INVESTIGATING EVOLUTIONARY TRADE-OFFS FOR DESIGNING NOVEL STRATEGIES TO SLOW DOWN EVOLUTION OF ANTIBIOTIC RESISTANCE

by

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ABSTRACT

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Antibiotic resistance is a global public health problem. The straightforward solution to this problem is developing new antibiotics that can kill all of the drug resistant bugs, alas; this has not been possible so far due to economic and natural limitations. Another plausible solution to this problem is the effective use of already existing antibiotics by designing novel treatment strategies. However, efforts towards finding such strategies have not been rewarding to the date due to our limited knowledge about the origins of antibiotic resistance at the molecular and population levels. In order to tackle this problem, we performed an extensive laboratory evolution experiment where we evolved drug sensitive *E.coli* populations against 22 different clinically important antibiotic compounds and systematically phenotyped and genotyped evolved populations. Benefiting from this extensive data set, we identified common genetic targets for resistance conferring mutations and resulting phenotypic changes. Our analysis allows us design effective multidrug treatments strategies that can slow down evolution of antibiotic resistance. We hope that, the methodologies that were developed throughout this study will also be helpful for finding effective therapies for combating cancer and immune disease.

ÖZET

EVRİMSEL ÖDÜNLEŞİMLERİN ARAŞTIRILARAK ANTİBİYOTİK DİRENCİNİ YAVAŞLATMAK İÇİN YENİ STRATEJİLERİN BELİRLENMESİ

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Anahtar Kelimeler: Antibiyotik, Antibiyotik Direnci, Çapraz Direnç, Fenotip, Genotip, Tüm Genom Dizilemesi, Mutasyon

Antibiyotik direnci küresel bir halk sağlığı sorunudur. Bu problemin en kolay çözümü tüm dirençli bakterileri öldürebilecek yeni ilaçlar geliştirmektir ama ne yazık ki bu çözüm ekonomik ve doğal kısıtlamalar sebebiyle mümkün olmamaktadır. Bir başka makul çözüm de alternatif tedavi metotları geliştirerek mevcut ilaçların daha etkili kullanılmasıdır. Henüz yeni strateji belirleme çabaları moleküler ve popülasyon düzeyinde antibiyotik direncinin sebepleri hakkındaki bilginin kısıtlı olması yüzünden faydalı olamamıştır. Biz bu sorunu çözmek için, ilaca duyarlı *E.coli* bakterilerilerini 22 farklı klinik olarak önemli antibiyotiğe karşı direnç kazandırdığımız ve sistematik olarak fenotip ve genotip değişikliklerine baktığımız geniş bir evrim deneyi uyguladık. Dirence sebep olan mutasyonların ortak genetik hedefleri ve neden olduğu fenotipik değişiklikleri oluşturduğumuz geniş veri setimizden faydalanarak belirledik. Analizlerimizi kullanarak antibiyotik direnci miktarını azaltabileceğimiz, çoklu ilaç tedavi stratejileri belirleyebiliriz. Umuyoruz ki bu araştırmayla geliştirdiğimiz yöntemler, kanser ve bağışıklık sistemi hastalıklarına karşı etkili tedavi bulmada da yardımcı olacaktır.

To my family with all my heart…

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LIST OF ABBREVIATIONS

CHL	Chloramphenicol
CLI	Clindamycin
ERY	Erythromycin
SPR	Spiramycin
FUS	Fusidic Acid
TOB	Tobramycin
AMK	Amikacin
KAN	Kanamycin
STR	Streptomycin
TET	Tetracycline
DOX	Doxycycline Hyclate
SPT	Spectinomycin
AMP	Ampicillin
PIP	Piperacillin
CEF	Cefoxitin
LOM	Lomefloxacin
CIP	Ciprofloxacin
NAL	Nalidixic Acid
TMP	Trimethoprim
SMO	Sulfamonomethoxine
SUL	Sulfamethoxazole
NIT	Nitrofurantoin
MIC	Minimum inhibitory concentration
SNP	Single Nucleotide Polymorphism

1. INTRODUCTION

1.1 Antibiotics

1.1.1 General Overview

The discovery of penicillin by Alexander Fleming is a milestone in modern medicine because it saved millions of human and animal lives by curing previously untreatable microbial diseases. Antibiotics are nanometer-sized small molecules that can cure bacterial infections by killing bacteria or inhibiting their growth. Antibiotics in nature are mostly produced by microorganisms Wi.e. *actinomycetes, streptomyces,* and fungiS and although there is no direct evidence, natural antimicrobial products are conventionally considered to be secondary metabolites that have roles in microbial communication. Biological and ecological roles of antibiotics are yet poorly understood, for example, antibiotic producing bacteria often secrete antibiotic molecules that have inhibitory effects on their competitors after forming spores as a result of starvation, when there is no competition for resources[1].Although several synthetic antibiotic molecules exist as of today, the majority of the clinically relevant antibiotics are derived from nature and further modified for higher efficacy and lower toxicity [1, 2]. With the advances in chemistry and structural biology, novel clinically important antibiotics were produced solely by chemical synthesis. Sulfonamides, quinolones and oxazolidinones are examples of synthetic antibiotics[2].

1.1.2 Mechanism of Action of Antimicrobial Agents

Antibiotics can be grouped into two main classes: bacteriostatic and bactericidal. Bacteriostatic antibiotics inhibit growth or proliferation of bacteria. This way, they give time to the immune system of host forremoving the infecting microorganisms from the body. Hence, complete removal of bacteria depends on strength of the immune system[3]. On the other hand, bactericidal antibiotics can kill bacteria when used in appropriate doses usually by breaking cell wall integrity. That being said, differentiating bacteriostatic and bactericidal antibiotics is not always possible because high concentrations of some bacteriostatic agents can also have bactericidal effects. Likewise,low concentrations of some bactericidal agents have bacteriostatic effects[4].

1.1.3 Major Antibiotic Classes

The mechanism of action of antibiotics is categorized based on the physiological functions affected in the presence ofdrugs or the structure of the bacteria. There are four major targets in bacterial pathogens according to mode of antibiotic action: cell wall biosynthesis, protein synthesis, DNA replication and repair, and folate metabolism[1].

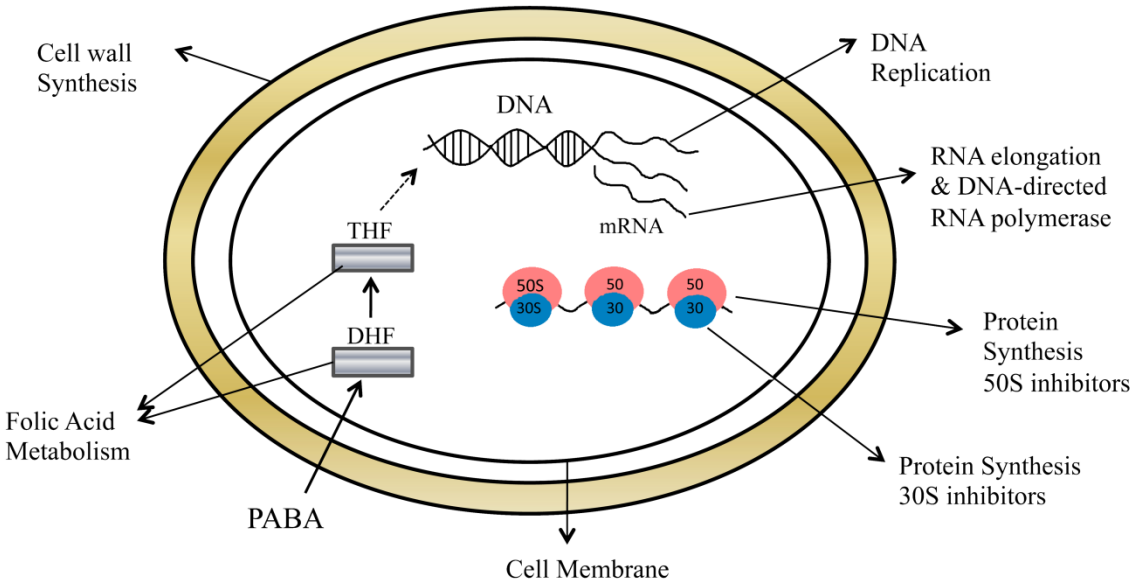


Figure1.1 Major targets for antibacterial action: DNA replication, protein synthesis, cell wall biosynthesis and folic acid biosynthesis are main targets for antibiotics. Protein synthesis is mainly blocked by 50S and 30S subunits binding inhibitors. In folic acid metabolism, dihydrofolate (DHF) reductase enzyme is blocked and formation of tetrahydrofolate (THF) is inhibited.

1.1.3.1 Cell Wall Biosynthesis Inhibitors

Gram staining is a method to differentiate bacterial species into two large groups, namely gram-negative and gram-positive, according to the chemical and physical properties of their cell walls by detecting peptidoglycan (PG) layer in the cell wall. Gram-negative and gram-positive bacteria both have PG layers as part of cell wall structure but gram-positive bacteria have thicker and multilayered PG layers, as shown in Figure 1.2. When these layers are corrupted by antibiotics or other chemicals, cells lyse and die consequently. Therefore, such cell wall structures are good targets for antibiotic therapies. The other difference between gram positive and gram negative bacteria is the existence of the second outer membrane in gram negative bacteria; hence, gram-positive bacteria are susceptible to some antibiotics that do not work against gram-negative bacteria because of the limited pore sizes of porin proteins of the gram-negative microorganisms' outer membrane[1].

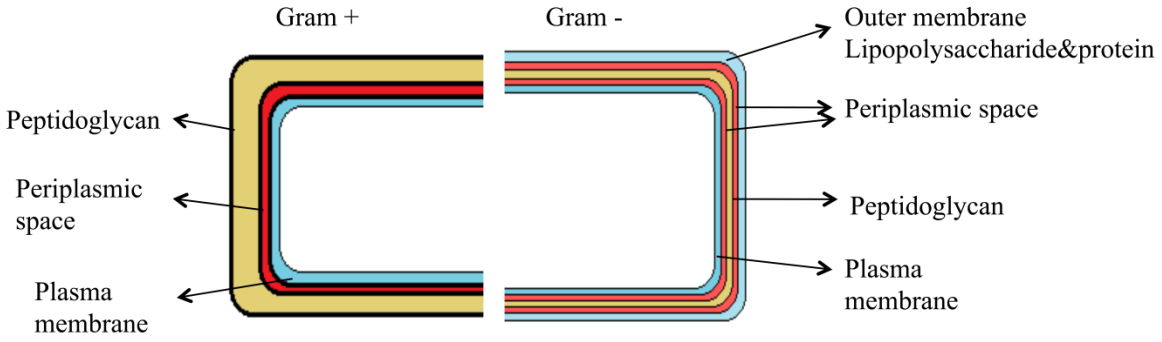


Figure 1.2 The cell wall structures in gram positive and gram negative bacteria. Gram positive bacteria have thicker peptidoglycan layer and gram negative bacteria have a second outer membrane.

Peptidoglycan, also known as murein, is a polymer consisting two hexoses: N-acetylglucosamine (GlcNac) and N-acetyl-muramic acid (MurNAc). The biochemicalsteps of peptidoglycan biosynthesis are catalysed by the enzymes MurA–F and MurG. Then, peptidoglycan units are transferred to the cell membrane by lipid-bactoprenol-phosphate for generating lipids I and II. Sugars and phosphates are added by transglycosylation and pyrophosphorylation, and finally, a peptide bond between the peptide chains is formed[5]. Several transpeptidases and transglycosylases connect the newly formed peptidoglycan structures to the cell wall peptidoglycan matrix[6]. All of these steps are targets for antibiotics, as shown in Fig.1.3. Specific antibiotics interfere with the synthesis of the cell wall, weakening the peptidoglycan scaffold within the bacterial wall so that the structural integrity eventually fails. For example, beta- lactams are bactericidal antibiotics that inhibit transpeptidase enzymes and prevent the assembly of the peptidoglycan layer in both Gram-positive and Gram-negative bacteria. Fosfomycin and tunicamycin inhibit MurA ang Mur G, respectively[5].

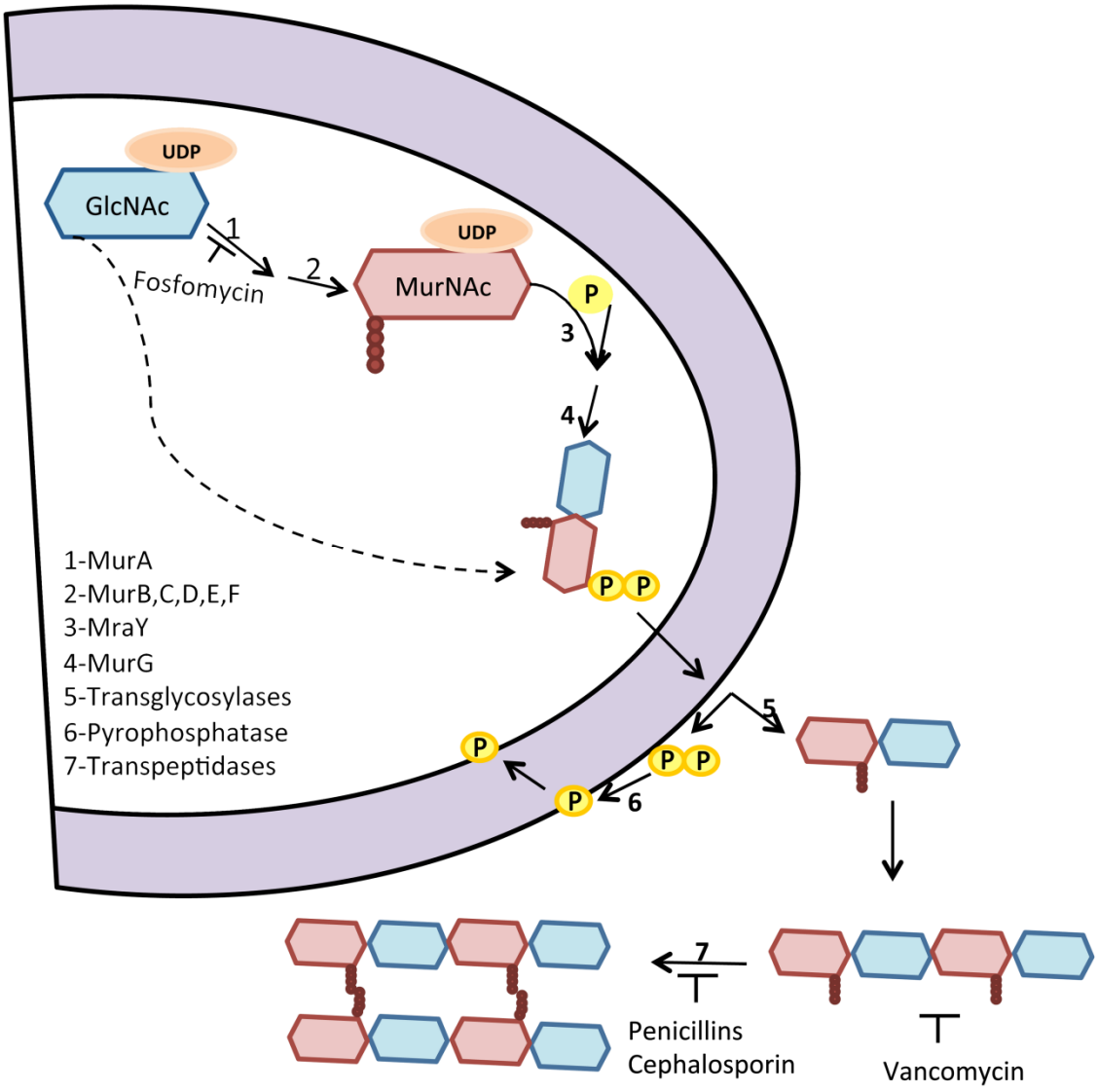


Figure 1.3 Targets for cell wall biosynthesis: Murein biosynthesis pathway is an important target for antibiotics. Fosfomycin blocks MurA in the first step. Penicillins and cephalosporin inhibit transpeptidases, adapted from reference [5].

β-Lactam antibiotics are broad class of antibiotics, which contain a β-lactam ring in their molecular structures. Some of β-Lactams are penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. Chemical structures of these antibiotics are shown in Figure 1.4.

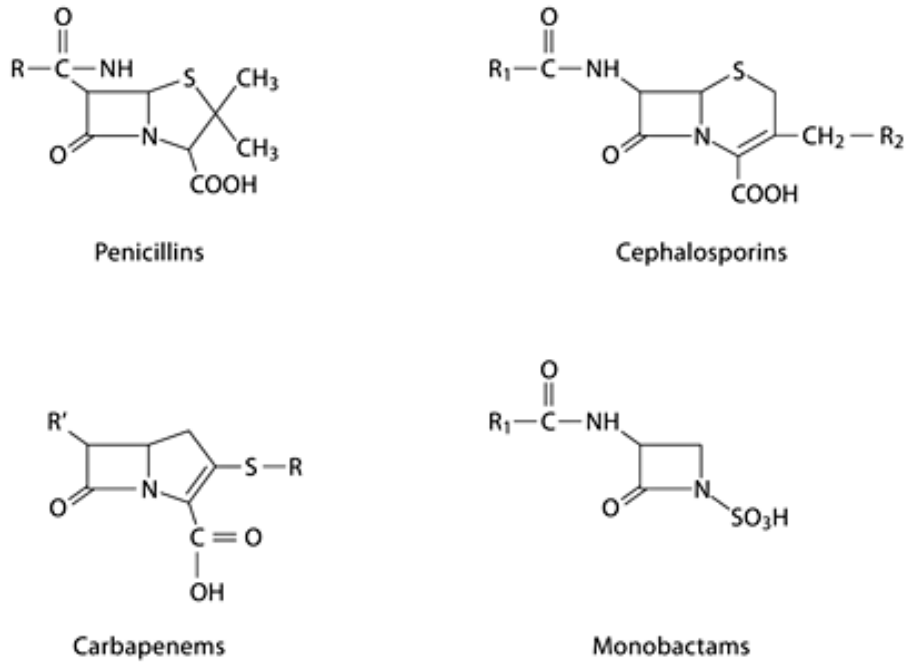


Figure 1.4 Chemical structures of beta-lactam antibiotics[1]

Vancomycin and teicoplanin, known as glycopeptide antibiotics, also prevent cell wall construction by interfering with transglycosylases steps. Their effectiveness is limited to Gram-positive bacteria because they cannot penetrate the outer membrane of Gram-negative bacteria due to their very large sizes. Size differences between different cell wall inhibitor antibiotics are demonstrated in Figures 1.4 and 1.5. When used in therapies, β-lactams selectively targets the infecting bacteria with almost no significant effect on the cells of the mammalian host since mammalian cells have a plasma membrane but lack the peptidoglycan wall structure[6].

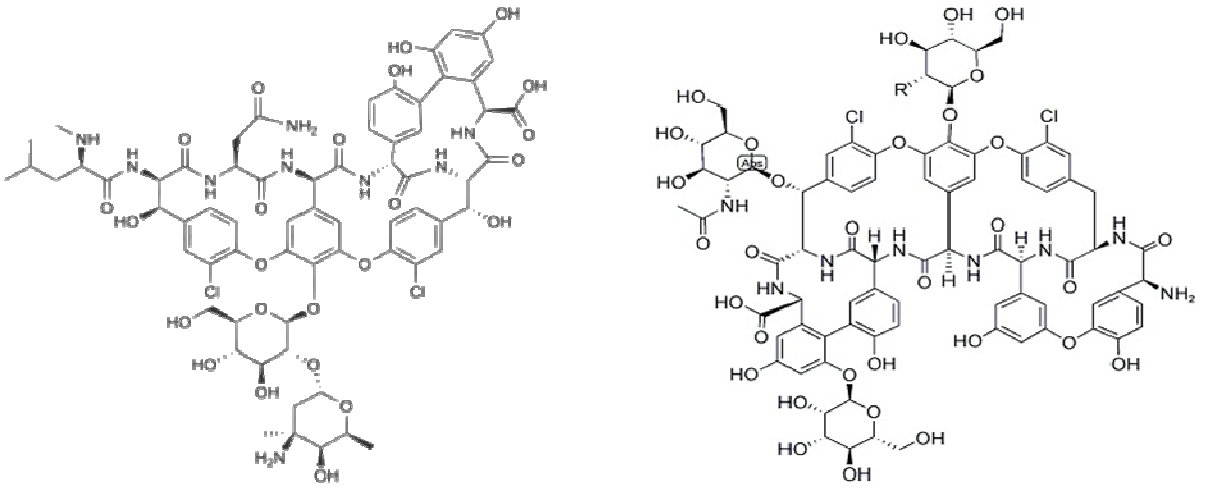


Figure 1.5 Structures of the glycopeptide antibiotics: vancomycin and teicoplanin [1]

1.1.3.2 Protein Synthesis Inhibitors

Bacterial protein synthesis is a highly complex, multi-step process involving many enzymes and conformational rearrangements of the ribosomal machinery. However, the majority of antibiotics that block bacterial protein synthesis interfere with the chemical reactions occurring at the 70S subunit of bacterial ribosome. In the bacteria, ribosome is gigantic machinery containing two large subunits, 50S and 30S, as shown in Figure 1.6. 50S, the larger ribosomal subunit, contains about 30 proteins, and ribosomal RNA structures known as 23S and 5S. 30S, the relatively smaller ribosomal subunit, has about 20 proteins and 16S, another ribosomal RNA structure [1].

The main targets of the antibiotics in the protein synthesis are:the messenger RNA (mRNA)-transfer RNA (tRNA) decoding region on the 30S subunit, the peptidyltransferase core on the 50S subunit, the formation of the 70S ribosome by the 30S initiation complex and the 50S ribosome, and the elongation process of amino acids into a polypeptide[7].

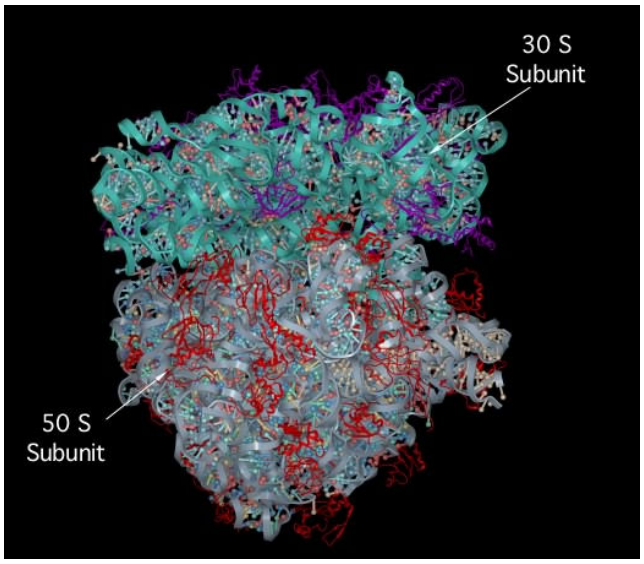


Figure 1.6 Crystal Structure of ribosome[8]

Antibiotics that block bacterial protein biosynthesis are often grouped as 30S subunit binding and 50S binding ribosomal inhibitors. 30S binding ribosomal inhibitors include aminoglycosides and tetracycline antibiotics. Tetracyclines, including doxycycline, prevent binding of aminoacyl-tRNA by blocking the A (aminoacyl) site of the 30S subunit of the ribosome. Aminoglycoside antibiotics, translation initiation inhibitors, have an affinity for the 30S subunit of the ribosome**.** Streptomycin, one of the most commonly used aminoglycosides in the clinic, interferes with the formationof the ribosomal 30S initiation complex**.** Kanamycin and tobramycin, the other two commonly used aminoglycosides, also bind to the 30S subunit of the ribosome and block the formation of the larger 70S initiation complex[9]. 50S binding ribosomal inhibitors include oxazolidinones, inhibitors of peptidyl transferases (amphenicols and pleuromutulins), macrolides, lincosamides, and streptogramins (shortly MLS) antibiotics. The inhibitory mechanism of oxazolidinones relies on targeting an early step involving the binding of N-formylmethionyl-tRNA to the ribosome[7]. Chloramphenicol, another ribosomal inhibitor, binds residues on the 23S rRNA (ribosomal RNA) of the ribosome and inhibits peptide bond formation [10].Macrolides inhibit ribosomal translocation by preventing peptidyl transferase[1, 9]. Some of the protein synthesis inhibitors and how they interfere with the translation process are demonstrated in Figure 1.7.

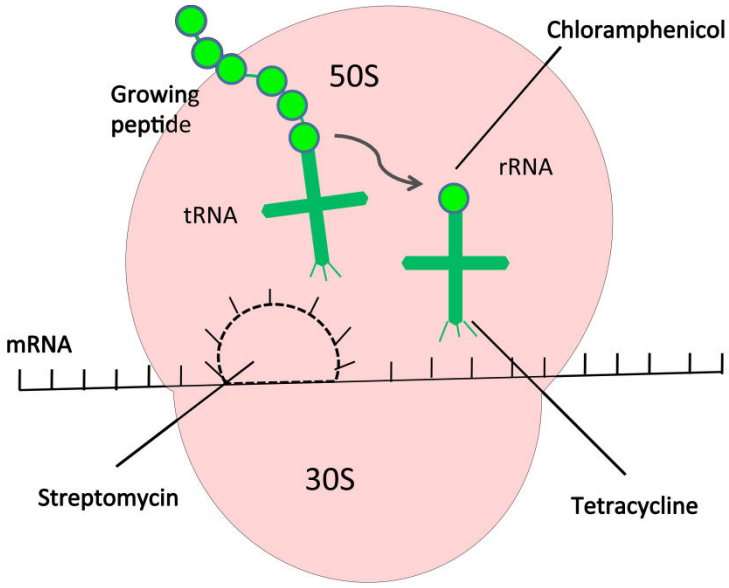


Figure 1.7 Antibiotics acting on the translational machinery.50S and 30S subunits of 70S prokaryotic ribosome and action of different antibiotics on protein synthesis is indicated in the figure. Chloramphenicol binds to 50S r-RNA and inhibits formation of peptide bond, streptomycin changes the shape of 30S portion and tetracyclines interfere with attachment of tRNA to mRNA-ribosome complex. Figure adapted from [17].

