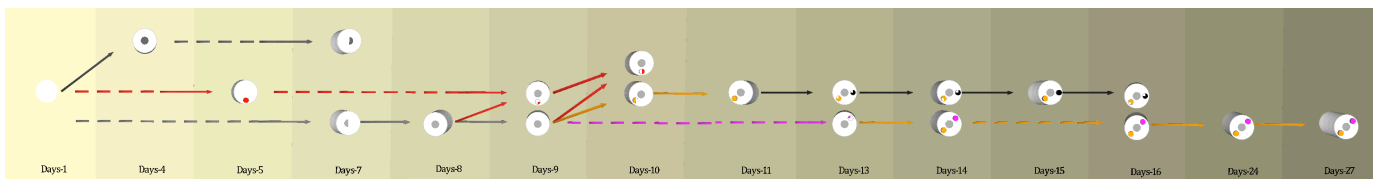
12.4. Diversity Scores by Cultures

Mild Cultures:
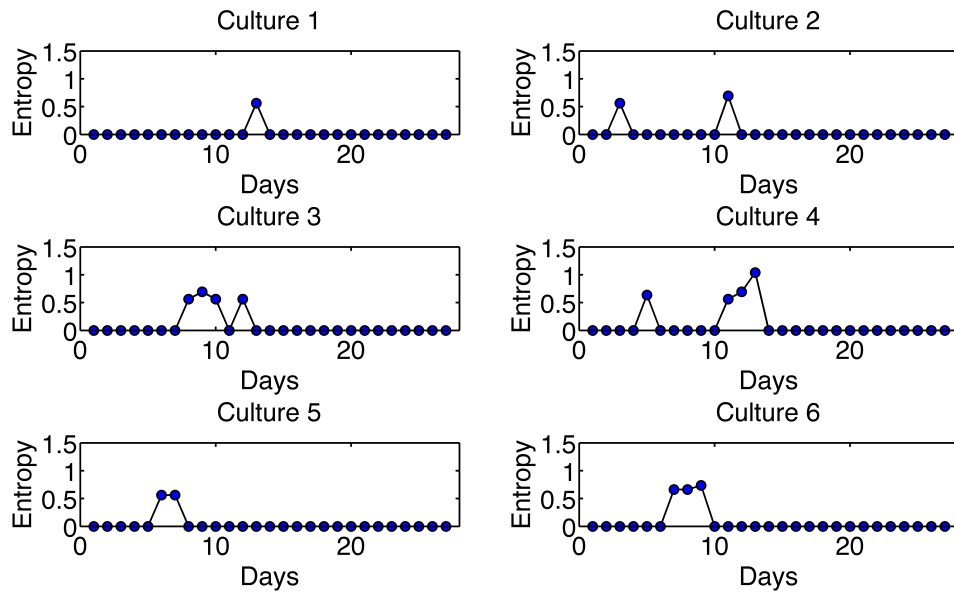
Strong Cultures:
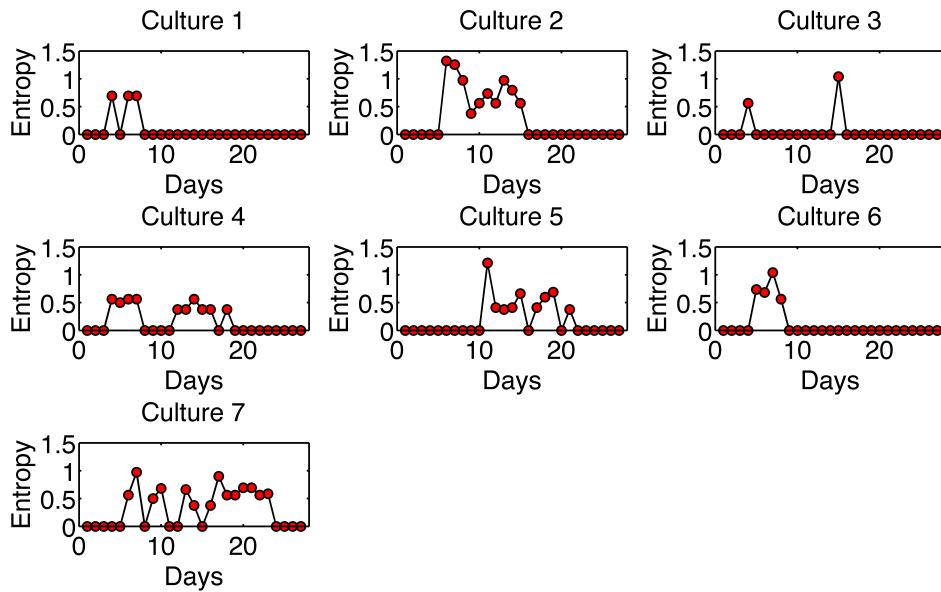
12.5. K_m Plots

WT protein
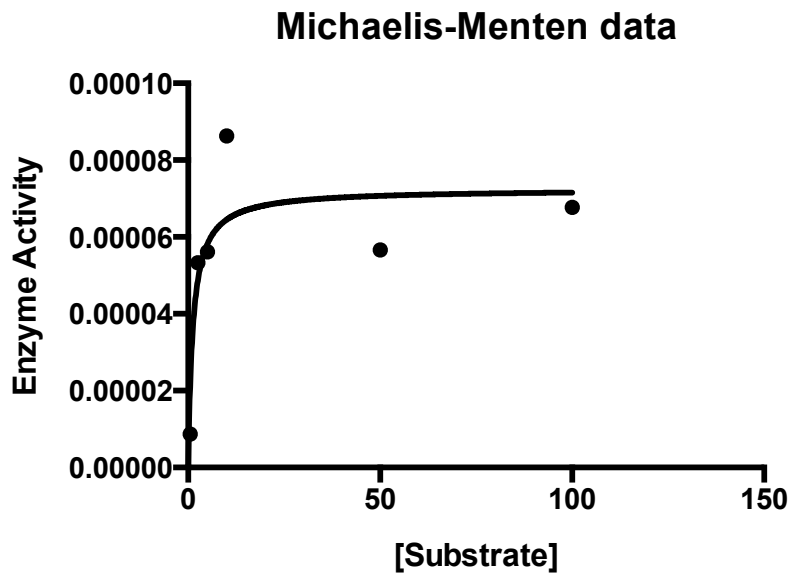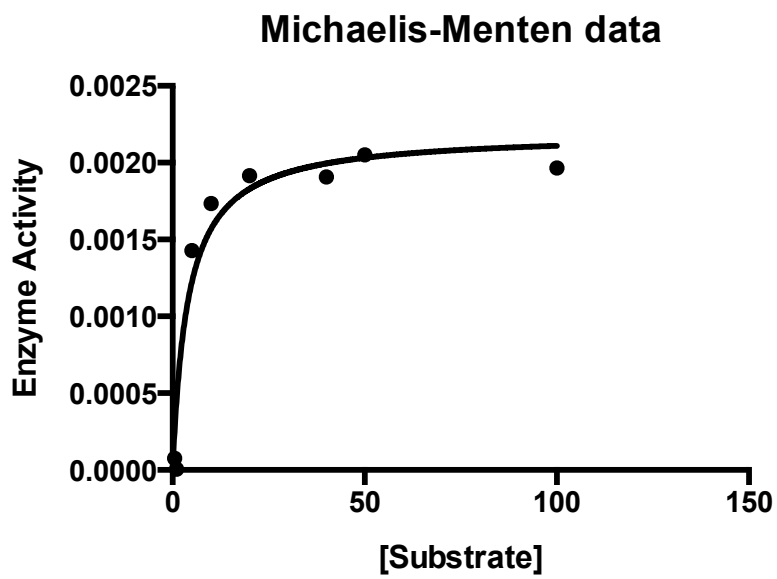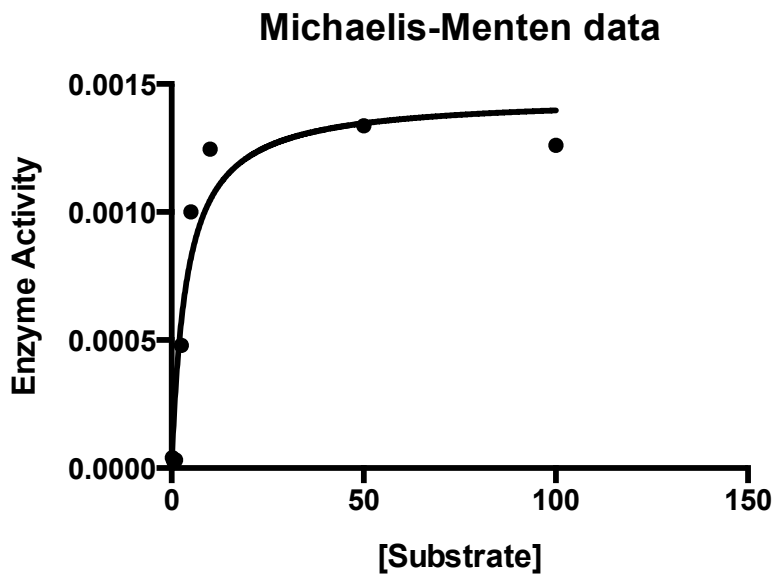