1.1.3.3 DNA Replication Inhibitors

Inhibition of DNA replication and transcription is an important target for antibacterial reagents. Quinolones, coumermycins and novobiocin are some of the DNA replication inhibitors. Quinolones are a class of synthetic antibiotics that interfere with DNA synthesis by inhibiting topoisomerase, an important enzyme involved in DNA segregation. Nalidixic acid is a member of the the first generation quinolones. Fluoroquinolones are the second-generation quinolones that include levofloxacin, norfloxacin, and ciprofloxacin. They are used against both Gram-negative and Gram-positive bacteria. These antibiotics selectively block DNA gyrase by binding to the A subunit of the enzyme and they induce formation of relaxation complex analogue [11, 12]. The coumermycins and novobiocin also inhibit DNA gyrase, however, they bind to the

ATP binding site on the B subunit[12]. Rifampicin (also known as rifampin) which is an important anti-tuberculosis drug blocks initiation of mRNA synthesis by specifically inhibiting bacterial RNA polymerase. This is the only drug in clinical use that blocks bacterial transcription[1]. Mechanism of action for DNA replication inhibitors is demonstrated in Figure 1.8.

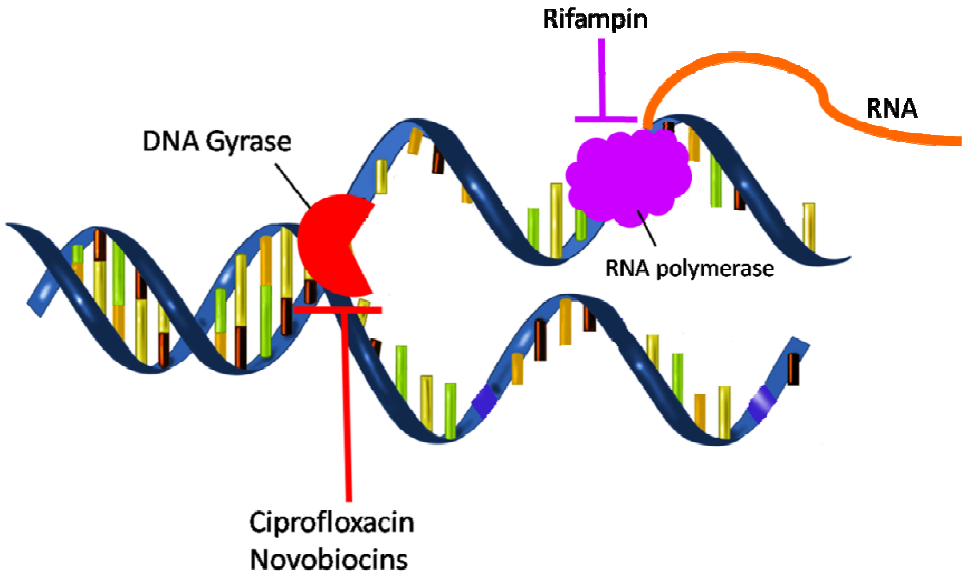


Figure 1.8 DNA and RNA replication inhibitors. Quinolones and novobiocins inhibit DNA gyrase, rifampin blocks RNA polymerase. Figure adapted from [5].

1.1.3.4 Inhibitors of Folic Acid Metabolism

Folate-dependent reactions are important in amino acid metabolism and in biosynthetic pathways leading to DNA, RNA, and membrane lipids[1]. Folate acts as a cofactor for enzymes involved in biosynthesis of DNA. The pathway begins when folate is reduced to dihydrofolate (DHF) which is then reduced to tetrahydrofolate (THF). Dihydropteroate synthase (DHPS) enzyme catalyzes the first step and dihydrofolate reductase (DHFR) enzyme catalyzes the later step. Both DHFR and DHPS are therefore

key enzymes involved in pyridime thymidylate for DNA biosynthesis [13]. Hence, folic acid biosynthesis pathway is an important antimicrobial target of the existing drugs and for pharmacological studies aiming to develop novel antibiotics.

Sulfamethoxazole and trimethoprim are two drugs targeting folic acid synthesis. Sulfa drugs are competitive inhibitors and alternative substrates for DHPS and they block the enzyme dihydropteroate synthase (DHPS) in the pathway to folate. Trimethoprim inhibits dihydrofolate reductase (DHFR) in the last step of biosynthesis pathway[1, 13]. Figure 1.9 shows the folic acid pathway and the enzymes inhibited by antibiotics.

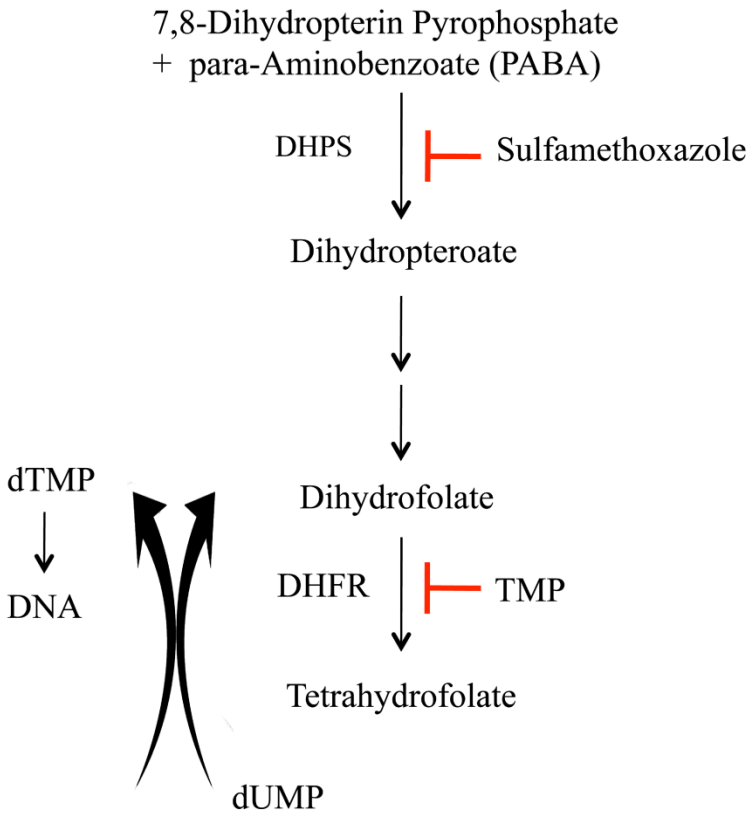


Figure 1.9 The bacterial folic acid biosynthetic pathway, adapted from [1].

1.2 Antibiotic Resistance

Antibiotic resistance is the ability of microorganism to tolerate the inhibitory effects of antibiotic drugs. Bacteria can develop resistance against drugs by spontaneous mutations and horizontal gene transfer. The evolution and spread of antibiotic resistance has become a major threat to public health since a significant portion of the hospital acquired bacteria are resistant to multiple drugs. Uncontrolled antibiotic use and increased mobility of humans and animals in the modern world are two major factors responsible for the increased resistance[3]. Antibiotics act as selective agents and impose a growth advantage to bacterials strains that carry resistance conferring mutations or genes[14]. In hospitals, there is intensive and constant exposure of bacteria to antibiotics. Thus, antibiotic-resistant bacteria are more abundant in hospitals compared to community and nature [14].

1.2.1 Mechanisms for Resistance

Resistance can be described in two ways: intrinsic and acquired resistance. Intrinsic resistance is the innate ability of a bacterial species to resist toxicity of antimicrobial agents through its inherent genetic toolbox, which allow tolerance of a particular drug or antimicrobial class. This natural insensitivity can be due to reduced affinity of the drug to target enzyme or molecule, innate production of enzymes that inactivate the drug, low permeability of drugs because of the pore sizes of membrane proteins, and reduced effective drug concentration as a result of overexpressed efflux pumps[15]. For instance, gram negative bacteria are naturally resistant to vancomycin, glycopeptides, due to limited uptake of these large-sized drugs[1]. Acquired resistance occurs when a naturally susceptible microorganism gets the ability to tolerate the toxicity of a particular antibiotic. Spontaneous mutations and the horizontal transfer of resistance conferring genes (mainly, transposable genetic elements) are two commons ways to acquire resistance[16]. Resistance genes have the ability of moving into other bacteria by different genetic mechanisms; such as, plasmids, bacteriophages and transposons[14]. For instance,

acquisition of resistance gene in *Staphylococcus aureus* causes methicillin resistance and nucleotide substitutions on the RNA polymerase genes give rise to rifampicin resistance in *Mycobacterium tuberculosis*[16].

There are three major mechanisms of antibiotic resistance; namely, enzymatic destruction or modification of the antibiotic by resistant bacteria, mutations or overexpression of efflux pumps, and replacement or modification of the antibiotic target(Figure 1.10) [17].

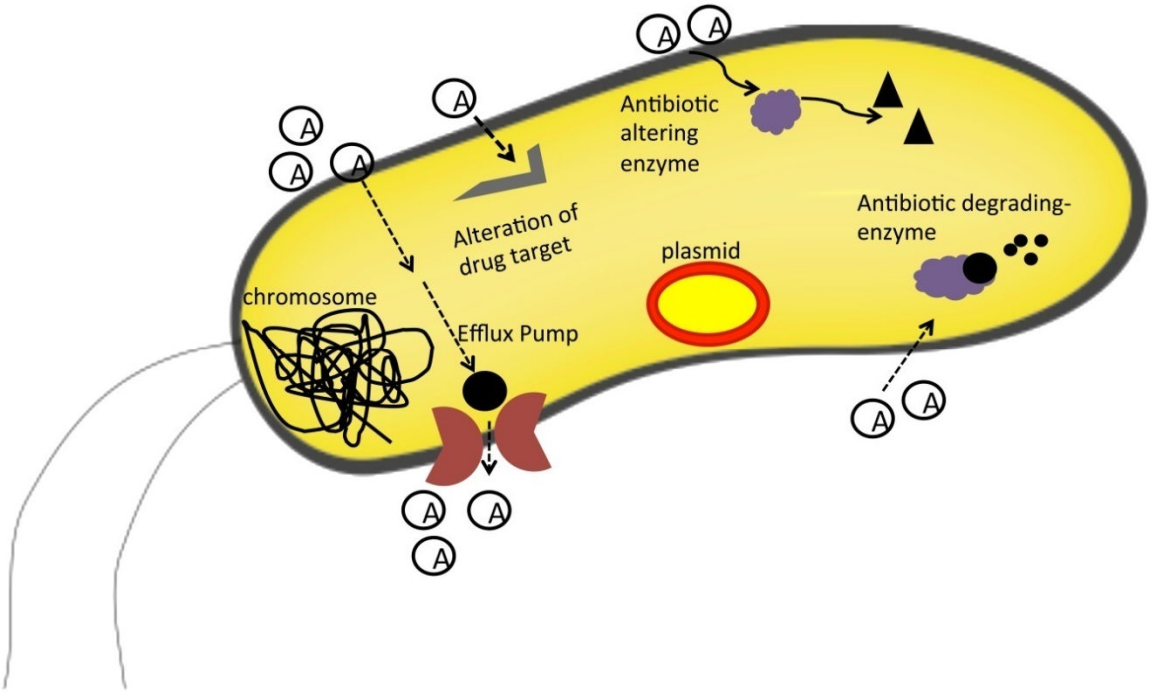


Figure 1.10 Mechanisms for Resistance. Antibiotic altering and degradingenzymes, alteration of drug target, resistance plasmids and upregulation of efflux pumps are major resistance mechanisms. “A” stands for antibiotic.

1.2.1.1 Enzymatic Destruction or Modification of Antibiotic

There are natural enzymes that can modify, cleave or inactivate some of the antibiotics. The most important example is the widespread occurrence of beta-lactamases in nature and clinic[18]. β- lactamase enzymes destroy the beta-lactam ring of penicillin and cephalosporin classes of antibiotics and render them useless [1, 18] . Today, more than 190 β-lactamase genes have been identifiedand categorized into different classes: A, B, C and D [19]. Enzymatic mechanisms to inactivate the antibiotics are shown in Table 1.1. For example, while beta lactamases destroy drugs by hydrolysis, aminoglycosides and chloramphenicol are inactivated by adding acyl group [20].

Some strategies were developed to overcome resistance of beta-lactam antibiotics. One of these strategies is production of semisynthetic beta lactams that are harder substrates for lactamase degradation [1]. Thienamycin, carbapenem group antibiotics, are such examples that are hydrolysed by lactamases in a relatively slower rate. Another approach is finding inhibitors or inactivators of lactamases and using these molecules with β-lactam antibiotics. Clavunic acid, sulbactam, and tazobactam are commonly used inactivators of beta-lactamases[20]. Interestingly, some of these inhibitors are originally derived from β-lactam producing microorganisms. The combination of amoxicillin and clavulanate, known as Augmentin brand, has been one of the most widely used form of penicilin[1].

Strategy	Type	Antibiotics Affected
Hydrolysis		Beta-lactams Macrolides
Group Transfer	Acyl	Aminoglycoside Chloramphenicol
	Phosphoryl	Aminoglycoside Macrolide Rifamycin
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycoside Lincosamide
	Glycosyl	Macrolide Rifamycin
Other	Redox	Tetracycline Rifamycin
	Lyase	Streptogramin (typeB)

Table 1.1 Different enzymatic strategies for inactivation of antibiotics[20]

1.2.1.2 Efflux Pumps

The second important mechanism for drug resistance is exclusion of antibiotics from the cytoplasm via overexpression of efflux pumps or more active mutant efflux pumps[17]. The reduced effective antibiotic concentration inside the cells often have almost no inhibitory effect on bacterial growth and imposes a significant selective advantage to resistant strains[21]. Some efflux pumps are specific to certain antibiotics or antibiotic

classes such as macrolides, lincosamides, streptogramins and tetracyclines, whereas several other pumps, also known as multiple drug resistance pumps, can pump out several structurally diverse drugs [21]. Such multidrug resistance proteins (pumps) are frequently seen in clinical isolates that are resistance to almost all available antibiotics. There are five families of efflux-pump proteins associated with multi drug resistance (MDR): the ATP binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and finally the resistance nodulation division (RND) family[22]. MDR efflux pumps are categorized according to the number of components these pumps carry (single or multiple), the number of transmembrane-spanning structures, the energy source that the pumpsutilize, and the types of substrate specific to the pumps. This kind of resistance mechanism can occur through spontaneous mutations in genes coding for efflux pumps or the transcription factors regulating the expression of these genes[22].

Figure 1.11 shows the five families of multidrug-resistance efflux pumps and common examples of the individual proteins that form each class of efflux pump in gram-negative and gram-positive bacteria. AcrAB are an example of RND type MDR efflux pumps and detailed structure and schematic model of AcrAB-TolC system is indicated in Figure 1.12. A single organism can express MDR efflux pumps of more than one family or more than one type of efflux pumps of the same family. For example, *Escherichia coli* have 20 MFS, 3 SMR and 7 RND members, for a total of 30 efflux pumps[1].

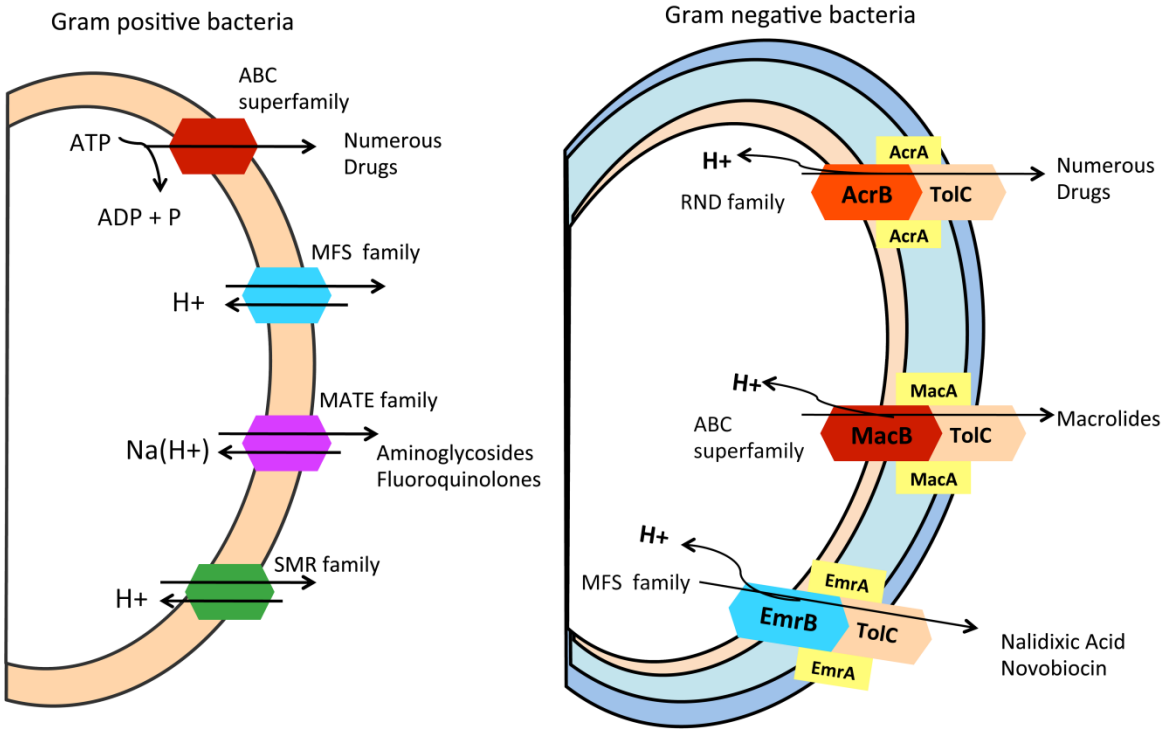


Figure 1.11 Diagrammatic representation of the structure and membrane location of efflux pumps from five families of multidrug-resistance efflux pumps: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family, adapted from [22]

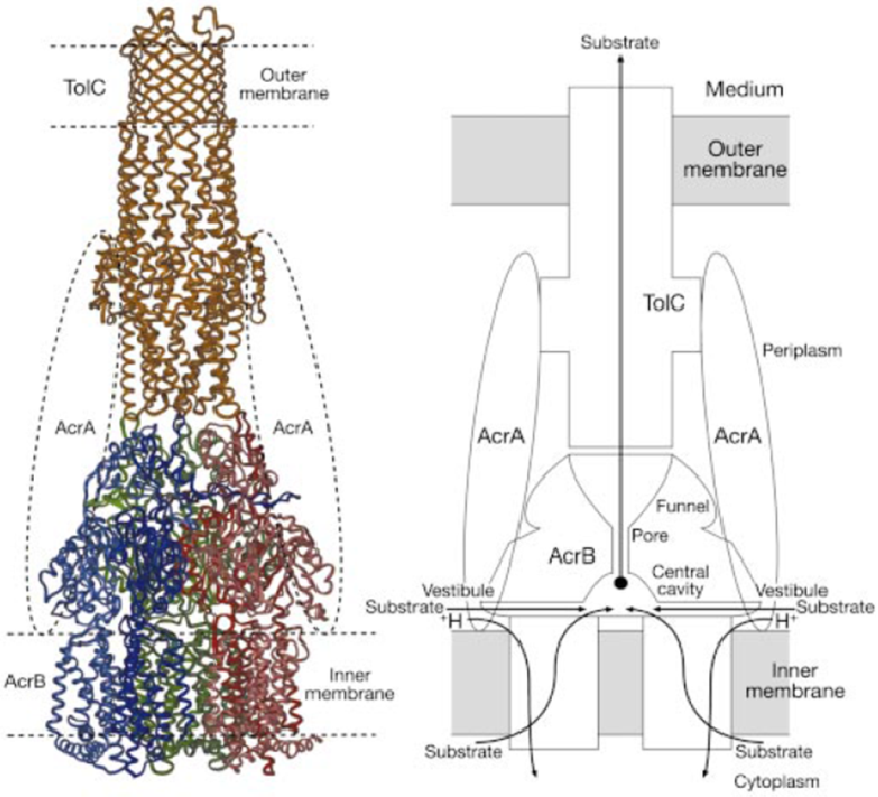


Figure 1.12 Detailed model of the AcrB–AcrA–TolC complex and the schematic mechanismof multidrug export mediated by AcrAB-TolC system[23]

1.2.1.3 Modification of the Antibiotic Target

Some resistant bacteria get rid of antibiotics when critical target sites are mutated and drug affinity is compromised. Most of the antibiotic targets have very important enzymatic functions so organisms cannot completely reduce antibiotics by getting rid of target enzymes via loss of genes. However, it is possible for them to reduce drug susceptibility via mutational changes in the target. In some cases, it was reported that the modification of target structures requires other changes in the cell genome to compensate for the reduced or altered activity of the target enzymes[24].

One of the most important examples of target modifications in the context of drug resistance is the altered transpeptidase, MecA in Staphylococcus aureus that confers resistance against methicillin (methicillin-resistant S. aureus, or MRSA) and many other β-lactam antibiotics [1]. Alterations in penicillin binding proteins (PBP) are other mechanisms observed in many bacteria and make them resistant to beta-lactam antibiotics; such as, *S. pneumonia, Streptococcus pyogenes, Neisseria meningitides, enterococc,*and *Helicobacter pylori*. The altered PBPs are usually generated by recombination events (transformation) between the PBP genes of *S. pneumoniae* and related PBP genes from its streptococcal relatives[24].

Mutations in RNA polymerase and DNA gyrases result in resistance to the rifamycins and quinolones, respectively. Fluoroquinolone-resistant bacteria generally have mutations in GyrA or GyrB subunits of DNA gyrase. Mutations in this region cause resistance due to reduced decreased drug affinity[25]. Mutations in the β-subunit of RNA-polymerase (rpoB) increase rifampicin resistance in *Mycobacterium tuberculosis* [24].

Resistance to MLS group antibiotics is observed in both gram-positive and gram-negative bacteria. Post-transcriptional modification of the 23S rRNA component of the 50S ribosomal subunit (methylation or dimethylation of key adenine bases in the peptidyl transferase functional domain) is responsible for the elevated resistance [1, 24]. Mutations on the 16S rRNA subunit of the ribosome cause resistance for aminoglycoside antibiotics. For example, amino acid substitutions are frequently observed in *Mycobacterium abscessus*

clinical isolates, and are responsible for phenotypes that are highly resistant against amikacin, gentamicin, kanamycin, neomycin, and tobramycin. Additionaly, trimethoprim resistance in bacteria occurs through mutation in the *DHFR* gene producing single amino acid substitution in the dihydrofolate reductase target enzyme [1, 24]. Such target mutations are found in almost all antibiotics and hence impose serious problems in microbial diseases. Chemical improvement of existing antibiotics is promising approach to tackle this problem.

1.2.2 Multidrug Resistance

Multidrug resistance (MDR) is a condition in bacteria that makes them resistant to different classes of antibiotics simultaneously. Use of vast amounts of antibiotics in hospitals, in husbandry, and agriculture has a significant contribution to this problem since many bacterial populations are exposed to different antibiotics several times within short time periods. For example, clinically isolated methicillin resistant *Staphylococcus aureus* (MRSA) strains are often found to be resistant not only to methicillin but also to many of the aminoglycosides, macrolides, tetracycline, chloramphenicol, and lincosamides[26]. Unfortunately, MRSA is not the only example for multidrug resistant bacteria;vancomycin-resistant enterococci (VRE), MDR–tuberculosis, *Acinetobacter, and Salmonella typhi*are other clinically important pathogens.

Multidrug resistance is often acquired by upregulation of genes that code for efflux pumps and drug target enzymes, and accumulation of various resistance conferring transposable genes[26]. Figure 1.13 shows accumulation of multiple genes, each coding for resistance to a single drug, on resistance (R) plasmids. R plasmids are well maintained genetic elements that are transferred efficiently from cell to cell. Assembly of resistance plasmids are done via accumulation of multiple resistance genes by mechanisms provided by transposons, and integrons[26].

The active pumping out of drugs by multidrug efflux pumps is the one of the key mechanisms regarding multidrug resistance. Resistance-nodulation-division (RND) pump

superfamily; such as, AcrB of *E. coli* and MexB of *P. Aeruginosa*, are two well known examples MDR related efflux pumps [26]. In addition to RND family, several multidrug efflux transporters belonging to the Multidrug and Toxin Extrusion (MATE) and ABC superfamily play important roles in MDR. Most gram-negative pathogens can contain several endogeneous genes coding for such pumps, and their expression may become upregulated via mutations on the regulatory regions, increasing resistance to many antimicrobial agents simultaneously with one or two small genetic changes.

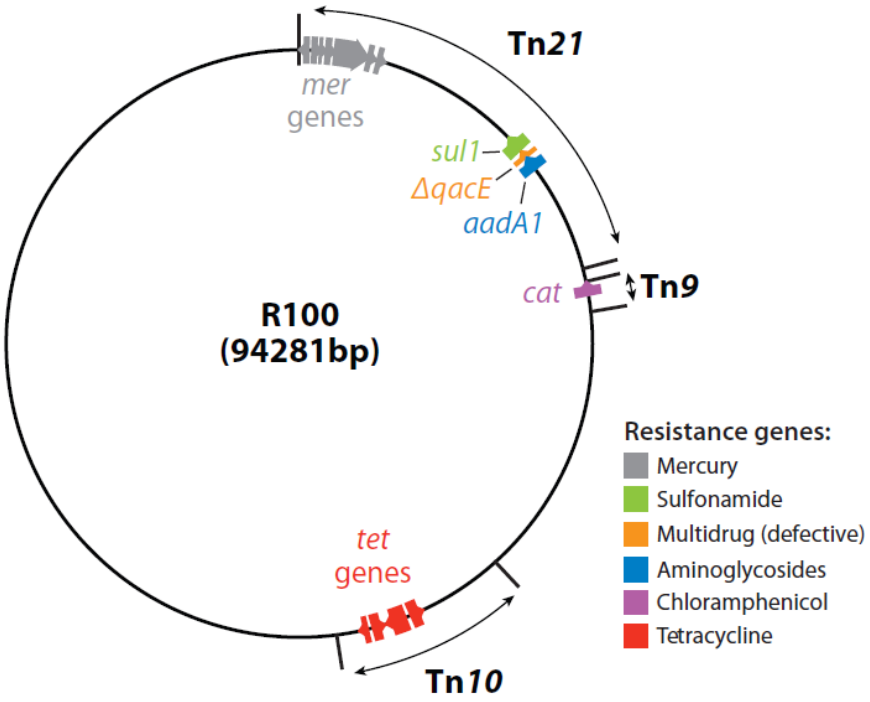


Figure 1.13. Map of an Resistance plasmid R100. Tetracycline resistance gene tetA is in the transposon Tn10, chloramphenicol acetyltransferase (cat) as a part of Tn9, and sulfonamide resistance gene sul1 and an aminoglycoside adenyltransferase gene aadA1 as a part of the large transposon Tn21. This figure is based on the nucleotide sequence deposited by GenBank sequence NC 002134[26]

1.2.3 Evolution of Drug Resistance in Laboratory conditions

The rate of adaptation to high doses of antibiotics and genotypic paths to resistant phenotypes can be investigated by well designed laboratory evolution experiments. Traditional selection experiments, continuous culture devices, and state-of-the-art microfluidic devices are currently used for studying evolution of antibiotic resistance in laboratory settings that mimic natural settings[16]. Conventionally these experiments are carried out by exposing bacteria to fixed drug doses until resistant mutants are observed. This is quite a touchy procedure since finding the appropriate mutant selection window is not straightforward; drug concentrations should be high enough to inhibit growth of the parental drug sensitive strain but yet low enough in order to allow some resistant mutants survive [27]. This approach usually reveals only a few initial adaptive steps. Continuous culture devices facilitate multistep experimental evolution and reveal genetic pathways when combined with whole genome sequencing[28]. For example, a recently developed continuous culture device, the morbidostat, continuously adjusts antibiotic concentration according to the actual rate in which resistance evolves to maintain nearly constant growth inhibition of an evolving microbial population[28]. Microfluidic devices can also carry out multistep experimental evolution in spatial drug gradients[16].

1.3 Drug combinations and evolution of resistance

Antimicrobial treatments increasingly rely on simultaneous use of multiple drugs because of rapid emergence and spread of antibiotic resistance in clinic and limited supply of new antibiotics. The idea simply comes from the expectation that the chance of developing two or three drugs simultaneously is slim. Thus, researchers have been trying to find drug pairs that would perform better when combined togetter. This approach is quite tedious in the sense that drug pairs often behave unexpectedly due to the interactions between them. Such interactions between drugs are classified as synergistic, antagonistic and additive according to whether the combined effect of the drugs is larger than, equal to

or smaller than the effect predicted by their individual activities[29, 30]. Figure 1.9 represents drug interactions schematically.

Clinicians have favored synergistic drug pairs for a long time since synergistic drug combinations generate increased efficacy at lower doses, and not surprisingly antagonistic pairs are generally avoided in clinical applications[27]. However, recent *in vitro* laboratory studies suggested that antagonistic drug pairs may have lower efficacy but have the potential to slow down the evolution of drug resistance, and synergistic drug combinations may be worse since resistance seem to evolve faster[29, 31]. An experimental study conducted by Hegreness et al (2008) showed that evolution in synergistic drug combinations is faster than evolution in antagonistic combinations and each drug separately[30]. Interestingly, this phenomenon was also predicted in an old study by Klein and Schorr (1952). According to Klein and Schorr, the rate of development of resistance to the antibiotics should be known for combined therapy and there is a correlation between the development of resistance and synergism between drugs that are combined. It was shown that in the cases when bacteria developed resistance after growing in each of two antibiotics, these two drugs were frequently found to be synergistic and never antagonistic. When the bacteria did not develop resistance in a rapid way, synergism rarely was the case and antagonism was observed frequently. This was indeed in good agreement with some clinical applications as well. For example, the combination of streptomycin and p-aminosalicylic acid is used in the treatment of tuberculosis although they are antagonistic drug pairs. In this case, the infectious pathogen can develop resistance to one of the chemotherapeutic agents, but since these drugs have separate modes of action,the other drug can still inhibit the resistant cells until the pathogen becomes resistant to both of the drugs simultaneously [32].

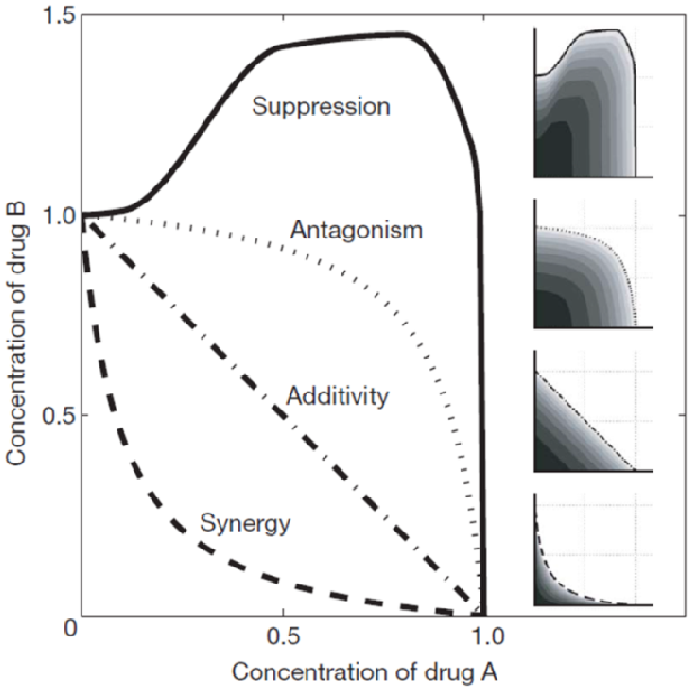


Figure 1.14 Synergistic, additive, antagonistic and suppressive drug pairs[29]

1.4 Cross Resistance

It is known and expected that bacteria develop specific resistance to the antibiotics they are exposed to. However, in some cases, when bacterial populations go through antibiotic therapies, it is observed that they pleiotropically develop resistance to some drugs which they were never exposed to. This phenomenon is named as cross resistance and it is a very important parameter for developing multidrug treatments. Some antibiotics are related to each other in terms of their mechanism of action and therefore resistance against one of these drugs help bacteria when they are exposed to similar drugs. For instance, cross resistance, mediated by beta-lactamases, is observed after therapies with beta-lactam antibiotics, including penicilins, cefalosporins, and monobactams[33]. However, more complicated scenarios were reported in the past where some strains that were resistant to DNA gyrase inhibitors were also resistant against some ribosomal inhibitors.

The first and simplest reason of cross resistance is close chemical similarity of antimicrobial agents, resulting in parallel biological effects. A second explanation can be that chemically different agents may interfere with the same metabolic pathway. For example, one agent may block an enzyme system, whereas another drug may form a complex with intermediary products in the same chain of biochemical events[34]. Other mechanisms for cross resistance can be activation of multi drug efflux pumps or decrease permeability of cell membranes.A study done by Syzbalski et al. (1952) also demonstrated that resistant strains of circulin and polymyxin B, which are microbiologically similar drugs, show 2 to 8 times higher resistance to three related actinomyces antibiotics and also to streptomycin. Their mechanism of action is alteration of structure of cell membrane that results in more permeability. They showed considerable degree of reciprocal cross resistance for each other[34].

Another recent research conducted by Dragosits et al. (2012) shows how cross-stress tolerance emerges during evolutionary adaptation. They used four different stress factors: osmolarity, acidity, oxidation, and n-butanol. As a result of the study, evolutionary cross-stress tolerancewas observed. For instance, n-butanol-adapted strains had very high fitness in hyper-osmatic conditions which was very close to the fitness of osmatic-adapted strains. They found stress factor specific mutations and also mutations in acrA gene that is involved in the acr multidrug efflux system. According to this study, if specific stresses had similar effects at a cellular level, high degree of cross-stress protection could be expected[35].

Systematic studies on evolution of cross resistance in the context of antibiotics are crucial both from basic science and clinical perspectives. These studies have the potential for providing genetic and biochemical information relevant to the development of resistance[34].

2.AIM OF THE STUDY

Understanding genetic changes that elevate antibiotic resistance is of great importance to develop alternative antibiotic therapies that aim to slow down evolution of resistance. In this study, our goal was to design and test novel strategies for minimizing the rate of evolution of drug resistance. In the first part of this project, we carried out long term evolution experiments to reveal genetic evolutionary pathways that lead to antibiotic resistant phenotypes. In the second part, we constructed a large and unique data set that will allow us map genetic changes responsible for elevated resistance by carrying out high throughput phenotyping measurements and whole genome sequencing of drug resistant strains. Successful completion of this project will guide us find generalized rules for designing drug therapies and provide a clear correlation between genotype and phenotype.

3. MATERIALS & METHODS

3.1 Materials

3.1.1 Chemicals & Media Components

Chemicals and Media Components	Supplier Company
Acetone	Merck,Germany
Agar-Agar	Merck,Germany
Antibiotics	Sigma, Germany
Chloroform	Sigma,Germany
DMSO	Sigma,Germany
Ethanol	Merck ,Germany
Glucose	Sigma, Germany
Hydrochloric Acid	Merck, Germany
Luria Broth	Merck, Germany
Magnesium Sulfate	Sigma, Germany
M9 Minimal Salts, 5X	Sigma, Germany
Protein Hydrolysate Amicase	Fluka,Germany
Sodium Chloride	Applichem, Germany

Table 3.1 Chemicals used in the study

3.1.2 Antibiotic and Chemical Solutions

1M $MgSO_4$ (MW:246,48)

12.324 gr $MgSO_4$ was dissolved in 50 mL distilled water.

1M $CaCl_2$ (MW:147,02)

1,47 gr $CaCl_2$ was dissolved in 10 mL distilled water.

Antibiotic solutions from Sigma were made from powder stocks as indicated in Table 3.1 and solutions were kept in -20 ^{0}C.

Antibiotics	**Solvent**	**Stock Solutions**
Chloramphenicol	Ethanol	20 mg/ml
Tetracycline	Ethanol	10 mg/ml
Nitrofurantoin	DMSO	20 mg/ml
Sulfamethaxozole	Acetone	20 mg/ml
Kanamycin	Water	20 mg/ml
Doxycycline	Water	10 mg/ml
Trimethoprim	DMSO	10 mg/ml
Streptomycin	Water	50 mg/ml
Ampicillin	Water	20 mg/ml
Nalidixic acid	Chloroform	20 mg/ml
Ciprofloxacin	0.05 M HCL	10 mg/ml
Amikacin	Water	20 mg/ml
Cefoxitin sodium	Water	10 mg/ml
Piperacillin	Water	10 mg/ml
Tobramycin	Water	10 mg/ml
Spectinomycin	Water	50 mg/ml
Lomefloxacin	Water	10 mg/ml
Fusidic Acid	Water	20 mg/ml
Erytromycin	DMSO	20 mg/ml
Clindamycin	DMSO	10 mg/ml
Spiramycin	Ethanol	20 mg/ml
Sulfamonomethoxine	Acetone	10 mg/ml

Table 3.2 All antibiotics and stock concentrations used in this study

3.1.3 Bacterial Growth Media

M9 minimal medium supplemented with 0, 4% glucose and 0, 2% amicase was used for liquid culture of bacteria. 11.28 gr M9 minimal salts was dissolved in 1 L of distilled water and autoclaved at 121^{o}C for 15 min. 50 gr glucose was dissolved in 500 mL distilled water and autoclaved at 121^{o}C for 15 min. Glucose solution was prepared as 25 X and diluted in media as last concentration will be 1X. Amicase was prepared as 10 X and filter sterilized with Corning, CA memrane 0,22 micron bottle top filters. 2 mL of sterile 1 M magnesium sulfate and 0.1 mL of 1 M sterile calcium chloride was added to 1L of M9 minimal medium.

Luria Broth from Merck was used for liquid culture of bacteria. 25 g of LB Broth was dissolved in 1 L of distilled water and autoclaved at 121^{o}C for 15 min. LB agar from Merck was used for preparation of solid medium for the growth of bacteria. 12-15 g of LB agar was dissolved in 1L distilled water and autoclaved at 121^{o}C for 15 min. Medium was poured onto sterile Petri dishes (20 mL/plate). Sterile solid agar plateswere kept at 4^{o}C.

3.1.4 Bacterial Strains

All experiments were performed with the drug-sensitive, wild type MG1655 *E.coli* strain.

3.1.5 Equipment

All equipment used in this study are shown in Table 3.3.

Equipment	Company
Autoclave	Priorclave, UK
Balance	Sartorius, BP610, Germany Schimadzu, TW423LV, Japan
Deep Freeze	-80 ^{0}C, New Brunswick Sci.,U410,USA -20 ^{0}C, Regal,Turkey
Distilled Water	Millipore, Elix-S, France
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 M200pro
Pinner	V&P Scientific,USA
Plate Shaker Incubator	Heidolph,Germany
Refrigerator	Regal,Turkey
Shaker Incubator	New Brunswick Sci., Innova 44, USA New Brunswick Sci., E24,USA
Spectrophotometer	Amersham Biosciences, UK
Vortex	VWR,USA
Whole-genome sequencing	Illumina Gene Analyzer IIx, USA

Table 3.3 Equipment used in the study

3.1.6 Software

MatLab and Samtools Software kit were used for data analysis.

3.2 Methods

3.2.1 Bacterial Cell Culture

E.coli (MG1655) strain was grown 24 hours at 30 ^{0}C shaking at 200 rpm in sterile M9 minimal medium supplemented with 0.4% glucose and 0.2% amicase. Bacterial strains either streaked or spreaded were grown on LB agar petri dishes overnight. Growth temperature on LB agar medium was 37^{o}C. Single colonies were picked from LB agar petri dishes. Experiments were started from single colony of *E.coli.* For the glycerol stock preparation of bacterial cells, glycerol was added to the overnight grown bacterial cultures to a final concentration of 15%. Cells were frozen and stored in multiple aliquots at -80^{o}C.

3.2.2 Measurement of Growth Rate

Growth rate was measured as prepared in 96-well plates, with 150 µl per well by TECAN. Matlab program was used to analyze the growth rate of strains.

3.2.3 Determination of Minimum Inhibitory Concentrations (MIC)

MIC line of antibiotics was measured by a standard overnight growth assay in liquid media, inoculating wild type *E.coli* in each of 96 wells. 150 µl minimal media was

added per well and antibiotic solutions was diluted serially with two fold intervals in each column. For each drug, typically 11 different concentrations were tested.

The MIC line was defined as the line separating regions of growth and no growth. Practically, the lowest drug concentration at which background-subtracted OD was less than 0.04 after 24 h was defined as MIC.

3.2.4 Evolution Experiments

MIC values of each drug were identified by serial dilution method as described above. Antibiotic solutions were prepared in different stock concentrations from power stocks and stored in -20 ^{0}C.

On the first day of the experiment, 4 replicates of six different concentrations of each drug with twofold intervals were prepared in 3 ml M9 minimal medium. Drug concentrations were prepared according the MIC values of antibiotics. Starting OD of the each culture was 0.00005; indicating that approximately $25x10^{3}$ bacteria were inoculated. On the second day, cultures were separated as duplicates for strong and weak selections and these parallel cultures were not mixed after the first starting culture. Growing cultures were identified by spectrophotometer and by visual examination. 100 µl of grown cultures were added to newly prepared 3 ml cultures; that is, 30 fold dilutions were made in each day. For strong selection, always last growing culture was continued to the next day. For weak selection, bacteria were chosen from two behind the last growing culture. By this way, different selection powers were applied on bacteria cultures. Drug concentrations were adjusted according to growth conditions of cultures in every day. If there was no growth in one day, same drug concentrations were applied in next day. If evolution was observed, drug concentrations were increased and glycerol stocks were prepared and stored. Evolution experiments were continued for 21 days. Figure 3.1 shows the schematic overview of evolution experiments.

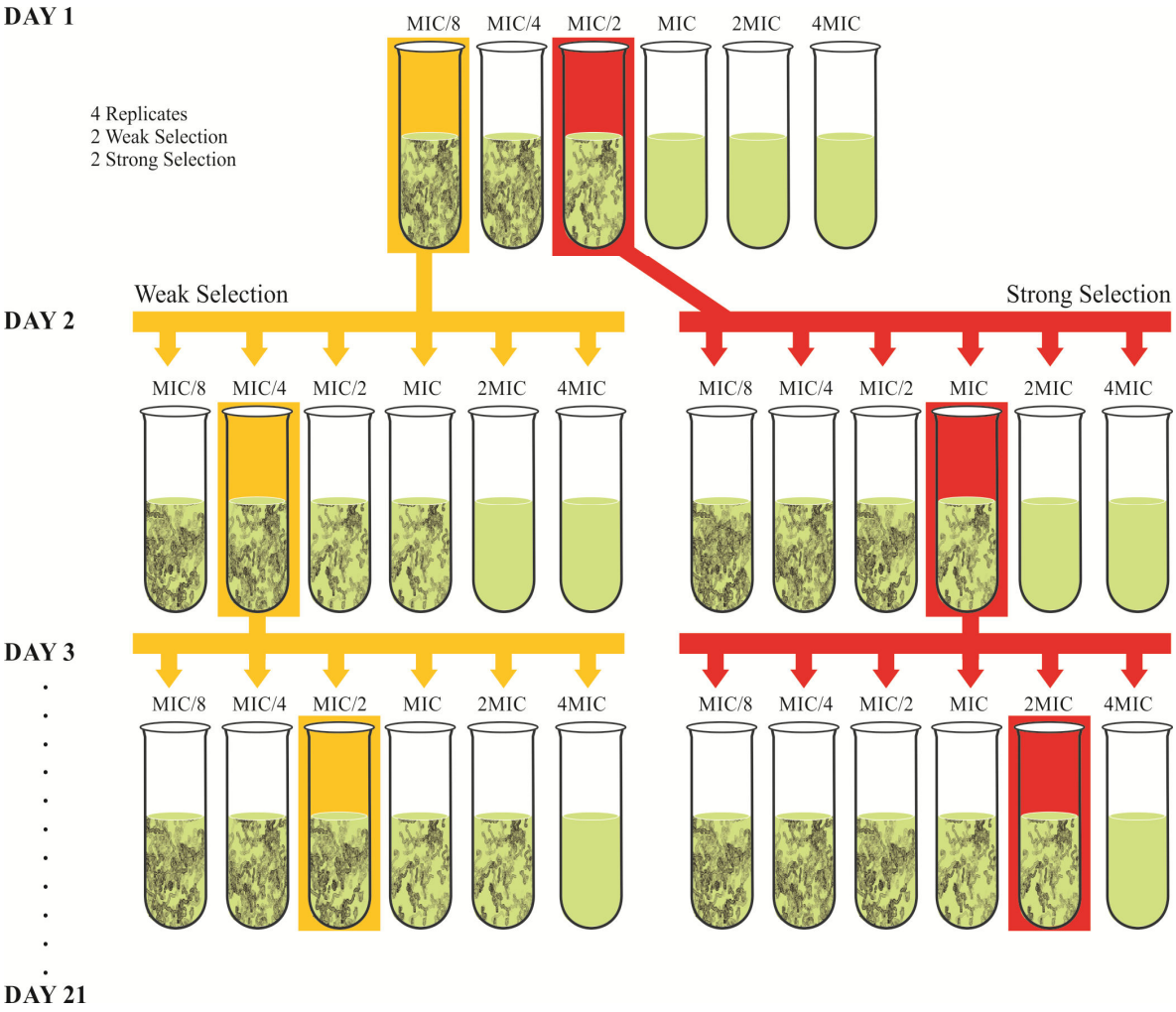


Figure 3.1 Schematic representations of evolution experiments. Yellow color shows weak selection and red color shows strong selection.

3.2.5 Phenotypic Characterization

3.2.5.1 Representative Colony Selection

After 21 days of evolution experiments, 88 evolved strains (4 strains for each antibiotic) were obtained. . Mixed cultures were streaked on LB agar medium without drug and grown at 37 ^{0}C. Ten representative colonies were picked randomly from each mixed cultures of evolved strains and cultured in liquid medium. Their MIC values were identified by serial dilution on 96-well plates. If MIC of a colony is same with MIC of mixed cultures, it was called as representative colony.

3.2.5.2 Growth Data

Master plates were prepared from representative colonies of evolved cultures. The cells in master plates were transferred into experimental plate, including 150 µl minimal media per well, using a 96-pinner. Cells in 96 well plates were grown in the TECAN-M200 for 24 hours at 30^{0}C, with taking data points in every 10 minutes. Growth curves and rates were determined for 88 evolved cultures.

3.2.5.3 Cross Resistance

For each antibiotic, several experimental plates were prepared in the range of MIC/4 to 2 fold of highest MIC of evolved strains with square root 10 (approximately 3, 16) intervals. The cells in master plates were transferred into experimental plates using pinner. Cultures were grown at 30^{0}C for 24 hours with rapid shaking. Experimental design was explained in Figure 3.2. Growth was measured in TECAN at 600nm and cross resistance data were analyzed in Matlab.

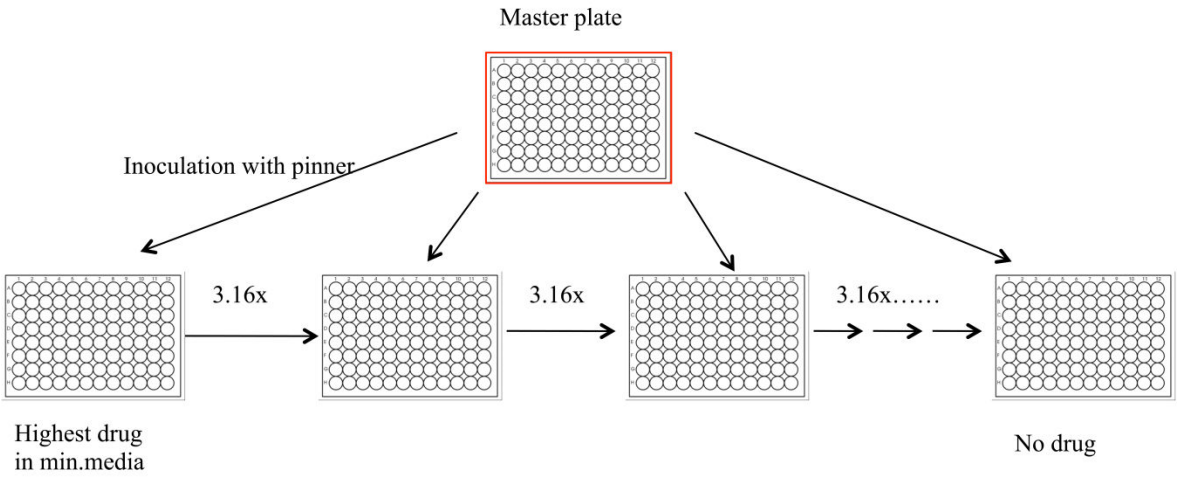


Figure 3.2 Experimental design of cross resistance. The plate on the left part has highest drug concentration. Drug concentrations are reduced 3.16 folds for each plate and last one has no drug. Bacteria are inoculated from master plate with pinner.

3.2.6 Genotypic Characterization

3.2.6.1 Next Generation Sequencing: Illumina

The automated Sanger method is considered as a 'first-generation' technology, and newer methods are referred to as next-generation sequencing (NGS). Currently, Illumina Genome Analyzer dominates the NGS market. It uses the clonally amplified template method coupled with the four-colour CRT method[36].

3.2.6.2 Whole Genome Sequencing

Representative colonies of each culture were sent to Genewiz NGS-Laboratory, USA in agar stabs for whole genome sequencing. DNA purifications of cultures were done

by Genewiz Company. Illumina genome analyzer IIx (101-bp single end reads, minimum coverage 100x per strain) was used for whole genome sequencing.

3.2.6.3 Mutation and Amplification Analysis

Reads of genome sequencing were aligned onto the MG1655 reference chromosome (NC_000913.2) using the Illumina pipeline. SNPs were identified with SAMtools.

4. RESULTS

In this study, 88 *E. coli* populations were evolved in increasing concentrations of 22 clinically relevant antibiotic compounds for ~100 generations. By using Illumina whole genome sequencing100 drug resistant strains and carrying out phenotyping measurements, we constructed a large data set that allows us to map genetic changes responsible for elevated resistance.

4.1 Bacterial Evolution Results

4.1.1 Evolution Strategy

In the first part of this study, we conducted microbial evolution experiments to generate antibiotic resistant strains which will be used to understand the genetic changes that elevate antibiotic resistance and compare level of cross resistance between antibiotics. A total of 22 different antibiotics from different classes were used in this study. Table 4.1 shows the antibiotics and their mechanisms of actions. We tried to choose at least 3 antibiotics from different classes except nitrofurantoin which carries multiple modes of action. As summarizedin methods section, selection procedure was carried at two different selection strengths in order to investigate the differences in the evolutionary process and

role of selection strength in the target specificity of drugs. For each antibiotic compound we used four replicates: two replicates for strong and two replicates for weak selection.Drug concentrations that applied relatively similar selection were updated and accordingly adjusted almost each day and evolved bacteria were transferred to the newly prepared cultures on a daily basis. Figure 4.1 shows our experimental design for microbial evolution.