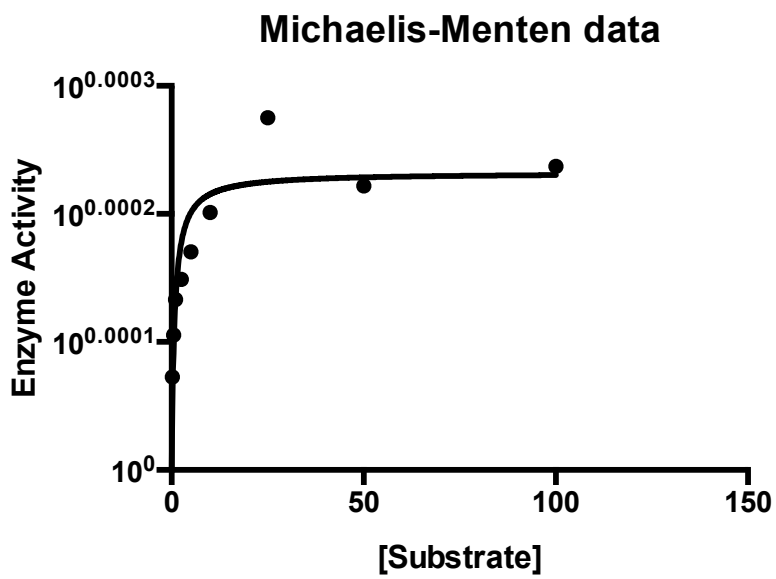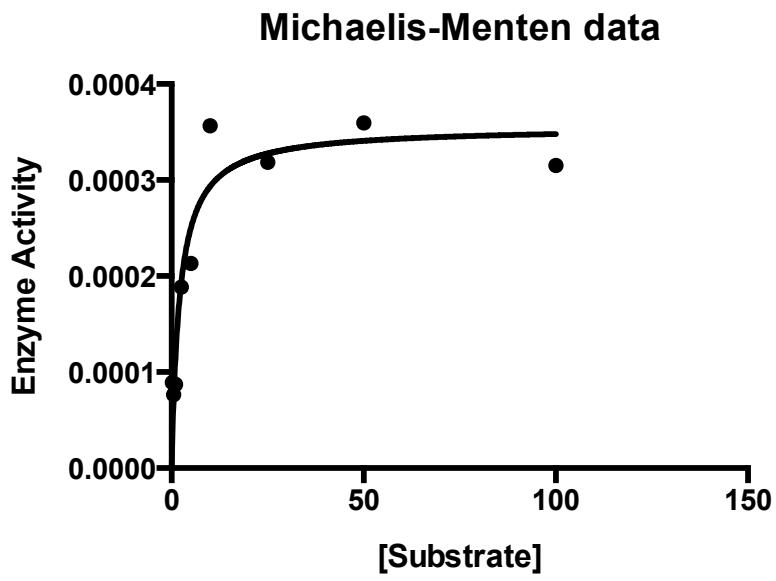
Michaelis-Menten data

P21L protein

Michaelis-Menten data

A26T protein

Michaelis-Menten data

L28R protein

Michaelis-Menten data

W30R protein

Michaelis-Menten data

W30G protein

Michaelis-Menten data

W30C protein

Michaelis-Menten data

I94L protein

Michaelis-Menten data
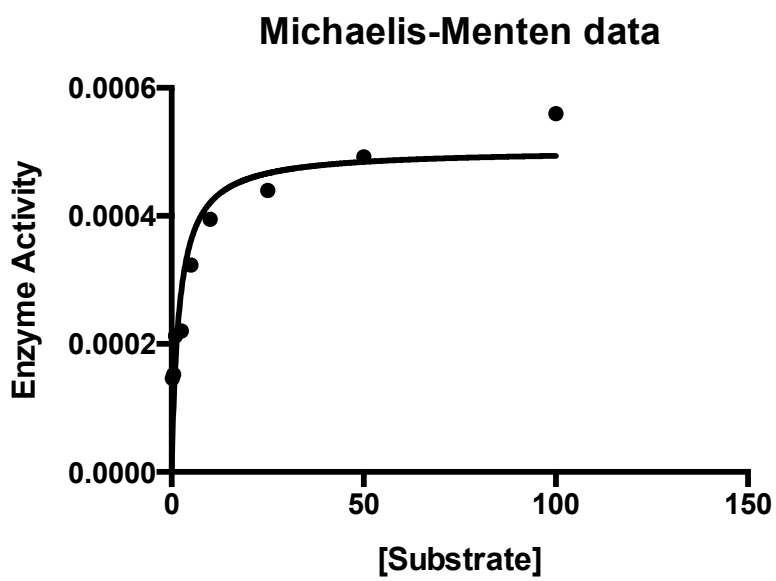
12.6. Ki Plots
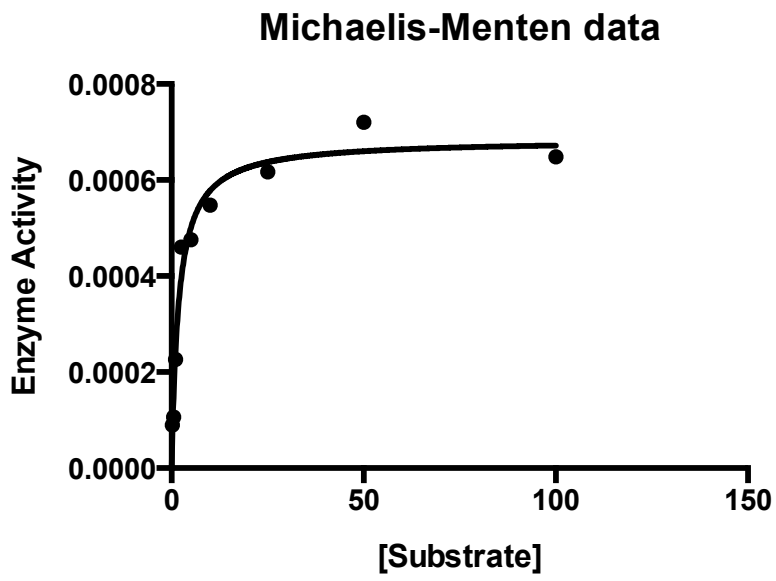
WT protein
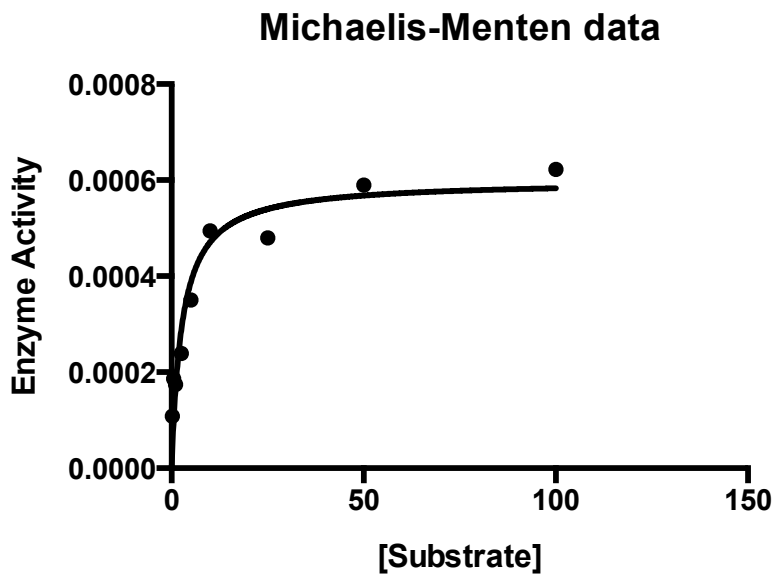
Michaelis-Menten
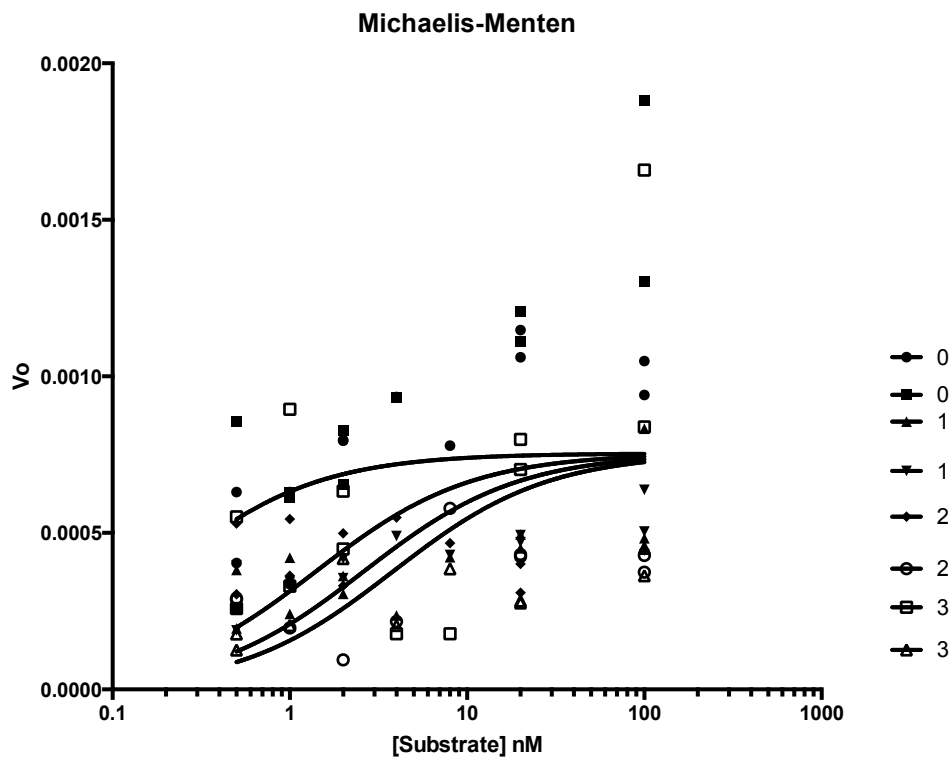
P21L protein
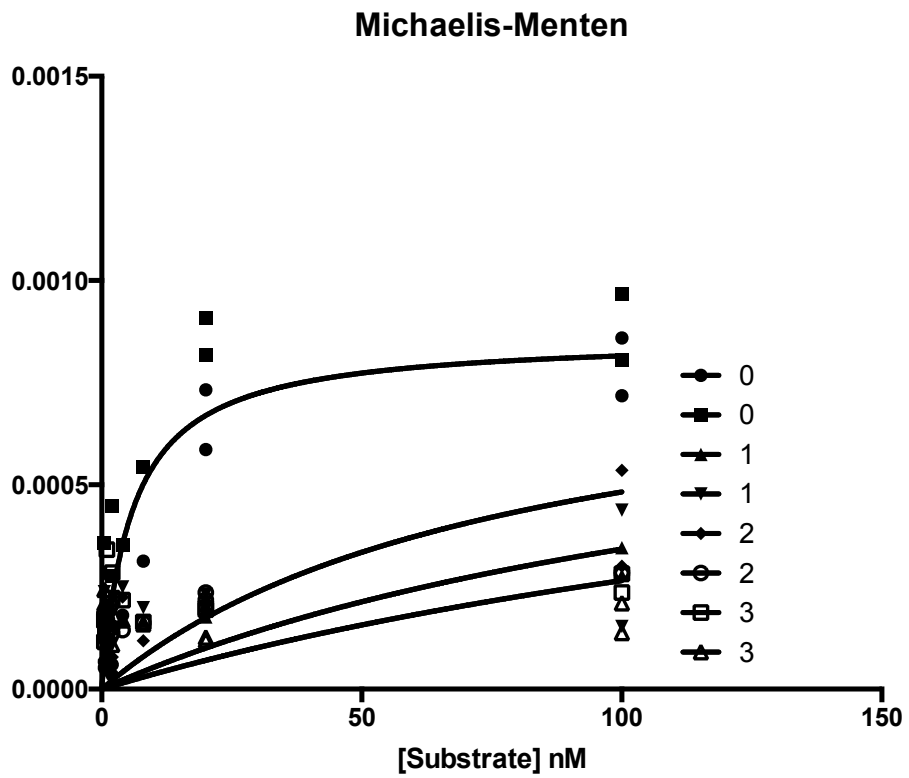
Michaelis-Menten