Antibiotics	**Main mechanisms of action**
Chloramphenicol	Protein Synthesis, 50S
Clindamycin	Protein Synthesis, 50S
Erythromycin	Protein Synthesis, 50S
Spiramycin	Protein Synthesis, 50S
Fusidic Acid	Protein Synthesis, 50S
Tobramycin	Aminoglycoside, protein synthesis,30S
Amikacin	Aminoglycoside, protein synthesis,30S
Kanamycin	Aminoglycoside, protein synthesis,30S
Streptomycin	Aminoglycoside, protein synthesis,30S
Tetracycline	Protein synthesis,30S
Doxycycline Hyclate	Protein synthesis,30S
Spectinomycin	Protein synthesis,30S
Ampicillin	Cell wall
Piperacillin	Cell wall
Cefoxitin	Cell wall
Lomefloxacin	DNA gyrase
Ciprofloxacin	DNA gyrase
Nalidixic Acid	DNA gyrase
Trimethoprim	Folic acid biosynthesis
Sulfamonomethoxine	Folic acid biosynthesis
Sulfamethoxazole	Folic acid biosynthesis
Nitrofurantoin	Multiple mechanisms

Table 4.1 All antibiotics used in this study and their mechanisms of actions

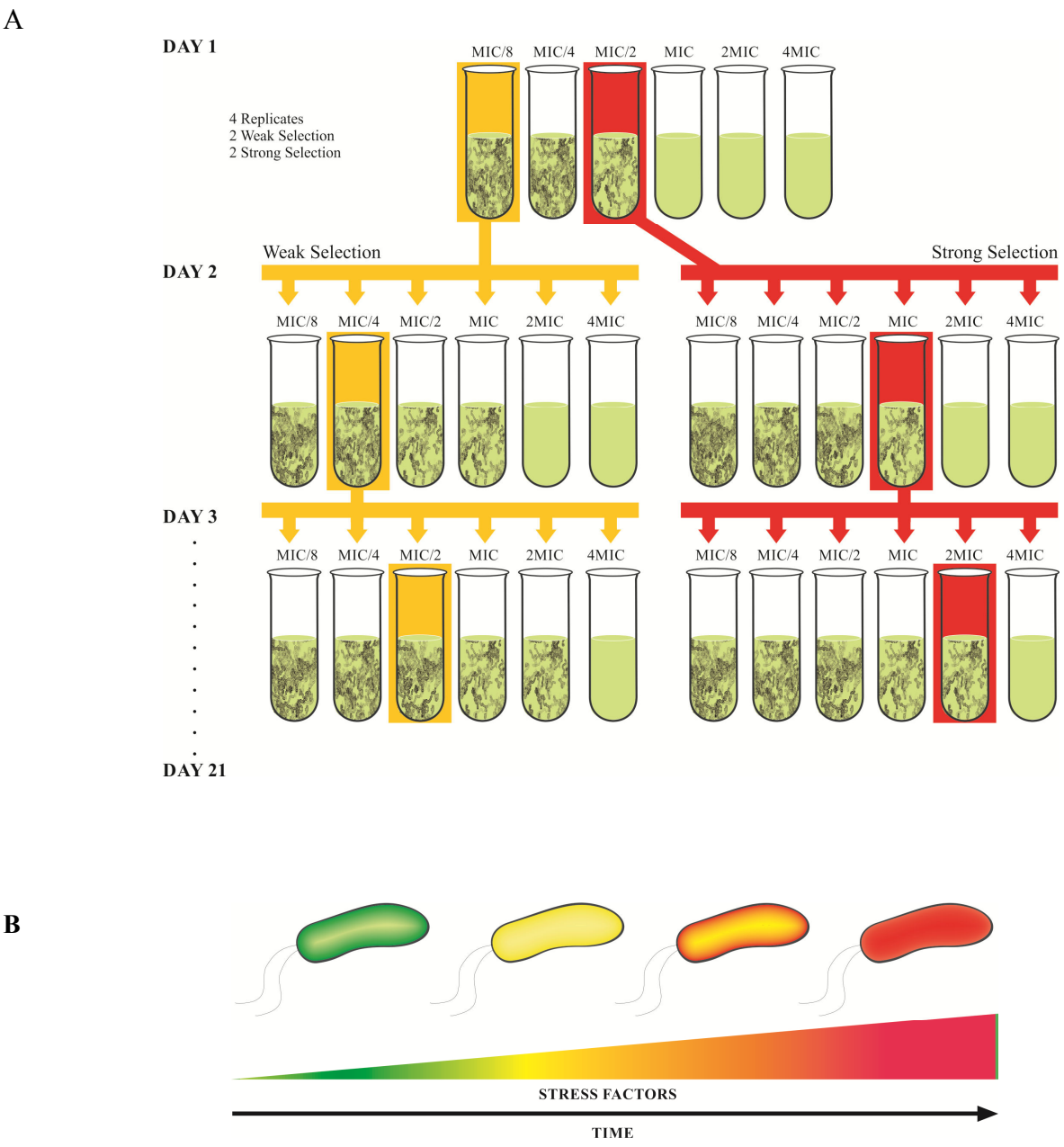


Figure 4.1 Experimental design. A) Red shows the strong selection, yellow shows the weak selection. For strong selection, always last growing culture was chosen and for weak selection two behind of the last growing culture was taken. Experiment continues for 21 days. B) With the increasing of time and stress factors, resistance levels of bacteria also increase.

4.1.2 Bacterial Evolution Results

88 isogenic wild type drug sensitive *E. coli* populations evolved in increasing concentrations of 22 clinically relevant antibiotic compounds for ~100 generations. Figure 4.2 exhibits all adaptation trajectories. Red lines represent the phenotypic changes of populations that were evolved under strong selection whereas black lines represent the phenotypic changes of populations that were evolved weak selection. Resistance levels of many populations increased several orders of magnitude after 21 days. These MIC values were further verified with measurements done in 96 well plates and a TECAN microplate reader. These populations were evolved in the presence of sulfamethoxazole, cefoxitin, tobramycin, streptomycin, and spectinomycin became super resistant. However, in some cases, resistance levels increased only ~ 10 folds. For example, MIC values for doxycyline and tetracycline resistant populations increased 8 and 16 folds, respectively.

Using the adaptation traces, we were able to compare levels of evolved resistance and rate of adaptation for all of the evolving populations. Our data suggest that in many cases, populations under strong selection reached to higher levels of resistance. Differences between selection strengths can be easily seen with the populations evolving in the presence of tobramycin, spectinomycin, and nitrofurontoin. Strongly selected resistant strains of these antibiotics have very high resistance levelscompared to the weakly selected ones. For instance, nitrofurontain resistance of strongly selected populations is approximately 50 times higher than the weakly selected populations. In fact, weakly selected populations only developed two fold increases in their MIC. On the other hand, selection strength did not affect the evolved resistance levels in some cases; such as: doxycycline, tetracycline, clindamycin, and nalidixic acid. Resistance to these drugs reached to same levels in both weak and strong selection strategies. Figure 4.3 shows the scatter plot of MIC values for strong versus weak selection for all 22 antibiotics we used. As anticipated, MIC values for strongly selected populations were higher in most cases. Finally, for some cases, we observed that the resistance levels of replicates, especially in strong selection mode, were significantly different than eachother. For example, for piperacillin, one of the strongly selected populations had ten fold higher resistance than the

other strongly selected population.Variation levels between replicates are represented by the error bars (standard deviation) in the scatterplot in figure 4.3.In summary, over time, the resistance level increased dramatically, with similar changes in parallel evolving populations.

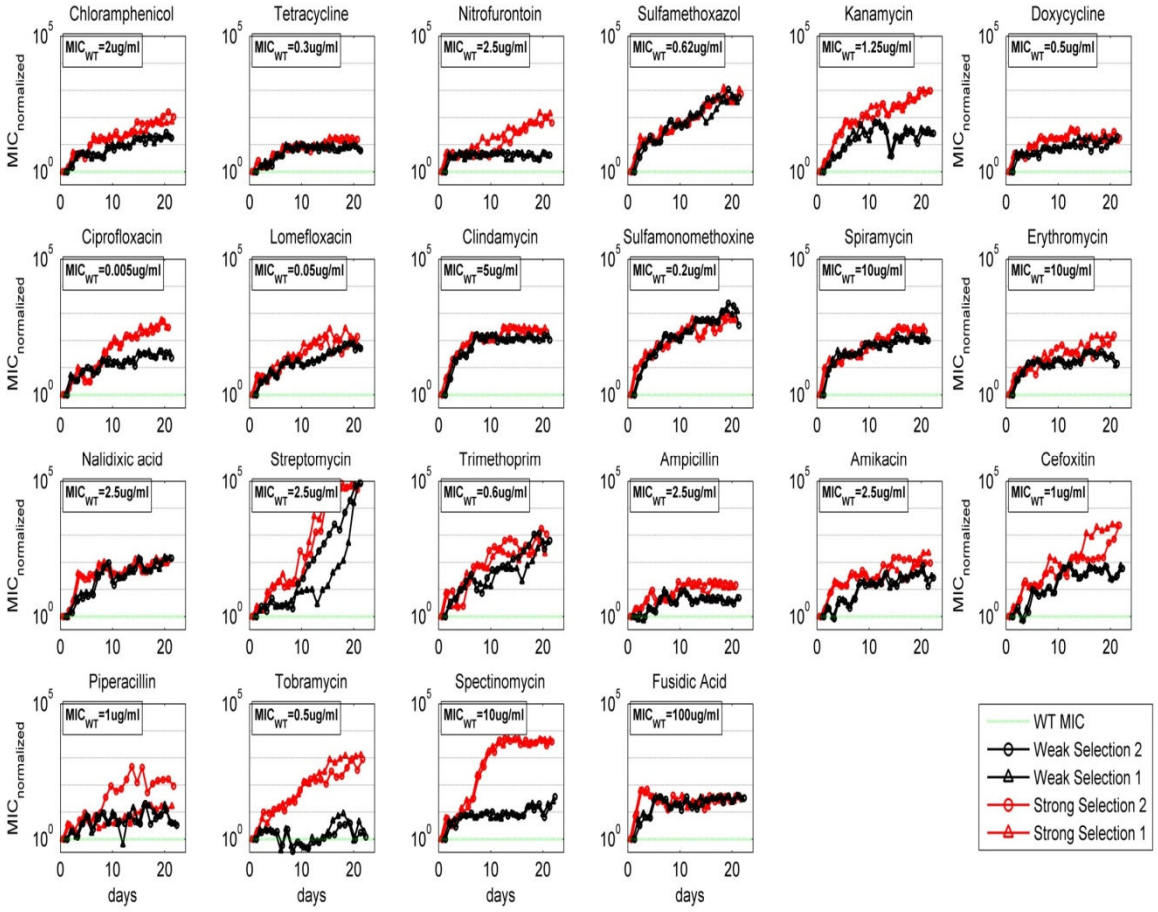


Figure 4.2 Microbial Evolution to 22 drugs.*E.coli* populations rapidly evolved high antibiotic resistance. Sample measurements of MIC versus time.

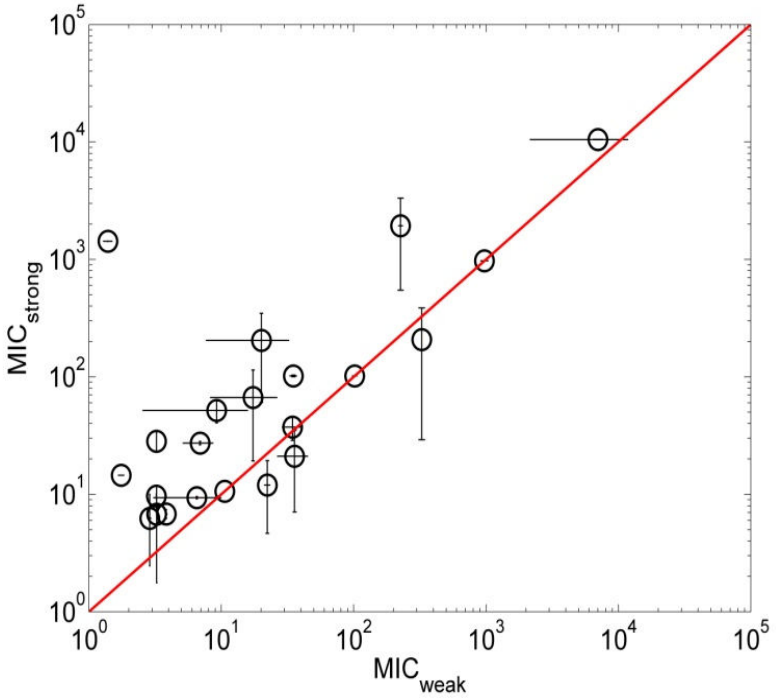
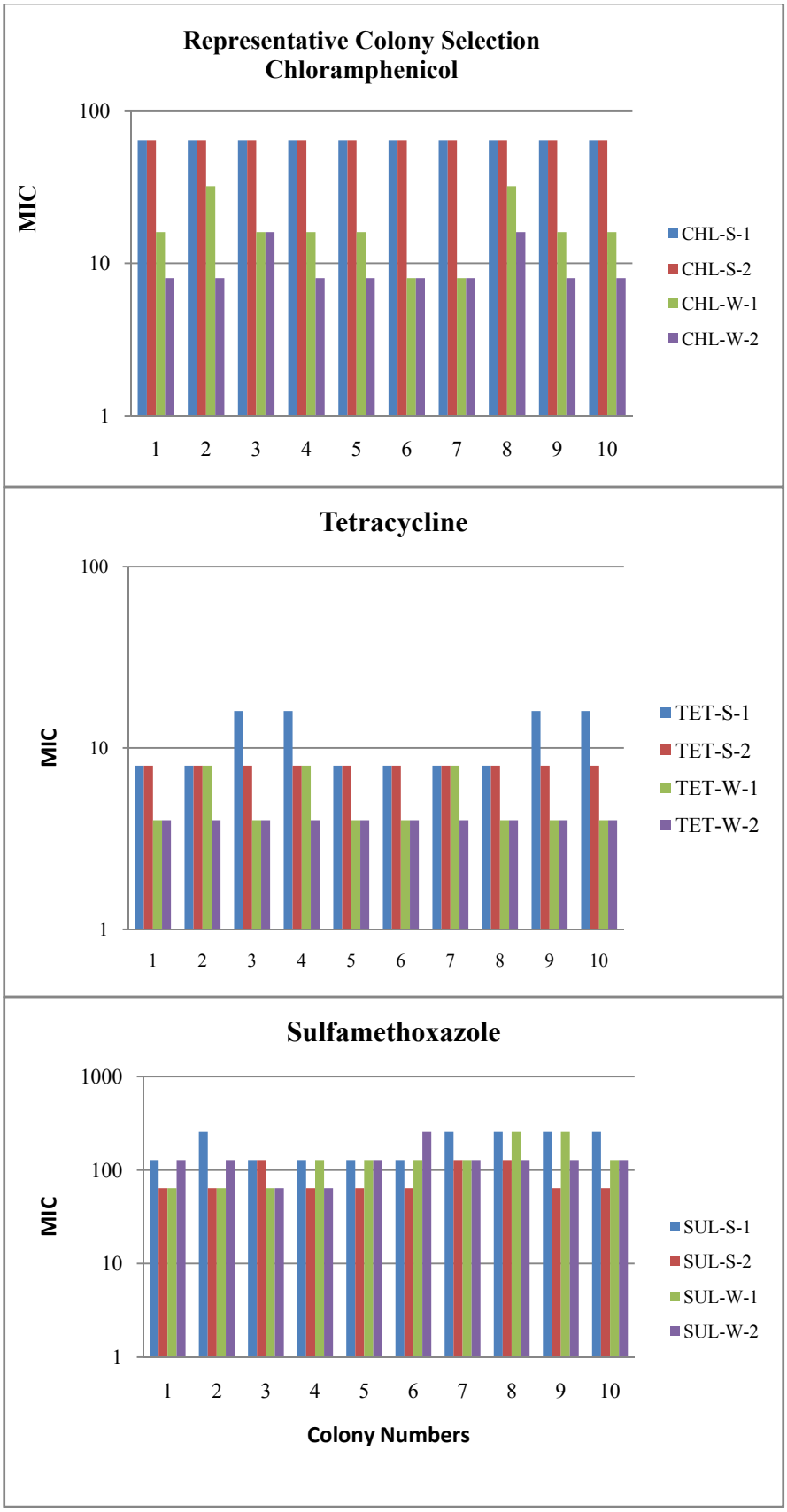


Figure 4.3 Comparison of MIC-Strong versus MIC-weak values. Error bars show variation levels between replicates. Red line indicates equality of MIC-strong and weak.

4.1.3 Representative Colony Selection

We plated population samples from the final day of the experiment and randomly selected ten colonies. We carefully quantified MIC values for each isolated colony; MIC values' distributions are shown in Figure 4.4. Although some minor fluctuations in MIC values were observed, majority of these colonies had similar phenotypes with the populations they were isolated from. One colony from each population that had the highest MIC value among all of the colonies was isolated as the representative colony for further genotypic and phenotypic investigation. In Figure 4.4, phenotypic distributions of the colonies for one example from each drug classare shown. The entire distribution data set can be found in appendix A. Representative colonies were sent for Illumina whole genome sequencing, and a master plate carrying all of the representative colonies was prepared to be used in phenotypic measurements.


Representative Colony Selection
Chloramphenicol
MIC
100
10
1
1 2 3 4 5 6 7 8 9 10
CHL-S-1
CHL-S-2
CHL-W-1
CHL-W-2
Tetracycline
MIC
100
10
1
1 2 3 4 5 6 7 8 9 10
TET-S-1
TET-S-2
TET-W-1
TET-W-2
Sulfamethoxazole
MIC
1000
100
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1
1 2 3 4 5 6 7 8 9 10
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SUL-S-2
SUL-W-1
SUL-W-2

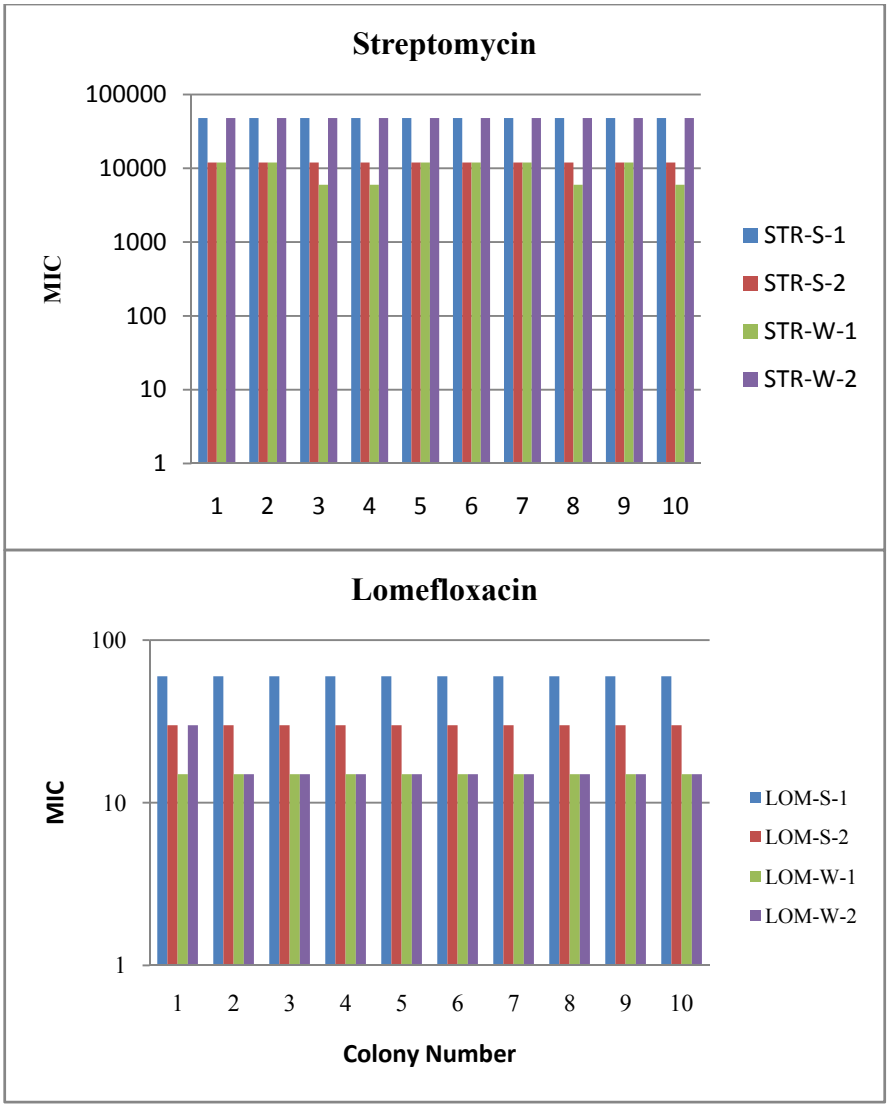


Figure 4.4 MIC values of colonies for each evolved strains of chloramphenicol, tetracycline, sulfamethoxazole, streptomycin and lomefloxacin antibiotics. 10 colonies were selected from each evolved culture of drugs and shown in x-axis. MIC values of colonies are indicated on the y-axis. Blue and red colors show strongly selected first and second strains, respectively. Green and purple colors are for weakly selected strains.Graphs are shown in logarithmic scale.

4.2 Cross Resistance

Resistance levels of all representative colonies against all 22 drugs were carefully quantified using a high-throughput assay as described in methods. Figure 4.5 shows sample phenotypic measurements where green line indicates the growth of wild-type, red line indicates the elevated resistance, and blue line indicates increased sensitivity. As expected, colonies had high resistance to the drugs they were evolved against. We call this type of resistance as "direct resistance". We observed cross resistance mostly within the strains that were evolved in drugs belonging to the same class but there were several exceptions showing increased cross resistance against antibiotics that belong to different classes as well. To our surprise, we also found many cases where some strains developed increased sensitivity to several other drugs. After repeating these measurements for all of the strains, we ended up having 1958 phenotypic measurements and combined all the data in a matrix shown in Figure 4.6 for better representation.

Figure 4.6 shows the full matrix of the phenotypying data. We placed evolved strains on the x- axis and the antibiotics they were phenotyped on the y-axis. Color map for increased resistance arranged between light pink and red. All of the resistance values were normalized with the highest direct resistance value; hence, the diagonal line from bottom left to upper right is mostly surrounded with dark red pixels. Similarly, phenotypes with increased susceptibility are represented with colors between light blue and dark blue. White colored pixels represent phenotypes that do not have any difference from the wild type parental strain in terms of MIC.

In order to better understand the data set, we divided the full matrix into strong and weak data as shown in Figure 4.7A and 4.7B. As expected, the levels of resistance in strong data is higher and this is reflected with the relatively darker colors compared to the weak matrix.

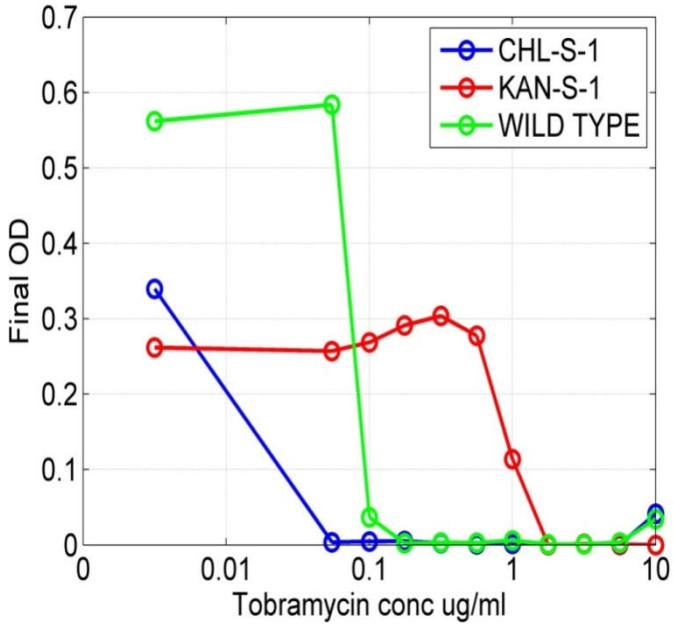


Figure 4.5 Identification of cross resistance of chloramphenicol and kanamycin strains to tobramycin. Green line shows the wild type, red line shows the increased resistance and blue line indicates increased sensitivity.