A26T protein
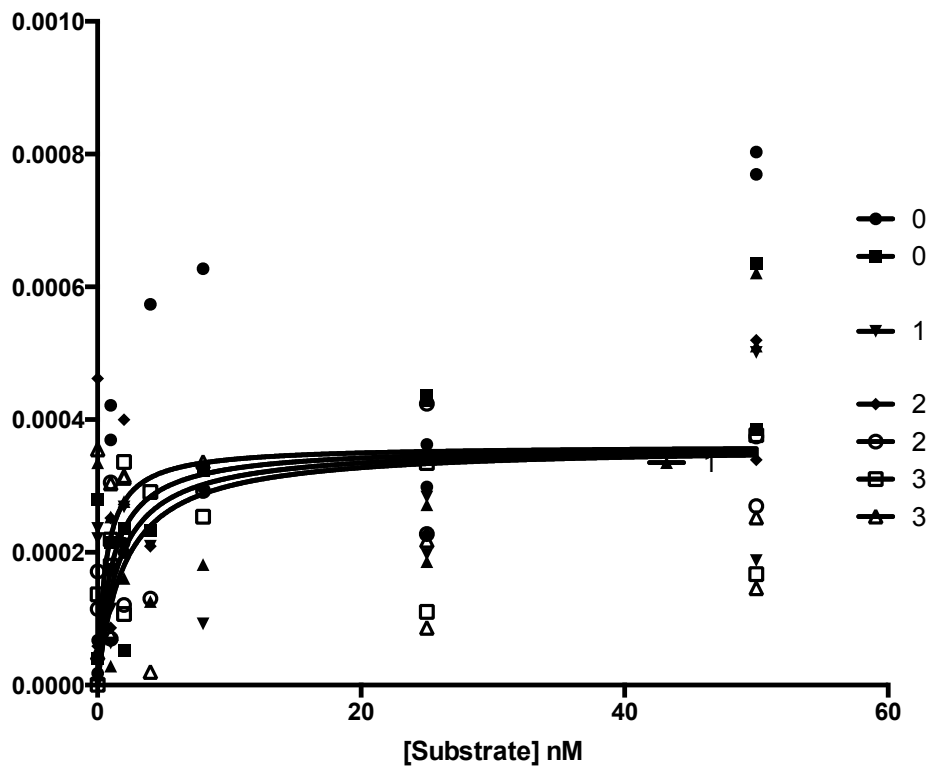
Michaelis-Menten

L28R protein

Michaelis-Menten
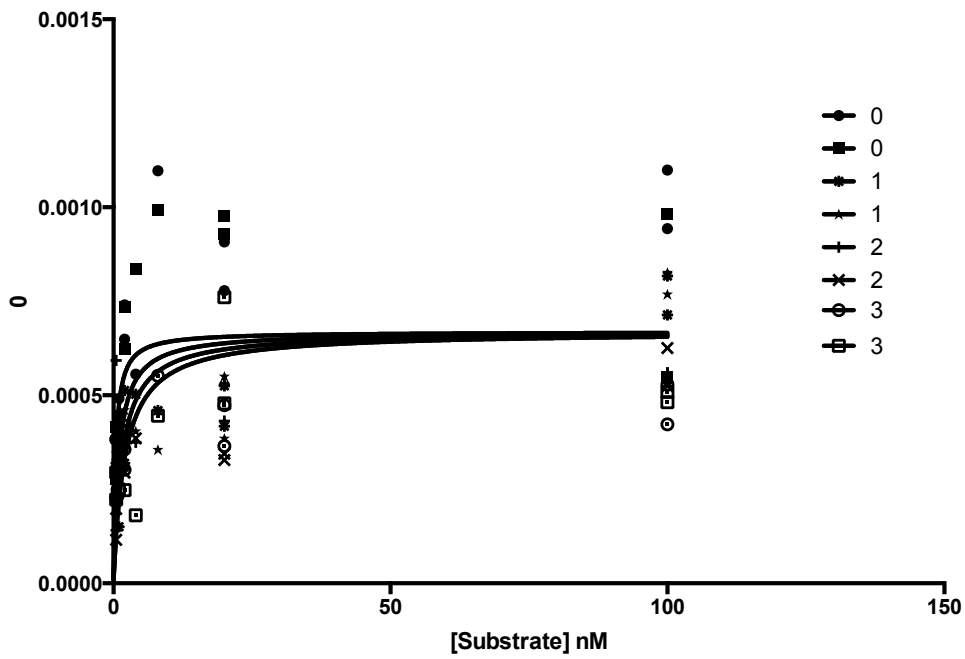
12.7. Stability Plots

WT protein

Data: Data7_CP
Model: MN2State
Chi^2/DoF = 3.551E4
T_m 54.64   ±0.054
ΔH 2.171E4   ±394
ΔH_v 1.279E5   ±2.88E3

P21L protein

Data: Data12_CP
Model: MN2State
Chi^2/DoF = 9.705E4
T_m 54.55   ±0.14
ΔH 4.324E4   ±952
ΔH_v 5.981E4   ±1.63E3

A26T protein

Data: Data7_CP
Model: MN2State
Chi^2/DoF = 4.190E5
T_m 54.12   ±0.20
ΔH 8.423E4   ±2.17E3
ΔH_v 4.951E4   ±1.58E3

L28R protein

Data: Data8_CP
Model: MN2State
Chi^2/DoF = 4.171E4
T_m 54.70   ±0.033
ΔH 4.043E4   ±432
ΔH_v 1.251E5   ±1.66E3

W30R protein

Data: Data8_CP
Model: MN2State
Chi^2/DoF = 1.531E5
T_m 49.42   ±0.27
ΔH 3.163E4   ±1.24E3
ΔH_v 5.343E4   ±2.60E3

W30G protein

Data: Data9_CP
Model: MN2State
Chi^2/DoF = 3.311E4
T_m 52.31   ±0.16
ΔH 2.266E4   ±560
ΔH_v 5.817E4   ±1.78E3

W30C protein

Data: Data10_CP
Model: MN2State
Chi^2/DoF = 2.971E4
T_m 54.38   ±0.27
ΔH 2.186E4   ±638
ΔH_v 4.077E4   ±1.47E3

I94L protein

Data: Data11_CP
Model: MN2State
Chi^2/DoF = 1.161E5
T_m 54.78   ±0.13
ΔH 4.639E4   ±1.02E3
ΔH_v 6.215E4   ±1.69E3