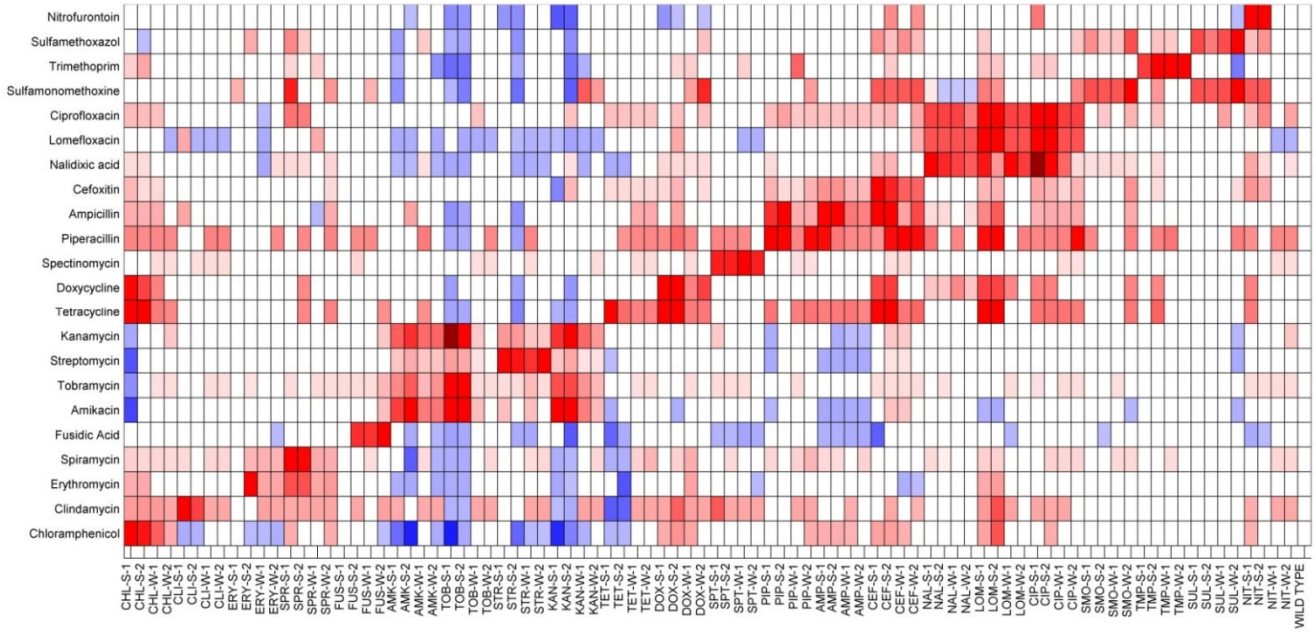


Figure 4.6 Full Matrix of Cross Resistance

We segmented the strong data shown in Figure 4.7A into drug classes to better understand the general evolutionary behavior and trade-offs. This way, we were able to find generalized rules for resistance evolution. The most striking observation we had was the overall increased antibiotic susceptibility of strains evolved in aminoglycosides (kanamycin, tobramycin, amikacin and streptomycin).These strains had at least 60% increased susceptibility against almost all of the drugs except aminoglycosides. Direct and cross resistances within aminoglycosides were generally reciprocal such that tobramycin resistant strains were resistant to amikacin and vice versa. Only streptomycin resistant strains did not show this feature that significantly. In DNA gyrase inhibitor antibiotics, we saw reciprocal resistance increase for all drugs. This was pretty common in all other drug groups as well suggesting that reciprocal behavior is common within same drug classes. Another interesting finding was that most of the evolved drug resistant strains became more sensitive to lomefloxacin but not to other gyrase inhibitors.

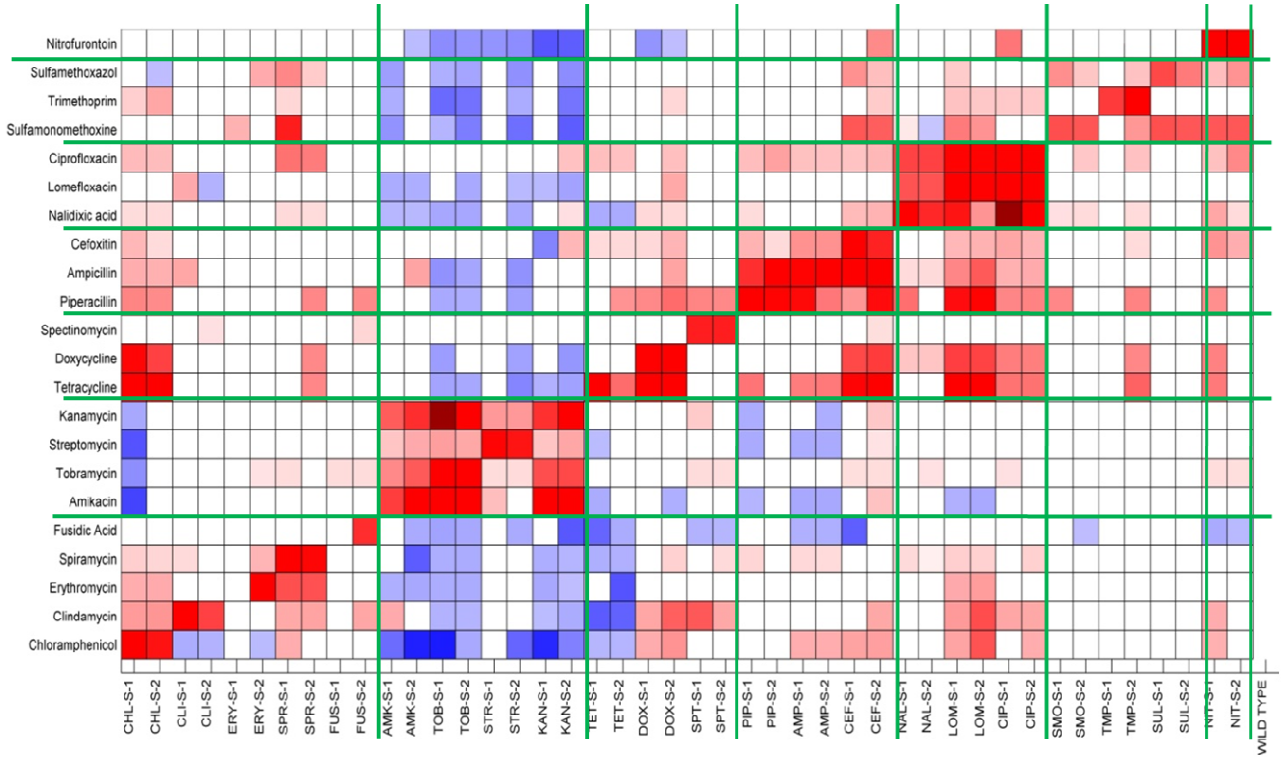


Figure 4.7 A) Cross resistance matrix of strong selection strains

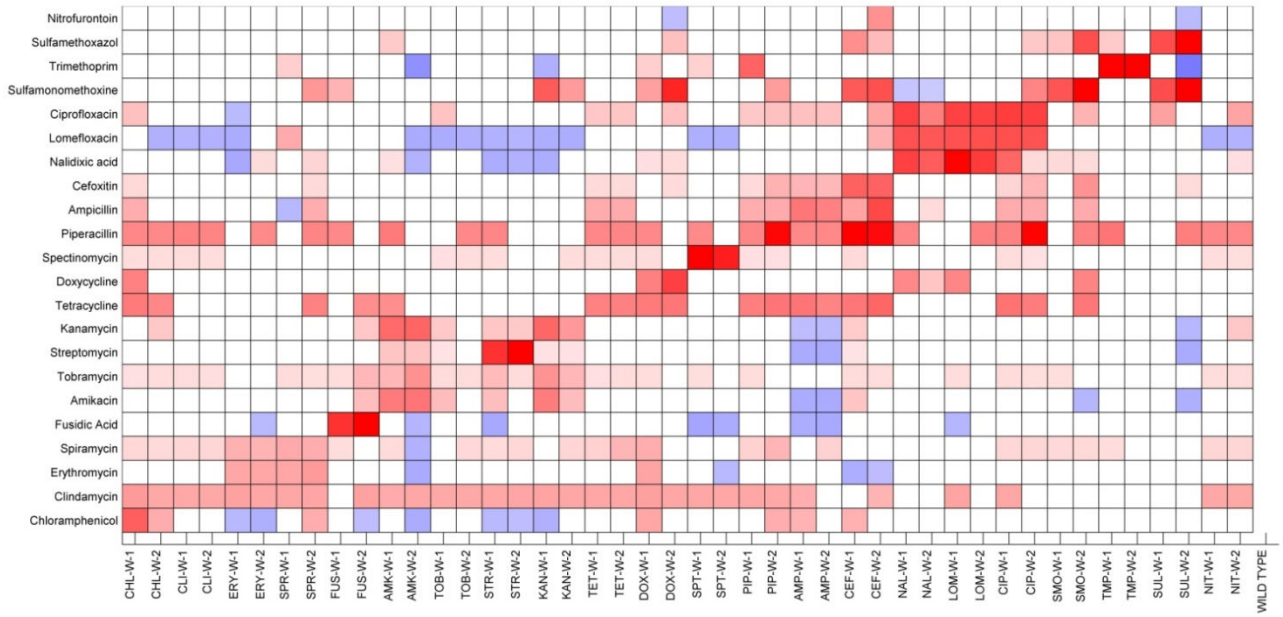


Figure 4.7 B) Cross resistance matrix of weak selection strain. Blue indicates sensitivity, red shows resistance and white means same with wild type. In the x axis, weakly evolved strains were place and in the y-axis, antibiotics were placed. All strains and antibiotics were arranged as same classes of drugs are close to each other.

Figure 4.7B shows the weak phenotyping data set. Colors are lighter than strong selection but general pattern is similar to strong selection. Reciprocal behavior was observed within the same drug groups not across different drug classes with a few exceptions. For instance, spectinomycin and clindamycin resistant strains reciprocally evolved cross resistance.

In order to test the reproducilibity of the phenotyping measurements, we repeated all of the measurements independently and compared them with the original measurments as shown in Figure 4.8 and found that data was reproducible for more than 95%.

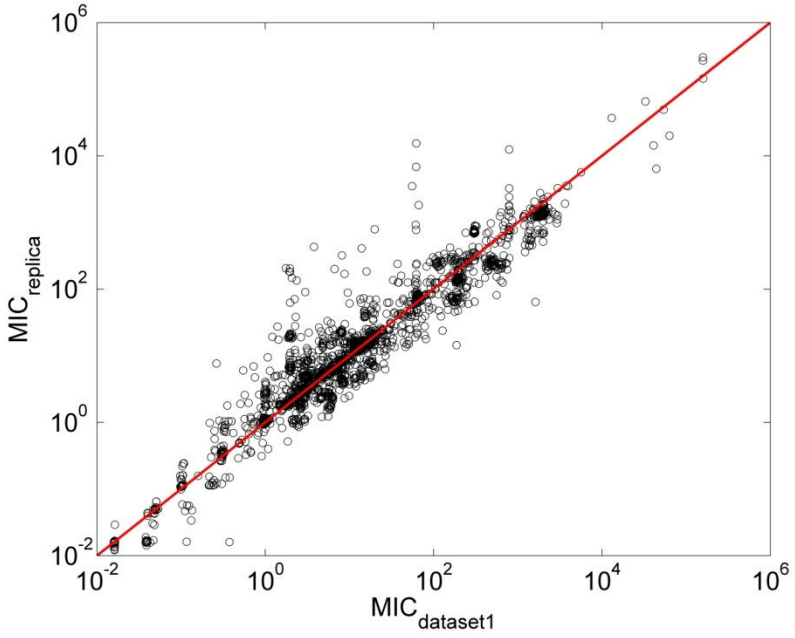


Figure 4.8 Quality control of cross resistance data set: More than 95% reproducibility

To better understand the mechanism of resistance, we made some generalization for drug classes and tried to form a simple view of the matrix, shown in Figure 4.9. Reciprocal evolution was observed in most of the same drug classes except protein synthesis-30S inhibitors. All drugs of DNA gyrase inhibitors and cell wall inhibitors had reciprocal evolution in their groups. We did not observe general pattern of reciprocal evolution across the groups. According to our cross resistance data, aminoglycoside resistant strains became more susceptible to nitrofurantoin, protein synthesis-50S, DNA gyrase and folic acid metabolism inhibitors. Resistant strains of protein synthesis-30S inhibitors did not show cross resistance behavior mostly. Only doxycycline resistant strains get resistant toward tetracycline but interestingly tetracycline resistant strains did not get any resistance to doxycycline. Additionally, Figure 4.9 shows that evolved strains of DNA gyrase inhibitors become resistant to cell wall inhibitors and evolved strains of nitrofurontain, a multiple mechanism drug, get resistant to folic acid biosynthesis inhibitors.

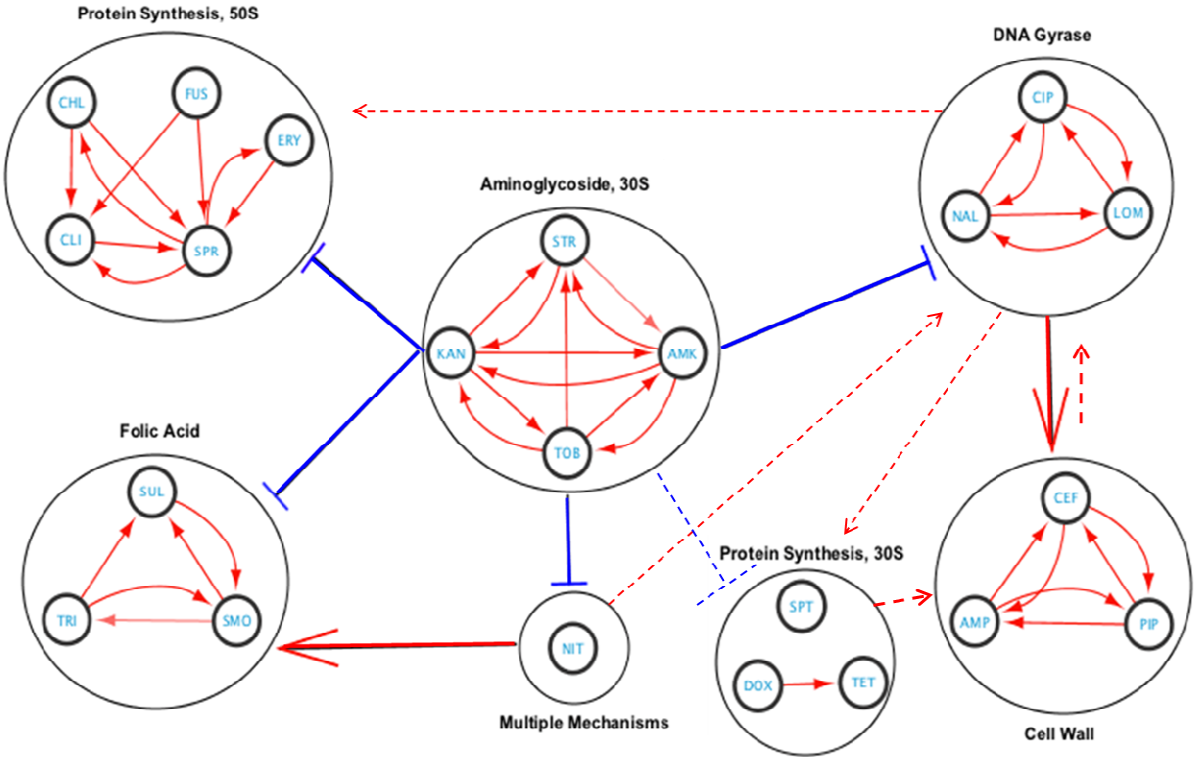


Figure 4.9 Cross resistance interactions by drug groups: multiple mechanisms, protein synthesis-50S, 30S& aminoglycosides, cell wall, DNA gyrase and folic acid biosynthesis inhibitor.

4.3 Whole Genome Sequencing

Representative colonies of each culture were sent to Genewiz NGS-Laboratory, USA for whole genome sequencing. Illumina genome analyzer IIx was used for whole genome sequencing. Properties of sequencing are 101-bp single end reads and minimum coverage 100 X per strain.

Whole genome sequencing revealed the genotypic characterization of evolved strains. Some strains were sent as replicates to make sure about the sequencing. We sent two wild type strains and compared our genome data of evolved strains with our wild type *E.coli* strain. For analysis we used SAMtools software kit.

4.3.1 WGS - Mutation (SNP) Results

Whole genome sequencing of 88 strains identified mutations and revealed that selection power affects the mutation types. By analyzing mutations, we tried to understand how selection power affects the target specificity. There were many drug-specific mutations, and some shared mutations.In some cases strong selection strains of same antibiotic had different mutations. Figure 4.10 shows the mutated genes for all drug resistant strains except from cefoxitin strong selection strains as a network. We could not show each mutated genes of cefoxitin strain since strong selection strains of cefoxitin drug had ~ 200 mutations. We saw that some genes are shared in different classes of antibiotics for mutations; such as, fusA, fis, marR, rph, trkH, ompR, ompF, acrR, mprA and gyrA. Table 4.2 shows the functions of these shared genes, mutation spots and number of mutations on the gene. All mutation positions and functions of mutated genes are given in appendix B as a table.

Mutated Gene	Function of gene	Mutation points	#of mutations
fusA	Protein chain elongation factor, Translation	G117C(2),V126G, R371L,L438Q(3),I545T, F605L, I654N, P659L,A678V	12
fis	DNA - binding transcriptional dual regulator	R5L, 69INDEL71, 39INDEL99	3
marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	R27P, L46H, 84INDEL103	3
rph	defective ribonuclease PH	207INDEL229	5
ompF	outer membrane porin,1a	10INDEL27, 158INDEL163, 191INDEL239, 213INDEL240, Q361X	5
ompR	DNA-binding response regulator in two-component regulatory system with EnvZ	E3X, 8IND44, R15C, E96D , 176IND183	5
acrR	DNA-binding transcriptional repressor	Q7X (2), S31T, 84IND103, A191D	5
gyrA	DNA gyrase , subunit A	S83L (8), D87N, D87T S464T, P738S(2)	13
mprA	DNA binding transcriptional repressor of microcin B17 synthesis and multidrug efflux	120INDEL134, 125INDEL176, 160INDEL176 (2)	4

Table 4.2 Shared mutated genes across the antibiotic classes

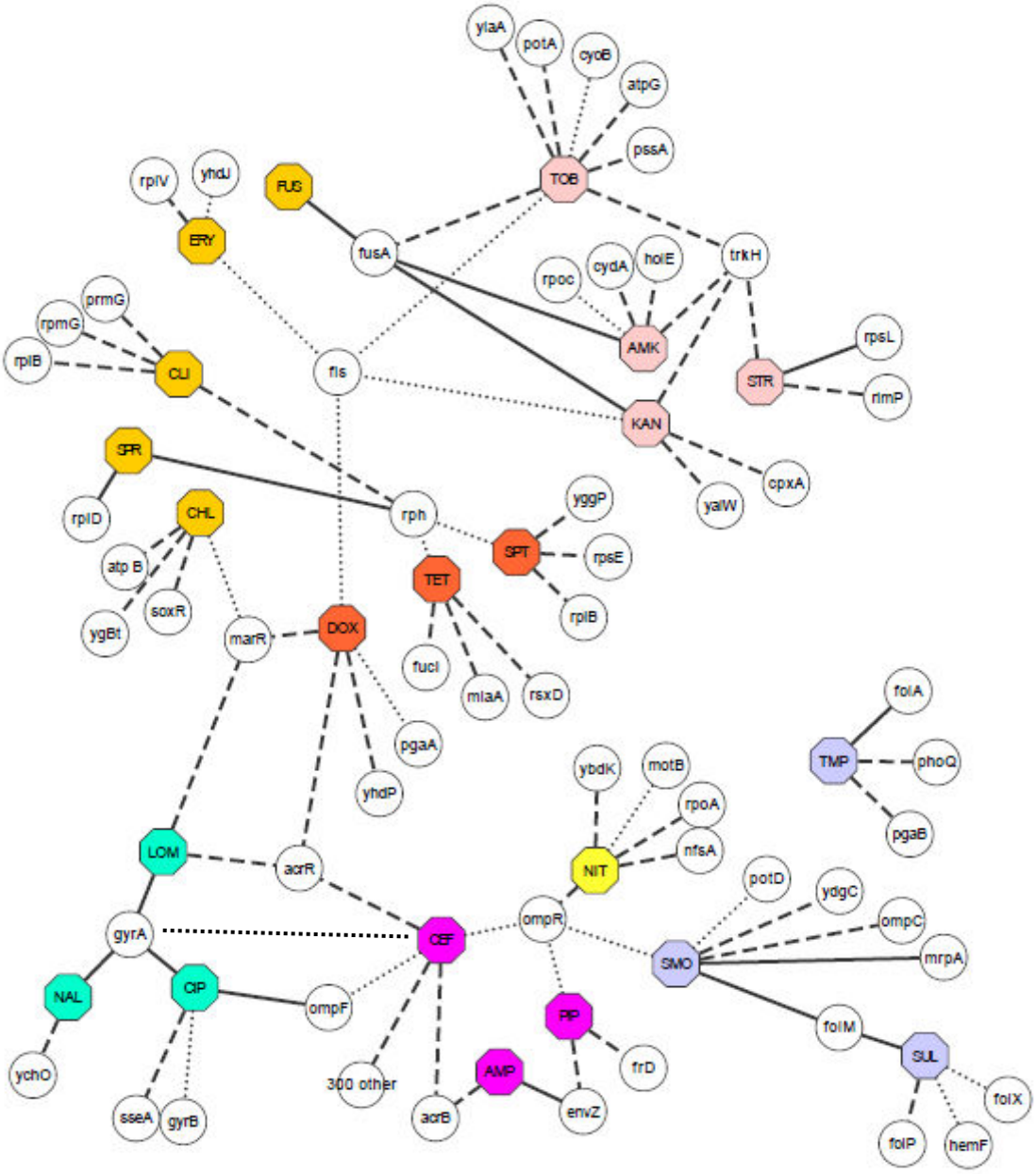


Figure 4.10 Mutation networks of antibiotic classes. Same drug groups are shown in same color and drugs are indicated as hexagon. Names of mutated genes are shown in circles. There are many drug specific mutated genes and some shared mutated genes.

We classified mutated genes according to their related functions; such as: translation, transcription, membrane proteins, DNA replication, folic acid metabolism and unknown functions. Antibiotics were also grouped according to their mode of actions, shown in Figure 4.11A. Evolved strains of all antibiotic classes have mutations on membrane proteins related genes except 50S inhibitors. Evolved strains of 50 S inhibitors have mutations on translation and transcription related genes. Additionally, all antibiotic groups have transcription related mutations; especially, they have acrR and marR mutations.

AcrR, local repressor, plays a modulating role in the regulation of acrAB genes of *Escherichia coli* by global stress signals and acrAB genes encode a multidrug efflux pump in *E.coli* [41]. The MarR is the repressor of the multiple antibiotic resistance (marRAB) operon in *E.coli.* Inactivation of marR results in increased expression of marA, which acts at several target genes in the cell leading to reduced antibiotic accumulation[37].

Strains of DNA gyrase inhibitors have mutations on transcription, cell membrane and DNA replication related genes. Mutations of folic acid inhibitors are related with folic acid metabolism, transcription and membrane proteins. As expected, all of the protein synthesis inhibitors have mutations on translation related genes but other drug classes do not have mutations on translation genes. All antibiotic classes including nitrofurantoin have unknown mutations.

Evolved strains of nitrofurantoin have mutations mainly on transcription related genes; such as: ompR, rpoA and mprA, and also mutations related with membrane proteins genes: motB. Only nitrofurantoin has mutation on motB gene. Product of this gene is MotB protein (motility protein B), an integral membrane protein, and required for rotation of the flagellar motor[38]. Nitrofurantoin is a multiple mechanism drug and works by damaging bacterial DNA, since its reduced form is highly reactive. The rapid reduction of nitrofurantoin inside the bacteria causes multiple reactive intermediates to attack ribosomal proteins, DNA, respiration, pyruvate metabolism and other macro molecules within the cell[39].

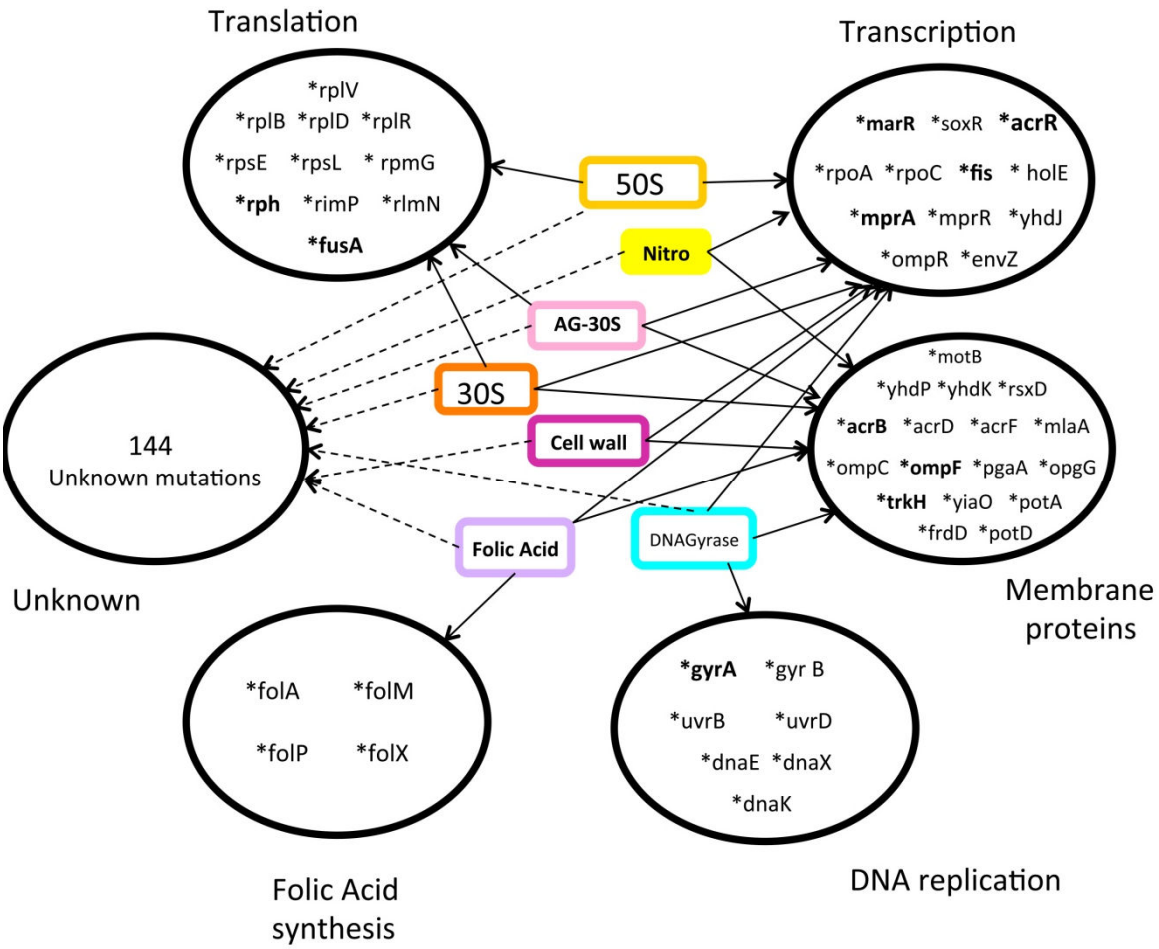


Figure 4.11 A) Overall mutations identified by whole-genome sequencing. Mutated genes are grouped as their related functions and antibiotics are also classified according to their mechanisms of actions.

Additionally, we identified reproducible and shared mutations from all mutations' data, shown in Figure 4.11B and 4.11C, respectively. If at least two mutations in the same or different positions of the same gene were observed, we called as reproducible mutations. Especially, mutations on multidrug resistance related genes; such as, marR, acrR and mprA were reproducible and also shared. We identified 31 unknown mutation places, which were seen at least two times. All of the mutated genes of folic acid metabolism were reproducible. However, only gyrA mutations were reproducible in the group of DNA replication.

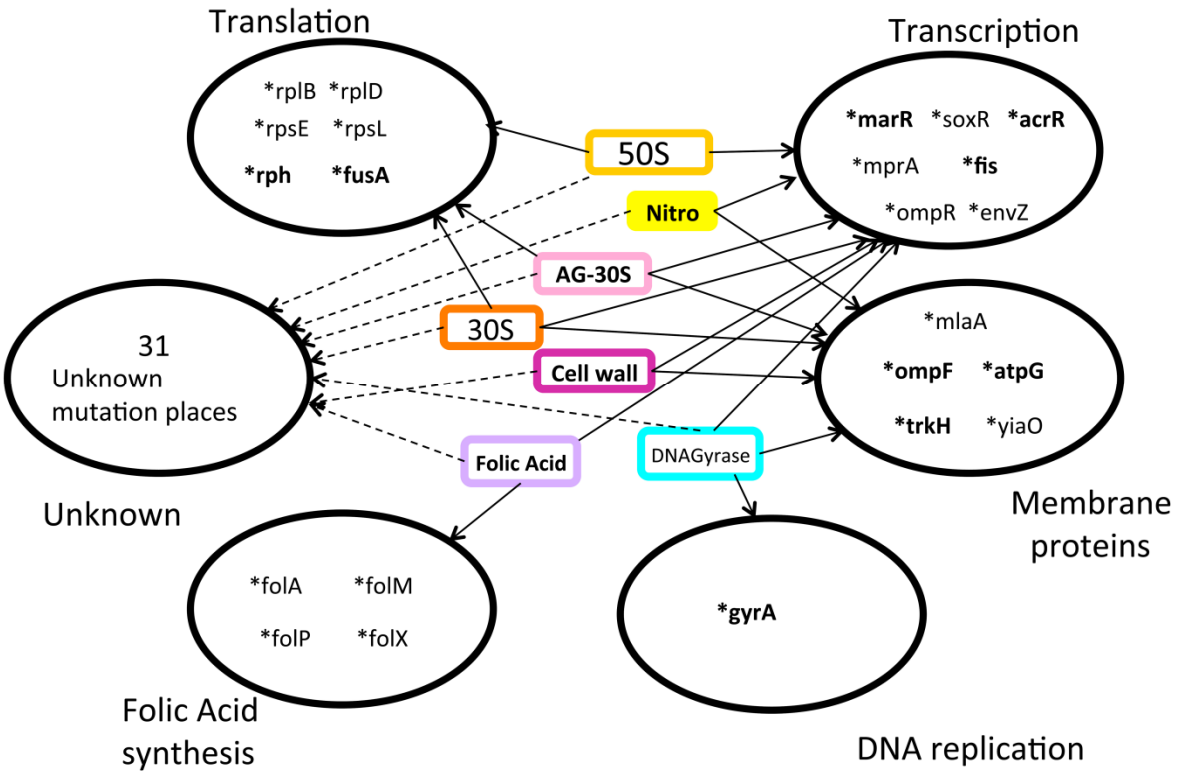


Figure 4.11 B) Reproducibly mutated genes involved in resistance. At least two mutations at the same position or different position were identifiedon the same gene.

Shared mutations are mainly responsible for multidrug resistance phenotype because different antibiotic groups have these mutations. For example, transcription related marR, acrR, mprA, ompR and fis genes are frequent mutational targets for different groups of antibiotics. We identified 7 shared unknown mutations from 31 reproducible unknown mutations. Mutations related with folic acid metabolism and DNA replication were not shared. By identifying reproducible and shared mutations, we defined hot spot mutation positions, some of them were shown in Figure 4.12.

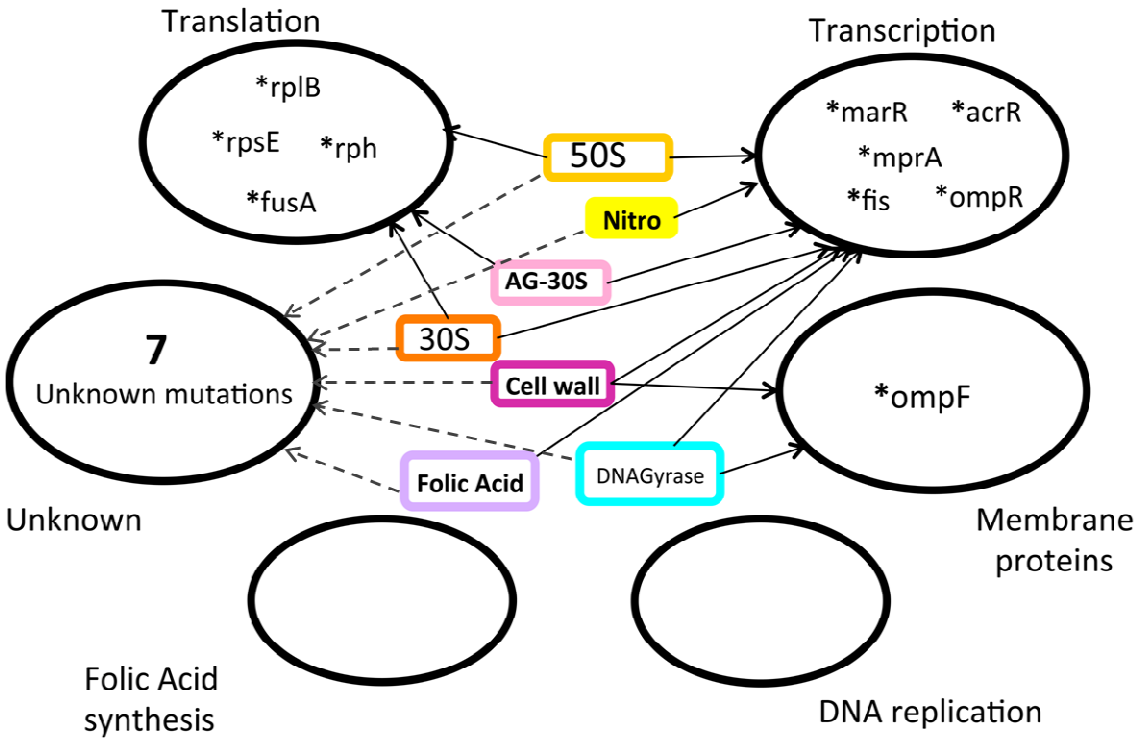


Figure 4.11 C) Shared mutations across different drug classes.

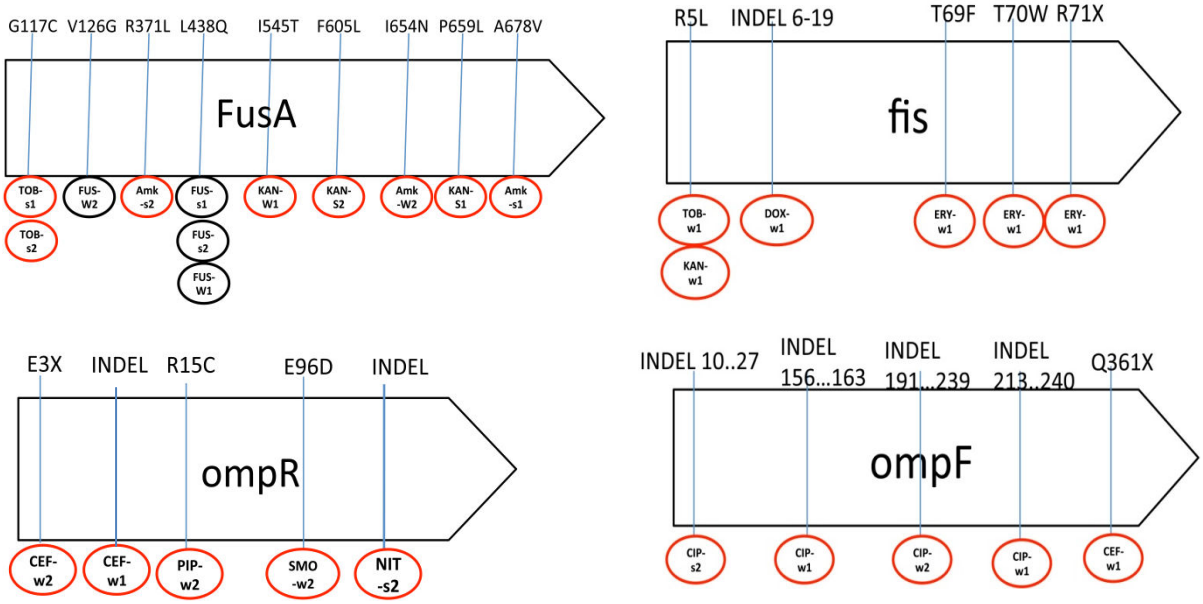


Figure 4.12 Hot spot mutation targets: FusA, fis, ompR and ompF genes. INDEL indicates frameshift mutations.

To further characterize the genotype data, mutations (SNPs or insertion-deletion mutations) of strong and weak selections of drug groups were classified.We observed totally 370 mutations on strongly selected strains, 24 mutations in weakly selected strains and 64 common mutations, shown in Figure 4.13. These mutations were grouped according to each drug classes to provide better understanding for the effect of different selection powers. As expected, there were more mutations on strongly selected strains than weak ones. In most drug groups, strong selection strains had more target specific mutations. For example,strongly selected strains of protein synthesis 50S binding inhibitors had mutations on the 50S subunit of ribosome genes but weakly selected strains usually had no mutions on ribosomal genes except one shared mutation with strong ones.DNA gyrase inhibitors had two hot spot mutation targets: gyrA and ompF and these mutated genes were common for both selection powers. Similarly, folM, folA and folP mutations were common for both strong selection and weak selection of folic acid metabolism inhibitors. DNA gyrase and folic acid metabolism inhibitors are very target specific drugs; thus, these results were expected.Mutations of cell wall inhibitors were mostly caused by strongly selected cefoxitin strains. Evolved strains ofcell wall inhibitors got mutations mostly related with membrane functions; such as, ompF, ompR, setB and acrB.Mutations of strong and weak selections are shown in Figure 4.14.

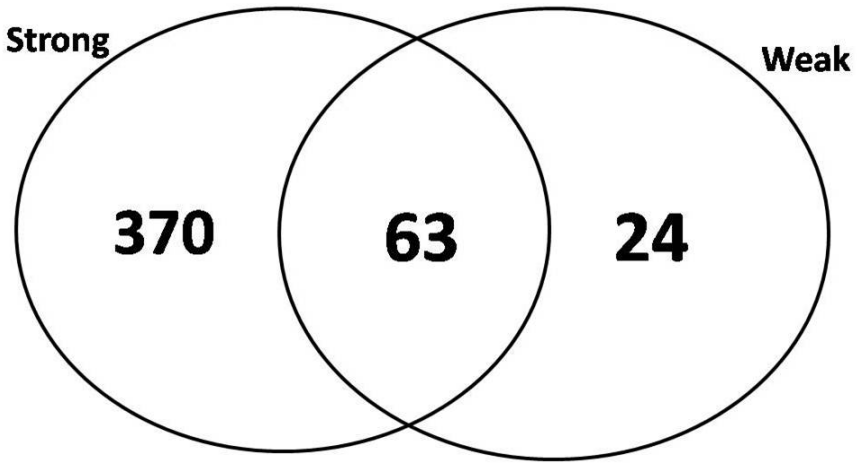
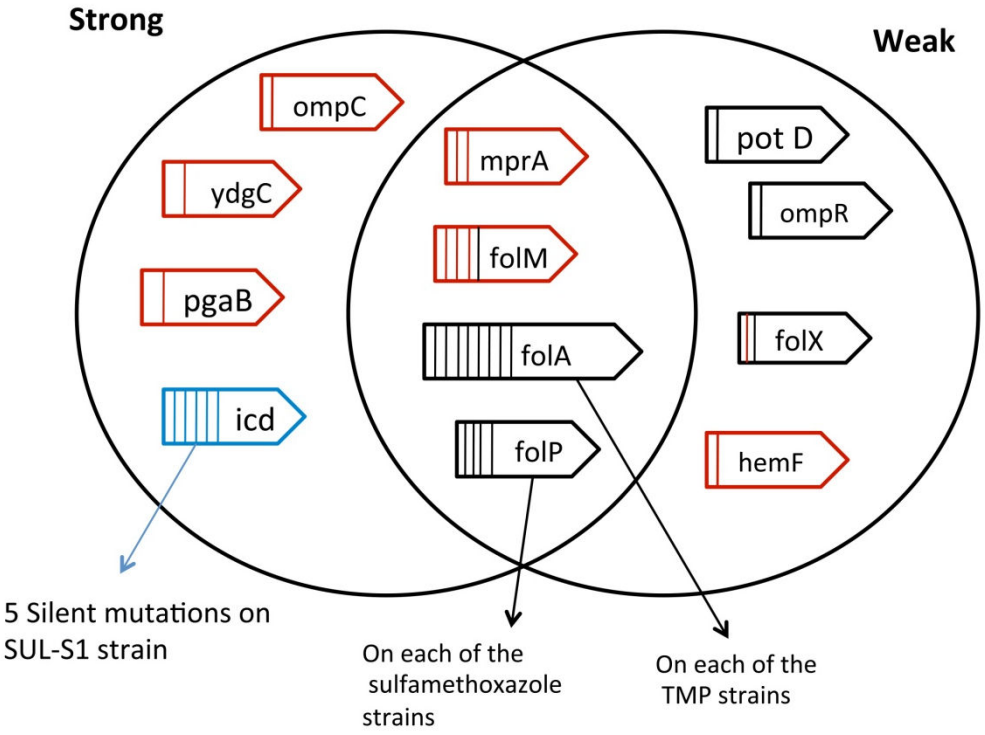
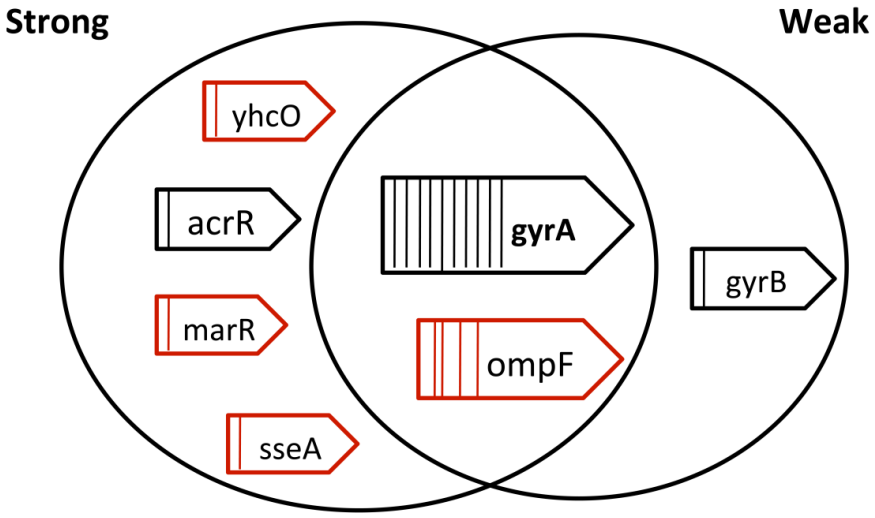


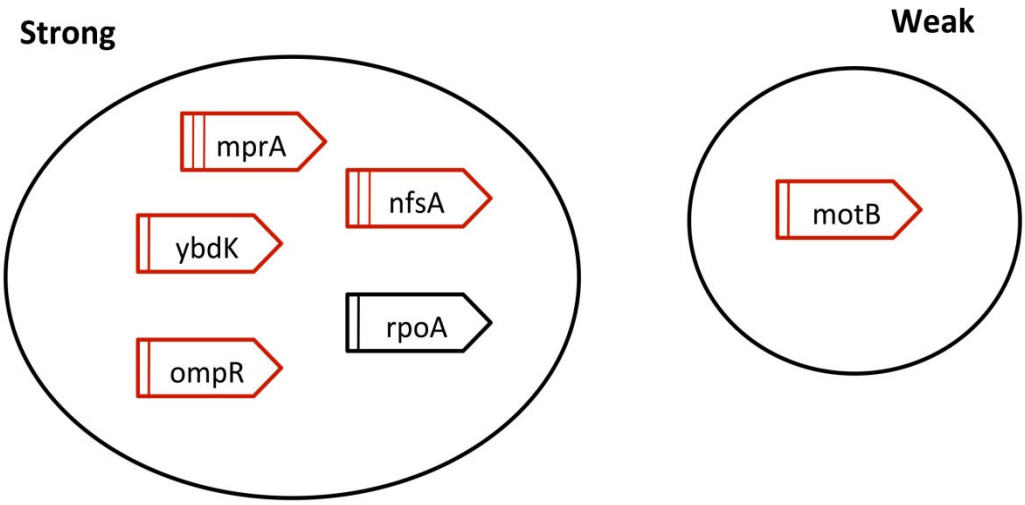
Figure 4.13 All number of mutations in strong and weak selection strains.



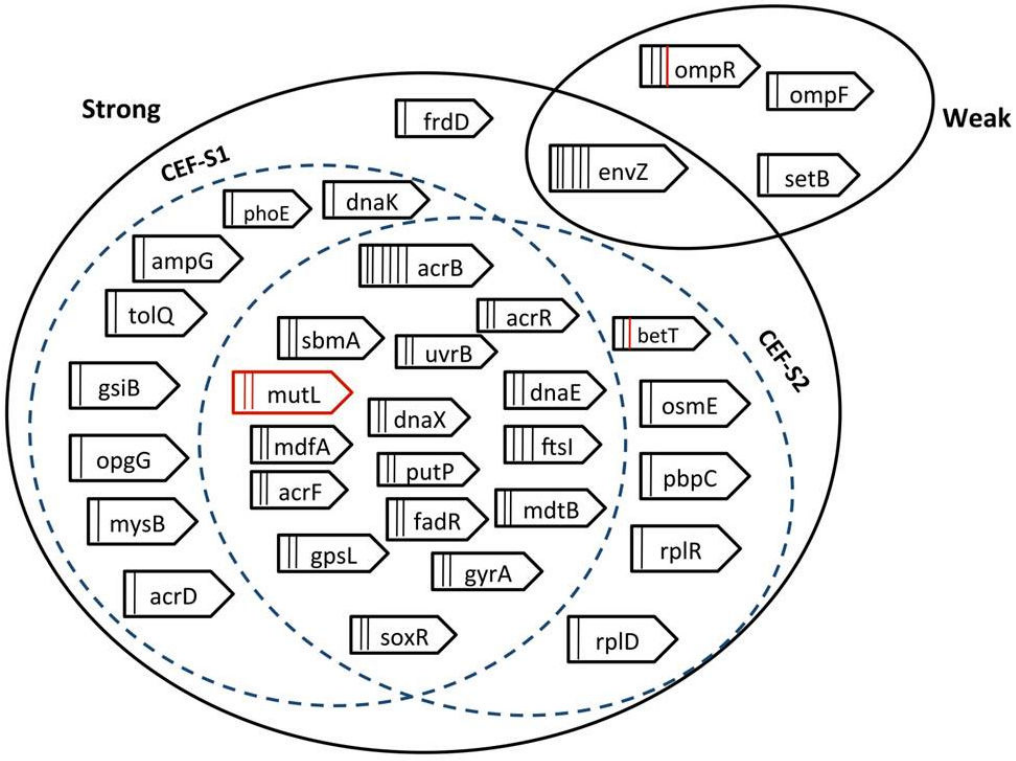
Mutations of folic acid biosynthesis inhibitors



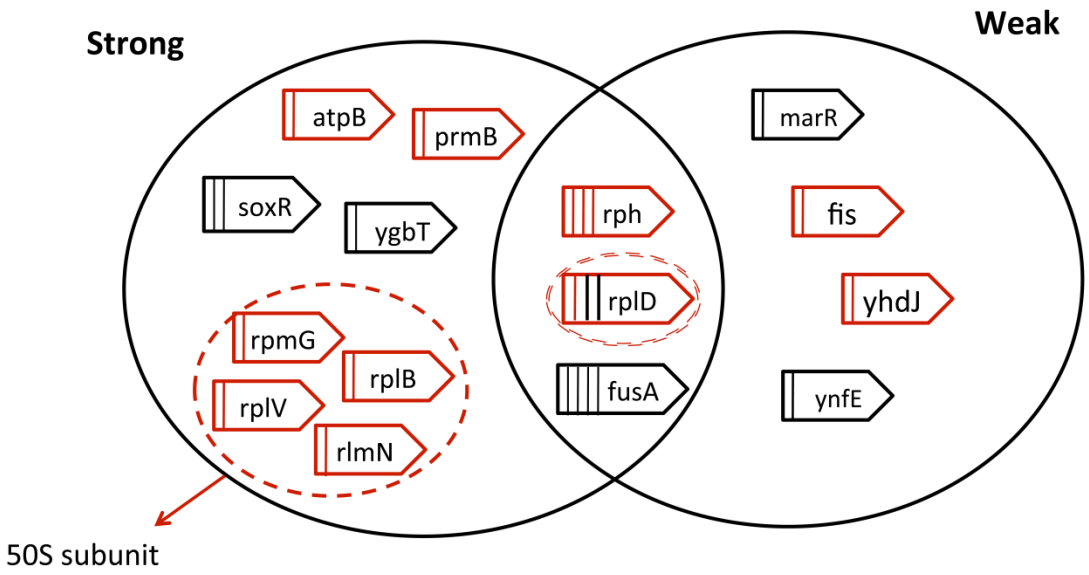
Mutations of DNA gyrase inhibitors



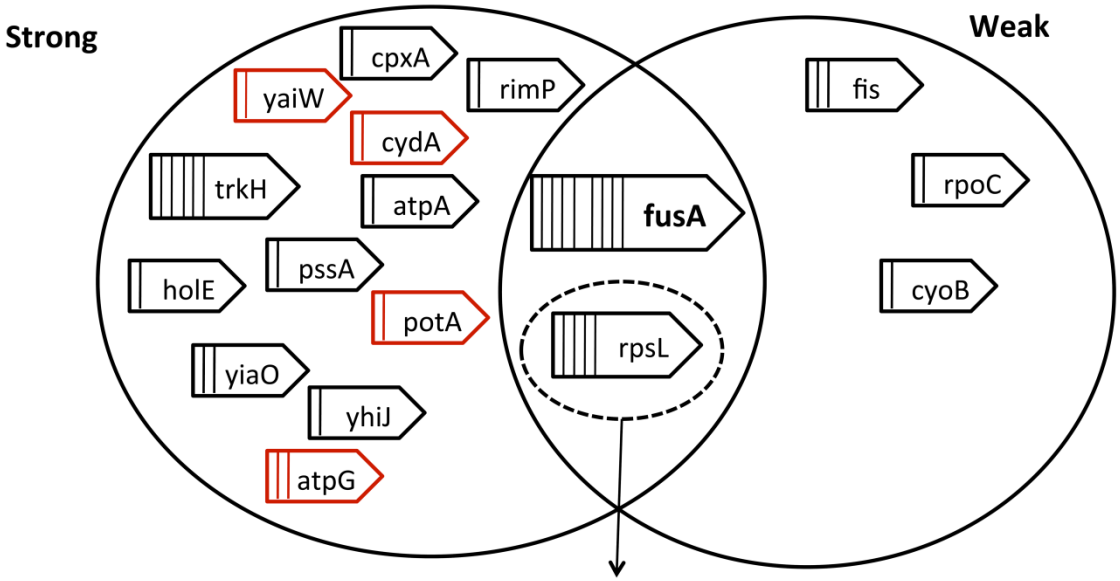
Mutations of Nitrofurantoin



Mutations of cell wall inhibitors

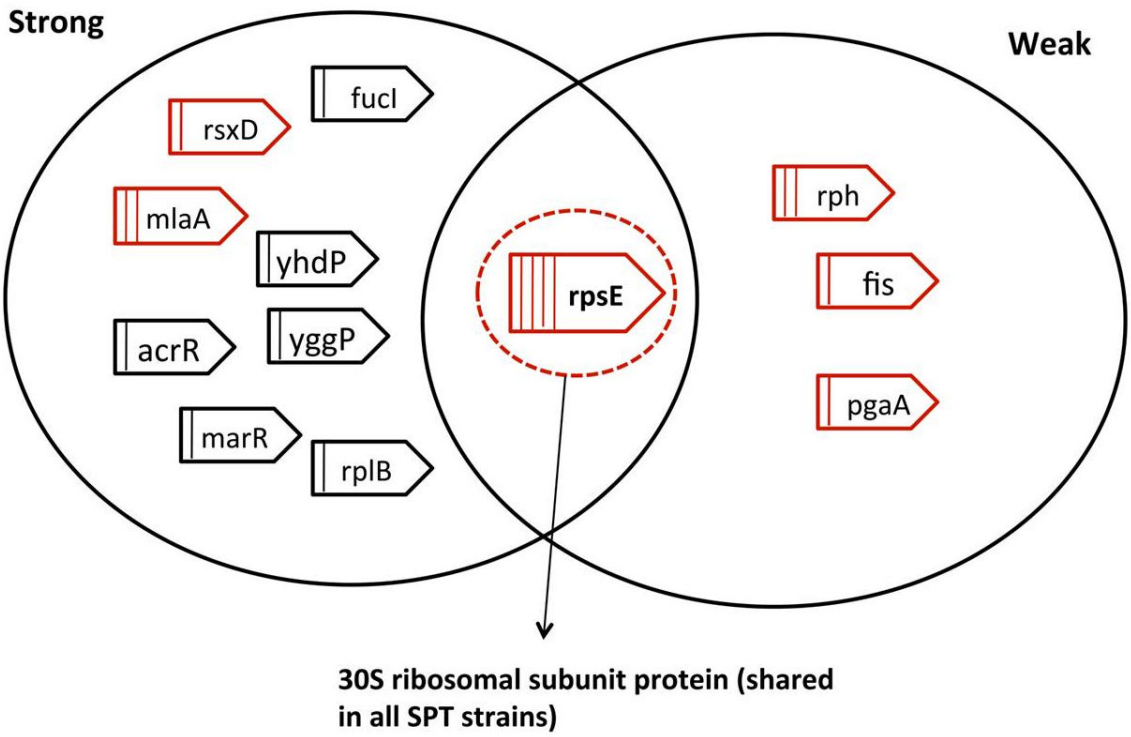


Mutations of protein synthesis-50S subunit binding inhibitors



30 S ribosomal subunit protein S12
Shared in all STR strains

Mutations of Aminoglycosides



Mutations of protein synthesis-30S subunit binding inhibitors

Figure 4.14 All mutations of each drug groups were classified according to strong and weak selections. Black color shows SNP and red color shows indel (frameshift) mutations. Straight lines on the gene figures indicate the number of mutations. Genes in the circle are related with ribosomal subunits.

4.3.2 WGS-Amplification Results

We also performed amplification analysis to our whole genome sequencing data. We defined length threshold as 5000 bp to be conservative enough. To identify regions likely to have been duplicated during the evolution process, the number of Illumina sequencing reads covering each position of the MG1655 genome were counted using the SAMTools software.

We found amplification on three strains: Spiramycin strong resistant strain 2, ciprofloxacin weak resistant strain 2 and amikacin weak resistant strain 1. Figure 4.12 shows amplified regions of these three strains and table 4.3 indicates the positions of amplification, length of amplified region and average counts for these regions. In only amikacin strain, we observed mutation on amplified region but this was an unknown mutation. For other two strains, amplified regions and mutations were not close to each other. In amikacin strain, very large region was amplified. When we analyzed the genes found in amplified regions, acrA, acrB and acrR genes related with multidrug efflux system were identified in these three strains. This could be also explanation for resistance of strain spiramycin-strong 2 which does not have a mutation on a region identified as a functional gene. Strongly selected spiramycin cultures had same resistance levels at the end of 21 days but only one of the strains had mutations on translation related rlmN, rplD and rph genes. Second strongly selected spiramycin strain had not a known mutation but had an amplified region. Additionaly, some genes related with transportation of molecules and regulation of beta lactamase synthesis were determined in these amplified regions; such as: sbmA, ampG,tsx, betT and ompT. All of the genes and functions of these genes found in amplified regions were indicated in appendix C.

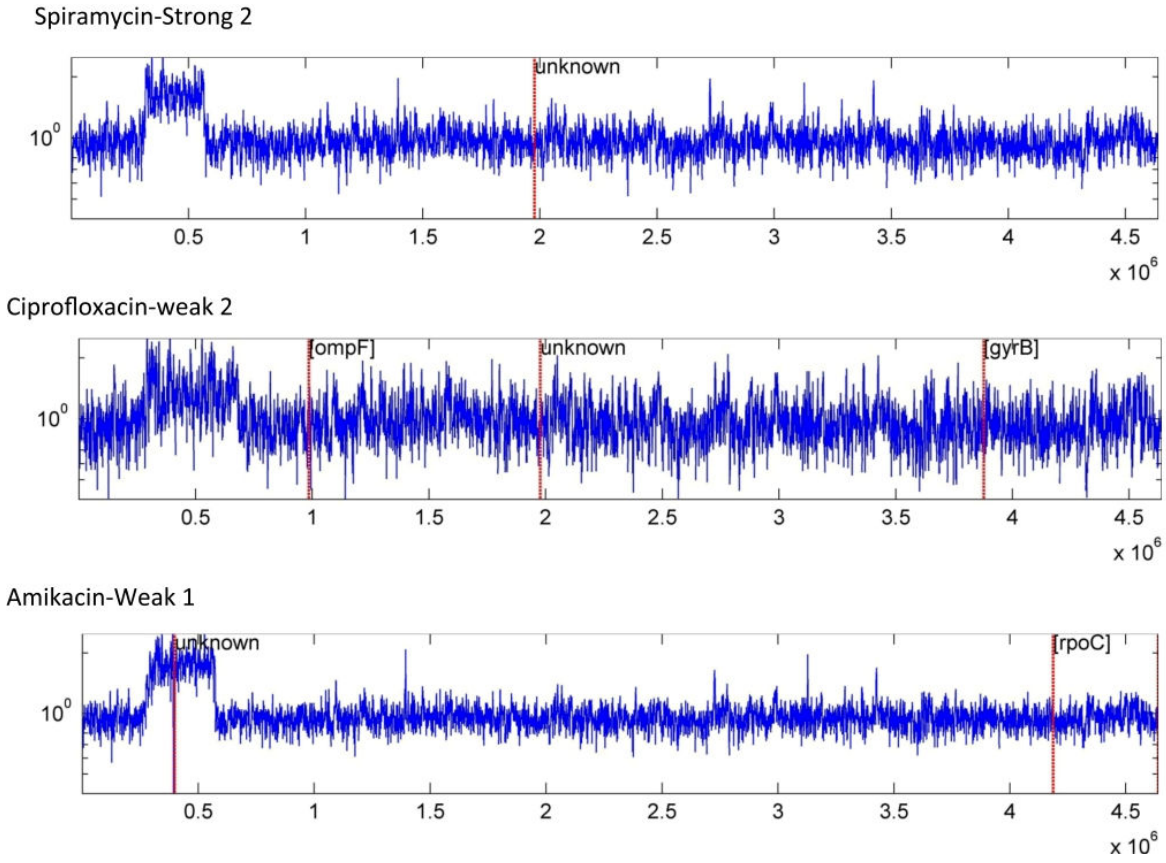


Figure 4.15 Amplification results. Strongly selected spiramycin second strain, weak selection of ciprofloxacin second strain and amikacin first strain has amplifications approximately at the same positions.X-axis shows the whole genome of resistant bacteria and y- axis shows average reads of genome. Red lines on the figure indicate mutated genes.

Strain name	Position	Length	count
Spiramycin_S2	318621	10493	183,3
Spiramycin_S2	331678	5421	178,3
Spiramycin_S2	343653	5402	182,8
Spiramycin_S2	389982	10584	197,1
Spiramycin_S2	429408	6417	179,5
Spiramycin_S2	449835	5679	178,5
Spiramycin_S2	479421	7507	178,8
Spiramycin_S2	557747	8955	188,3
Ciprofloxacin_w2	311848	11185	177,4
Ciprofloxacin_w2	390681	7152	174,3
Ciprofloxacin_w2	560749	5700	164,1
Ciprofloxacin_w2	569504	5366	166,4
Ciprofloxacin_w2	579044	5827	173,9
Amikacin_w1	317334	20504	178,3
Amikacin_w1	343388	6069	175,8
Amikacin_w1	360880	5298	165
Amikacin_w1	419295	7442	172,2
Amikacin_w1	428808	7981	169,9
Amikacin_w1	447529	8165	172,5
Amikacin_w1	456002	10259	171,7
Amikacin_w1	478002	11729	174,9
Amikacin_w1	499724	5956	169,3
Amikacin_w1	526336	6239	175,7
Amikacin_w1	535725	5845	163,5
Amikacin_w1	556440	10764	176,7

Table 4.3 Amplication table. Amplification positions, length of each amplified regions and average counts of this regions are indicated.

5. DISCUSSION

Antibiotics are nanometer sized small molecules that can cure bacterial infections and the discovery of the first antibiotic compound by Alexander Fleming is a milestone in modern medicine However, use of antibiotics gave rise to resistance problem because bacterial populations have a significant capacity to deal with stressful conditions and antibiotics act as selective agent for resistant strains. Hence, antibiotic resistance is a global public health threat. As indicated in the introduction part, resistance can be intrinsic or acquired and there are many mechanisms for resistance; such as: enzymatic destruction or modification of the antibiotic by resistant bacteria, increase activation of efflux pumps and replacement or modification of the antibiotic target. Multi drug resistance and cross resistance are very important parts of antibiotic resistance because today we have a list of organisms resistant to many different antibiotics in both hospitals and the community. Over the years, many attempts have been made in the medical fields to resolve the mentioned problem. The most widely applied proposals to this problem are developing novel antibiotics and using the existing ones more effectively. The number of new antibiotics has declined significantly in recent years because of financial and natural constraints. Thus, the effective use of available drugs seems to be the most reasonable option. In this respect, applications of drug combinations have been mostly suggested. Due to lack of knowledge on bacterial drug resistance at the molecular and population levels, there is a growing need of knowledge on phenotypic and genotypic characterization of bacteria while developing resistance to antibiotics.

In this project, the main aim was designing and testing novel strategies for minimizing the rate of evolution of drug resistance. Through integration of phenotypic analysis of resistant strains and sequencing, we were able to give an idea of the evolutionary pathways for different classes of antibiotics, cross resistance between twenty two drugs, general patterns of cross resistance in drug groups and reasons for resistance mechanisms: mutated genes and amplified regions of evolved strains. In order to understand the mechanisms of bacterial resistance against drugs, we performed long term adaptation experiments by using classical methods and we phenotyped and genotyped evolved strains. For phenotypic characterization, we searched for cross resistance between drugs. Cross resistance was especially between the drugs of same classes and we observed this phenomenon for all drug class except protein synthesis, 30S inhibitors. This can be because of different mutations. Drug specific and shared mutations were observed in this study. When we combined our phenotypic analysis with whole genome sequencing, we got lots of knowledge about antibiotic behaviors.

Reciprocal evolution was observed mostly within drug classes, but not across different classes. However, there were some exceptions seen in protein synthesis 30S subunit binding inhibitors; such as: DOX resistant strains evolved resistance to TET but we did not observe the opposite. Mutations on AcrR and marR genes may explain this result since these two genes are related with multi drug resistance. These results were a bit of surprise but when we searched all mutations, we generally understood reasons. For example, ampicillin resistant strains became resistant to tetracycline and spiramycin mostly because they got acrB and envZ mutations. There are some unexplained results of our study and we still continue to analysis our genotype data.

Another interesting result of our research was evolved strains of aminoglycoside group antibiotics showed increased susceptibility towards most of the other drugs. When we looked into mutations, we realized that aminoglycosides have trkH mutated gene. Most of these mutations were on the same place and caused same amino acid substitution. TrkH is a potassium transporter and mutation on this gene could be a reason for increased

sensitivity. Additionally, most of the evolved strains, except gyrase inhibitors, behaved as wild type to lomefloxacin, which is also a kind of gyrase inhibitor.

The bacterial evolution patterns do not resemble each other. Spiramycin evolved strains get same amount of resistance at the end of evolution experiment. According to whole genome sequencing, first strong selection strain of spiramycin get mutations on translation related genes but second strong selection strain of spiramycin did not get any known mutation. Instead of mutation, this strain had amplified region (duplication) in its genome. However, mutation places of first strain are quite far away from amplified regions of second strain. AcrA,acrB and acrR genes, multidrug resistance genes, in the amplified regions was found. These multi drug efflux system related genes can be an explanation for cross resistance behavior of spiramycin second strong selection strain to other drugs.

Evolved strains of folic acid inhibitors had mutations on folic acid biosynthesis pathway; such as, folA, folX, folP and folM. We expected to find mutations on DHFR regions but mutation on folM was surprising since it is dihydrofolate reductase isozyme.

We observed many mutations on strongly selected strains of cefoxitin. ~200 mutations were count for these strains but weak selection strains only have two or three mutations. These strains got mutations on their DNA polymerases. They both have mutations on dnaE and dnaX genes, DNA polymerase (III) subunit. May be these two mutations resulted in many replication errors and many errors cause too many mutations on various genes. We are not sure about what happened in the cell so we will repeat evolution experiment for cefoxitin as twenty replicates: 10 replicates for weak selection and 10 replicates for strong selection to be sure about results. If we see too many mutations again in the last day cultures, we will sequence evolved strains from different days to understand which mutation on a specific gene causes many mutations and mutation orders. In our experiment design, we only sequenced cultures of last day so we could not understand mutation order; therefore, we should make sequencing from various days of the culture for cefoxitin.

Additionally, we identified mutations according to selection powers for each drug classes. Much more mutations were observed in strongly selected strains than weakly selected strains. There were also many shared mutations between strong and weak selections. When we searched mutations carefully, we observed that strong selection strains mostly had target specific mutations. For example, strongly selected strains of protein synthesis 50S subunit binding inhibitors had mutations in the 50S subunit related genes and one shared mutation in this subunit; however , we did not observe such specific mutations in the weakly selected strains. Interestingly, mutations in the 30S subunit related genes were not observed frequently. Only two 30S subunit genes: rpsL and rpsE were mutated in all of the streptomycin and spectinomycin, respectively. Small size of 30S subunit when compared with 50S may be an explanation for this genotype. Additionally, 31 reproducible and 7 shared unknown mutation were identified but we do not know anything about functions of these genes. 7 shared unknown mutation places can be thought as hot spot target places for mutations because 2 of these unknown mutation places were seen 9 and 8 times, respectively across the different antibiotic groups. There can be an undiscovered gene in that region related with multiple drug resistance.

Lastly, we want to see whether there is a relationship between cross resistance and drug interactions. We used data set from Yeh et al. (2006) to make comparison shown in Figure 5.1. We could not see general pattern since our cross resistance is not reciprocal. Interactions between drugs have one direction but cross resistance has two directions. For instance; evolved strains of chloramphenicol are resistant to tetracycline but evolved strains of tetracycline became more susceptible to chloramphenicol. According to Yeh et al (2006), interaction between chloramphenicol and tetracycline is antagonism so we cannot make generalization about interactions and cross resistance. However, if we observe reciprocal cross resistance, we can look for drug interactions. We observed reciprocal resistance mostly between same drug classes, especially DNA gyrase inhibitors, cell wall inhibitors and aminoglycosides, and we searched interactions within same classes of antibiotics. For example, DNA gyrase inhibitors had reciprocal cross resistance and we searched for interaction types between these three antibiotics (ciprofloxacin, lomefloxacin and nalidixic acid) and observed that there is an additive interaction between ciprofloxacin and

lomefloxacin, nalidixic acid and ciprofloxacin, lomefloxacin and nalidixic acid. Additionally, there is a reciprocal cross resistance between all cell wall inhibitors (piperacillin, ampicillin and cefoxitin) and we observed a synergistic interaction among these three drugs. For aminoglycosides, there is also synergistic interaction between them. Then, we searched for antibiotics showing reciprocal cross resistance and interactions between them. There is a reciprocal cross resistance between tetracycline and ciprofloxacin and interaction between these two drugs is antagonistic. We could not make a general assumption for cross resistance and drug interactions.

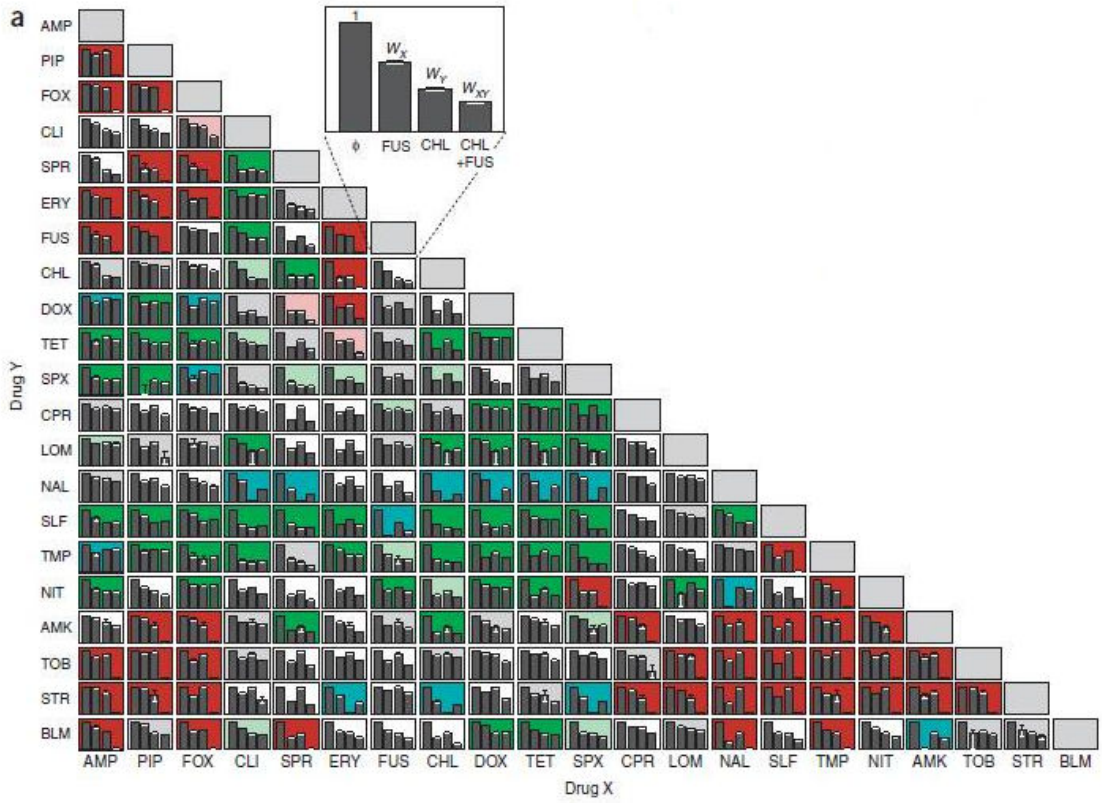


Figure 5.1 Systematic measurements of pairwise interactions between antibiotics. Growth measurements and classification of interaction for all pairwise combinations of drugs X and Y. Synergistic interactions are shown red-pink, antagonistic buffering is green- light green, antagonistic suppression is blue and additive is white color. Cases that do not fall into any of these categories are labeled inconclusive: gray background[40].

6. CONCLUSION

Antibiotic resistance is a serious problem in hospitals and in community; therefore, understanding the evolutionary biology and genomics of resistance can give information about therapeutic strategies. Antibiotic resistance can evolve through accumulation of mutations. Indeed, only one mutation can be sufficient for bacteria to overcome antibiotic induced stress. In this project, microbial evolution techniques, phenotypic measurements and whole genome sequencing were successfully used to reveal phenotypic and genotypic changes that cause to resistance.

In summary, we designed an evolution experiment for twenty two drugs and observed evolution of bacteria for 21 days. After generation of 88 evolved strains, we performed phenotypic and genotypic characterization for these strains to investigate cross resistance between antibiotics. As a result of this study, same groups of antibiotics showed increased cross resistance for each other. We could not find relationship between drug interactions and cross resistance. Lastly, we identified mutations and amplifications of all evolved strains. When we combine our phenotypic and genotypic data, we may give some recommendations for therapeutic implications. We learned that reciprocal cross resistance is common in same drug classes so we should avoid choosing drugs from same classes in multidrug treatments. Antibiotics causing mutations on multidrug efflux pump related genes should not be used in long time treatments. We can choose drugs according to mutation pathways for multiple drug treatments.

7.FUTURE WORK

In the light of the results reported here, we would like to suggest the some future work. According to our phenotypic measurements, mathematical pair wise scores may be given for cross resistance matrix. By this way, we can score drug behaviors and identify the group of an unknown drug by using our master plate and cross resistance experiments. General pattern of mutations can be examined by analyzing cross resistance data more carefully according to sequencing data. In this study, we performed whole genome sequencing and revealed mutations and functions of mutated genes. However we did not search the effect of mutations in transcriptional level. Expression levels of mutated genes may be searched as future work.Also, in our study, mutations occurred naturally but we do not know whether it will be different with site directed mutagenesis or not. Therefore; well-known mutations on specific genes may be carried out again with site directed mutagenesis and the differences can be compared. Because of the very interesting genotypic results in cefoxitin strains, evolution experiments for cefoxitin will be repeated with twenty replicates to make sure about increased number of mutations and sequencing will be performed from different days to analyze the mutation pathway. According to our phenotype data, aminoglycosides got resistant to each other but they became more susceptible to other antibiotic classes. Genotype of aminoglycosides should be analyzed more carefully. Lastly and most importantly, specific drug pairs can be identified from our data for alternative treatment strategies and these drug pairs can be tested for both treatment and resistance.

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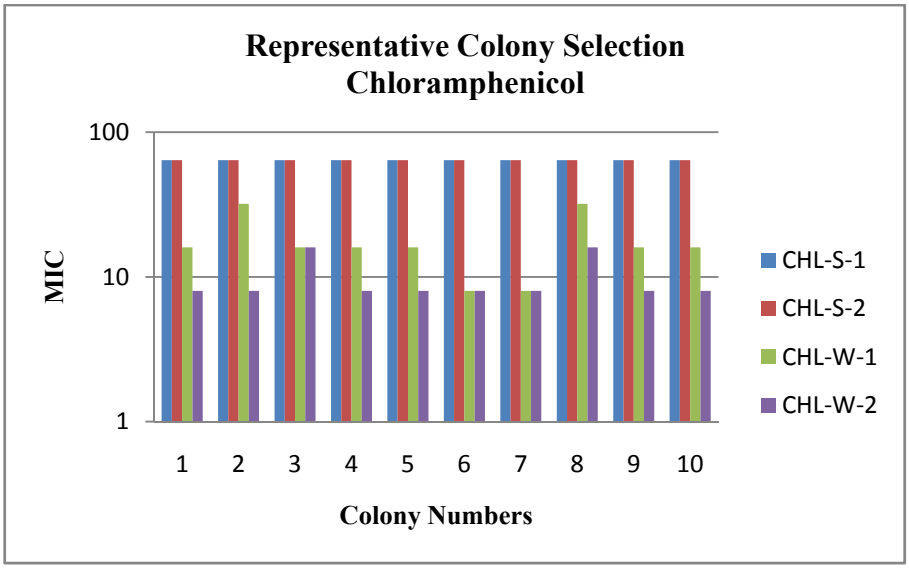
APPENDIX

Appendix A: All Graphs for Representative Colony Selection



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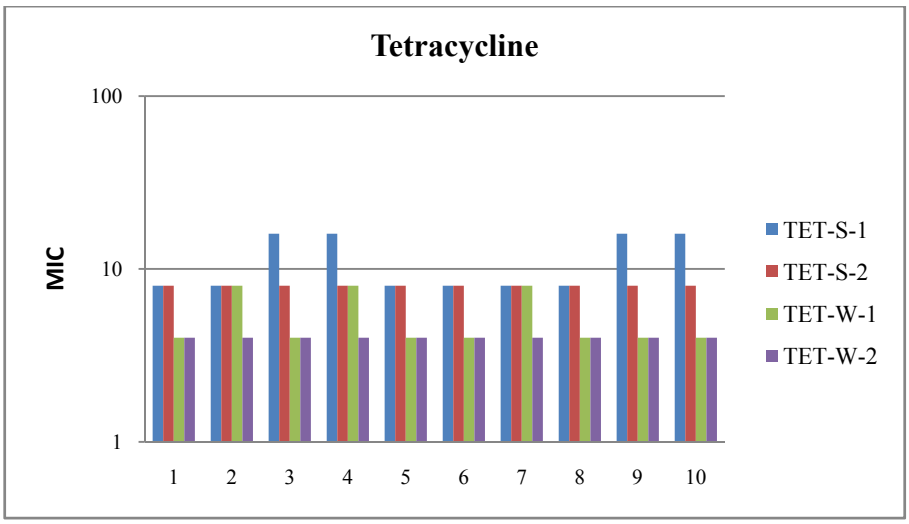
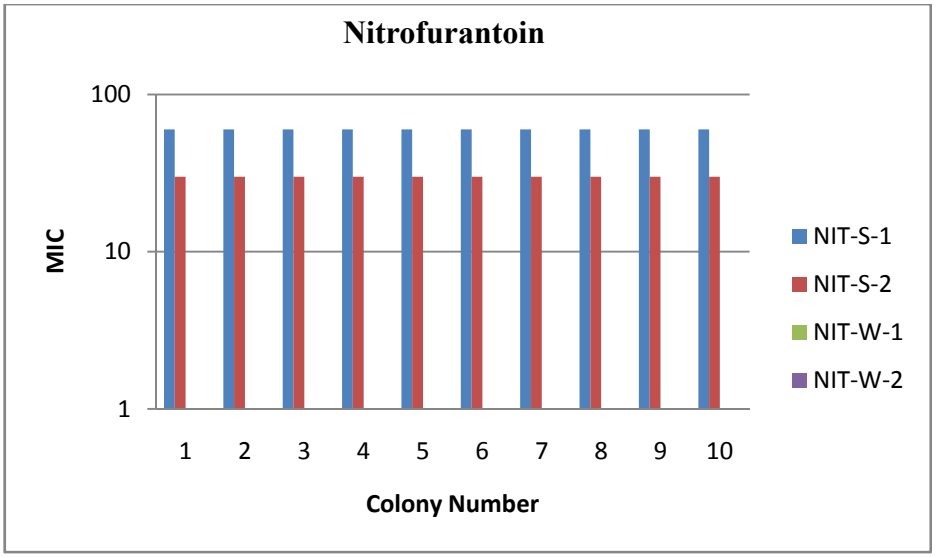
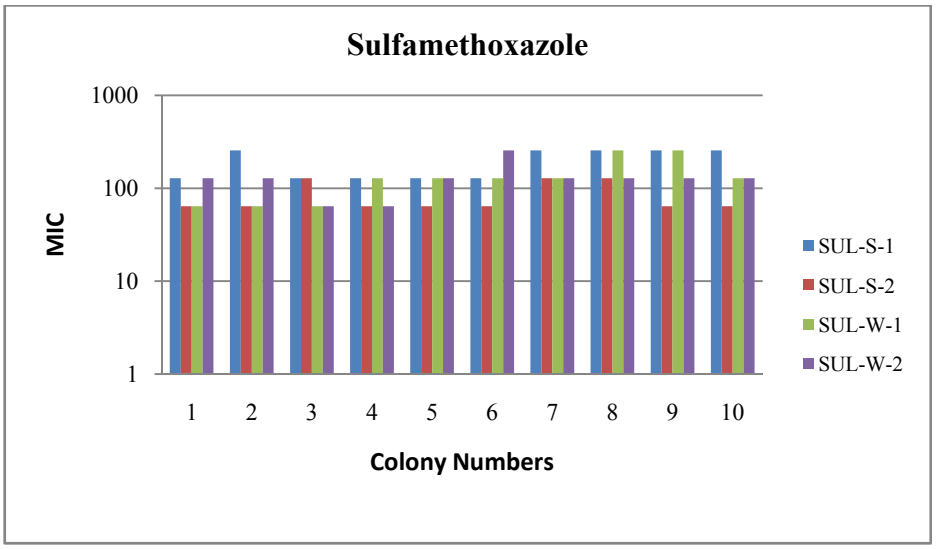
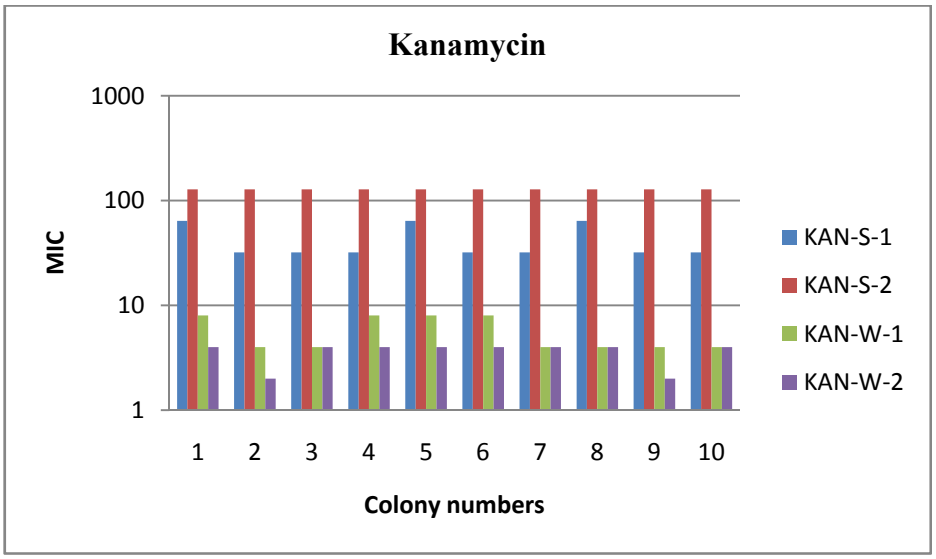
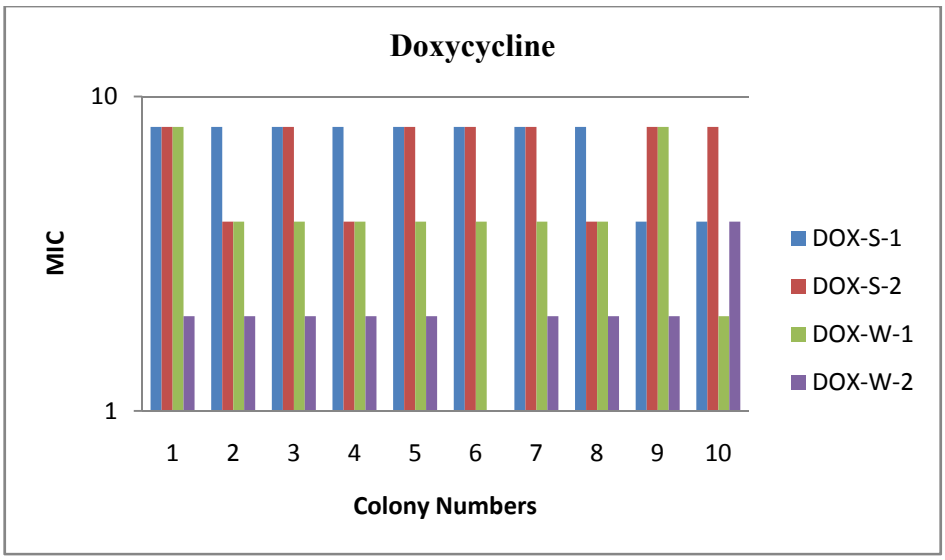
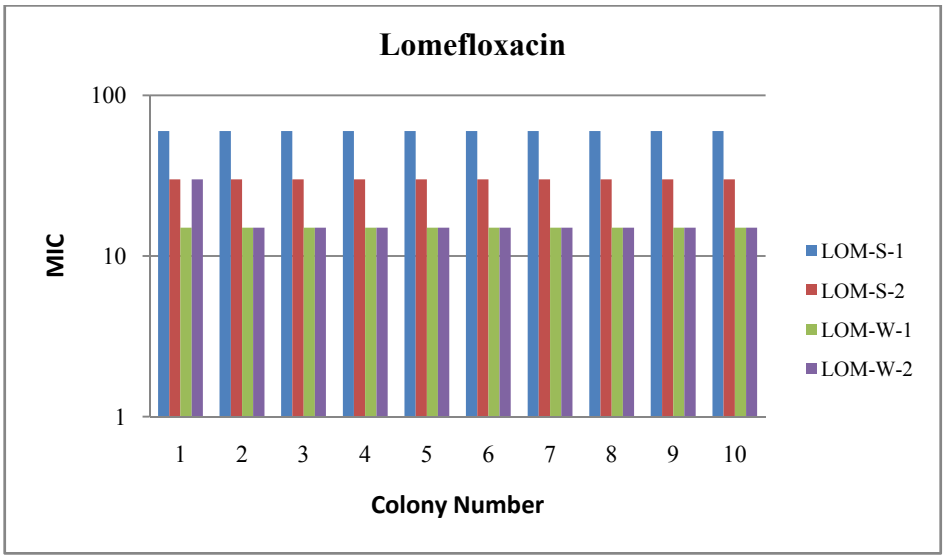


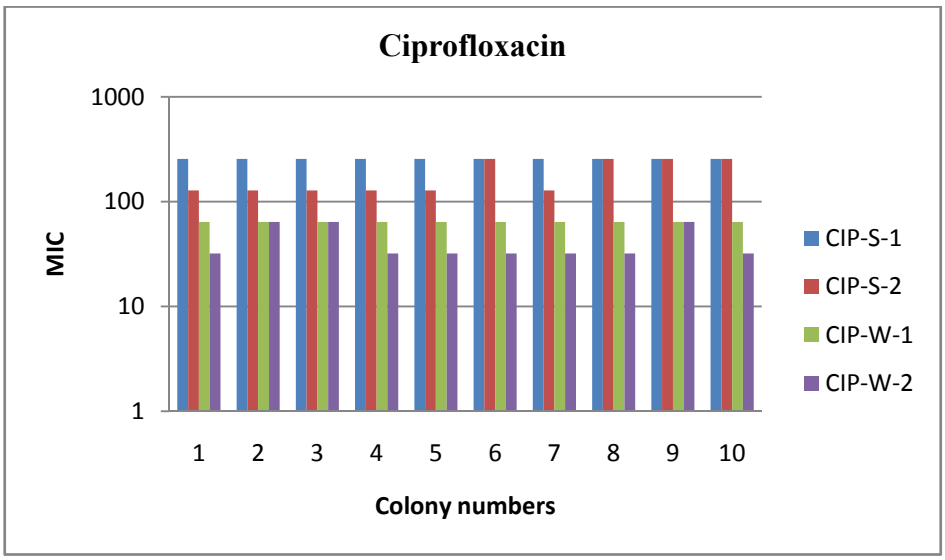

Nitrofurantoin
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Number
NIT-S-1
NIT-S-2
NIT-W-1
NIT-W-2

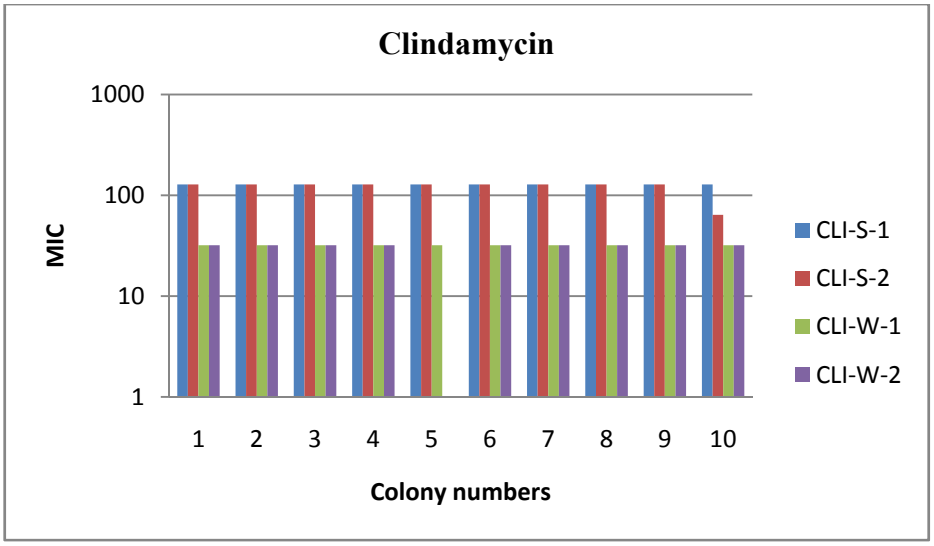

Sulfamethoxazole
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
SUL-S-1
SUL-S-2
SUL-W-1
SUL-W-2

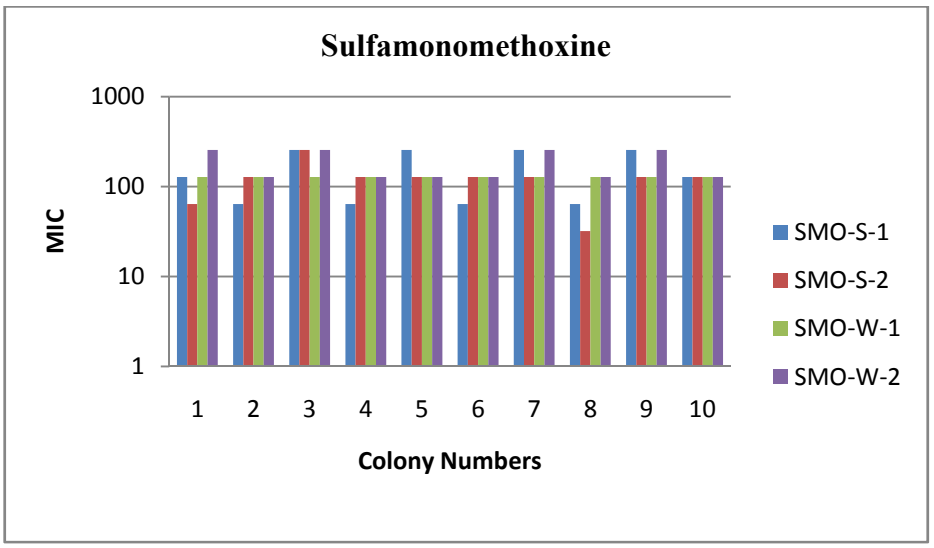

Kanamycin
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony numbers
KAN-S-1
KAN-S-2
KAN-W-1
KAN-W-2


Doxycycline
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
DOX-S-1
DOX-S-2
DOX-W-1
DOX-W-2


Lomefloxacin
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Number
LOM-S-1
LOM-S-2
LOM-W-1
LOM-W-2


Ciprofloxacin
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony numbers
CIP-S-1
CIP-S-2
CIP-W-1
CIP-W-2


Clindamycin
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony numbers
CLI-S-1
CLI-S-2
CLI-W-1
CLI-W-2


Sulfamonomethoxine
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
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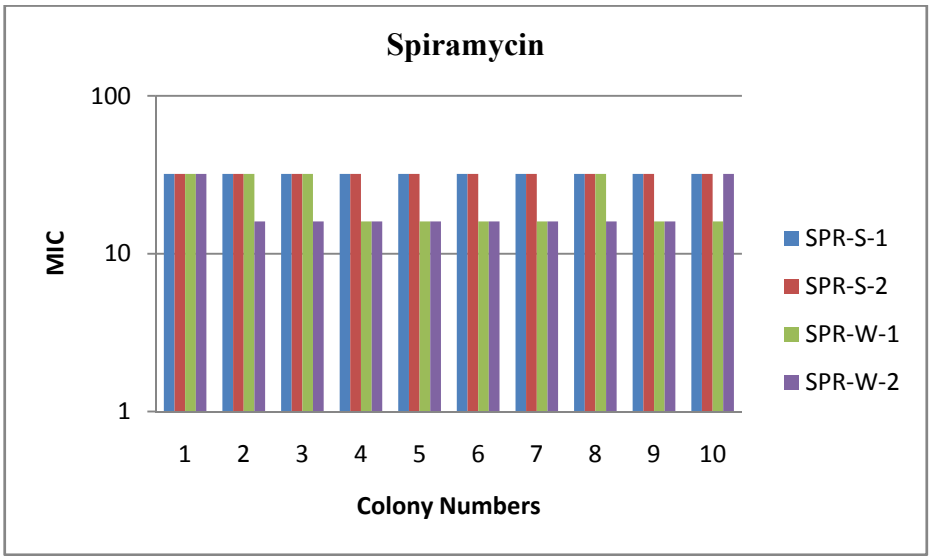
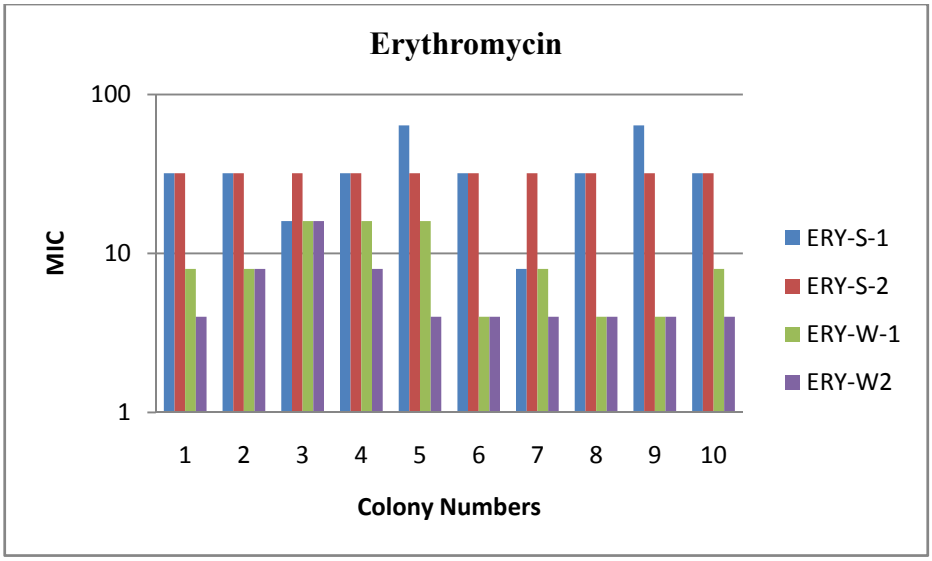
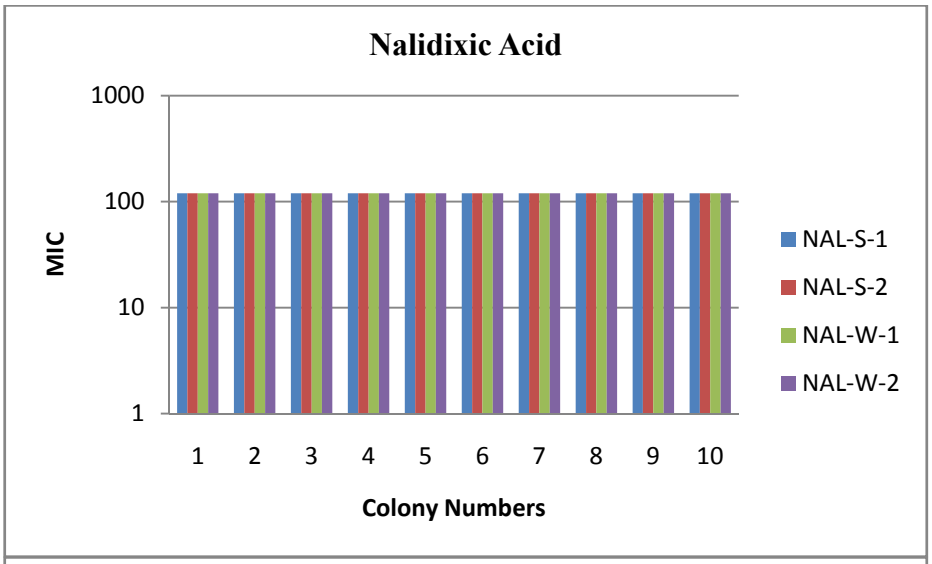
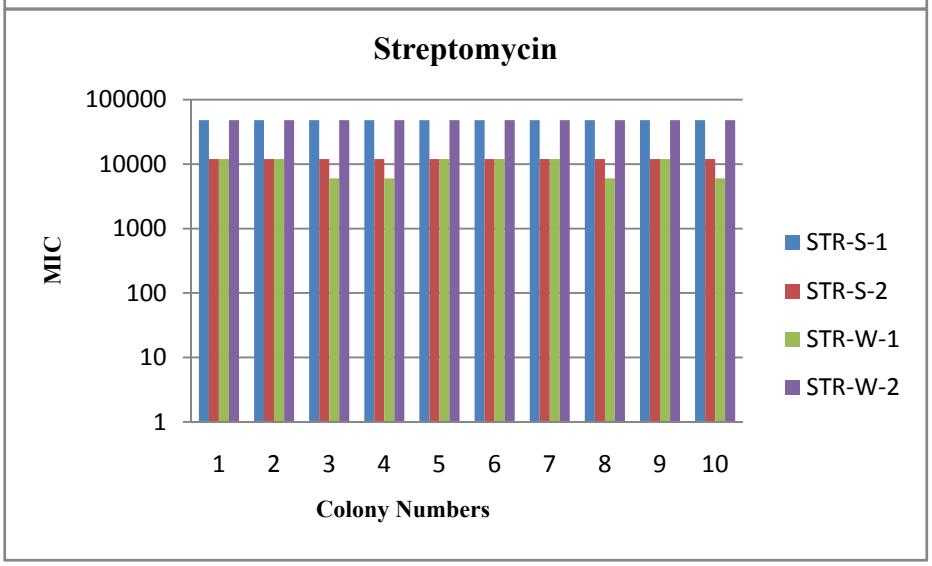
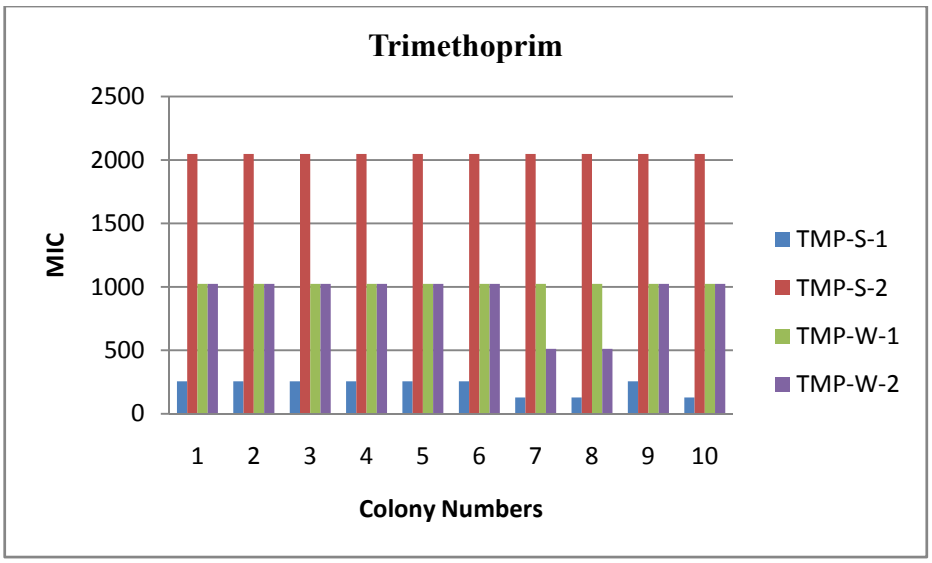
SMO-S-2
SMO-W-1
SMO-W-2

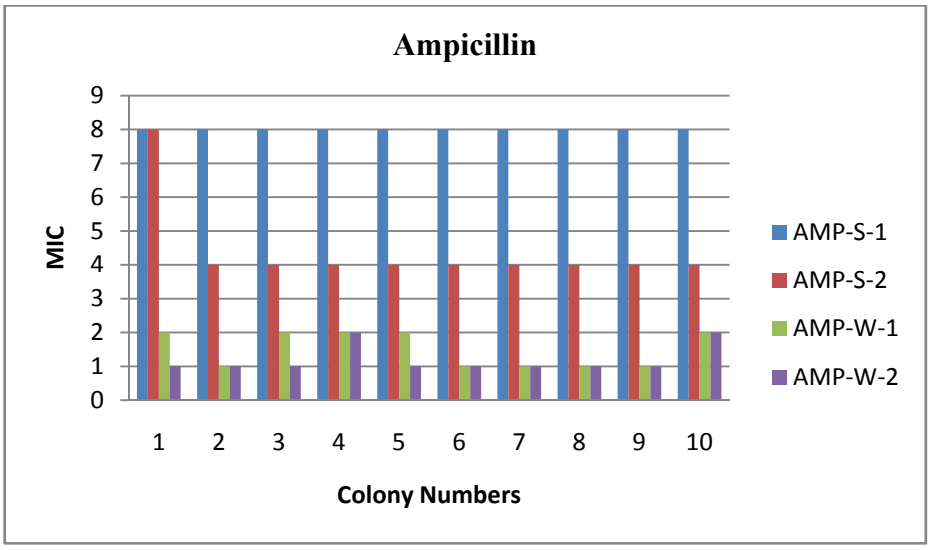

Spiramycin
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
SPR-S-1
SPR-S-2
SPR-W-1
SPR-W-2

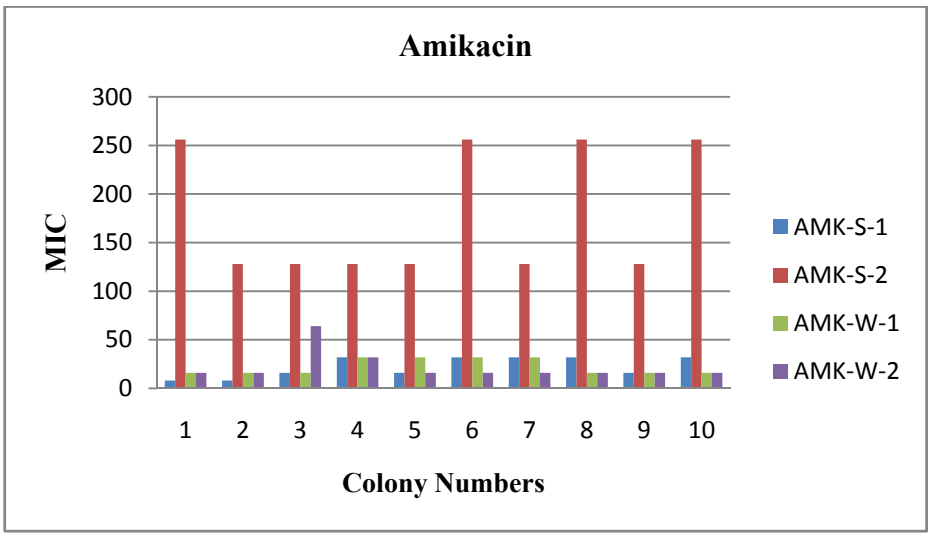

Erythromycin
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
ERY-S-1
ERY-S-2
ERY-W-1
ERY-W2

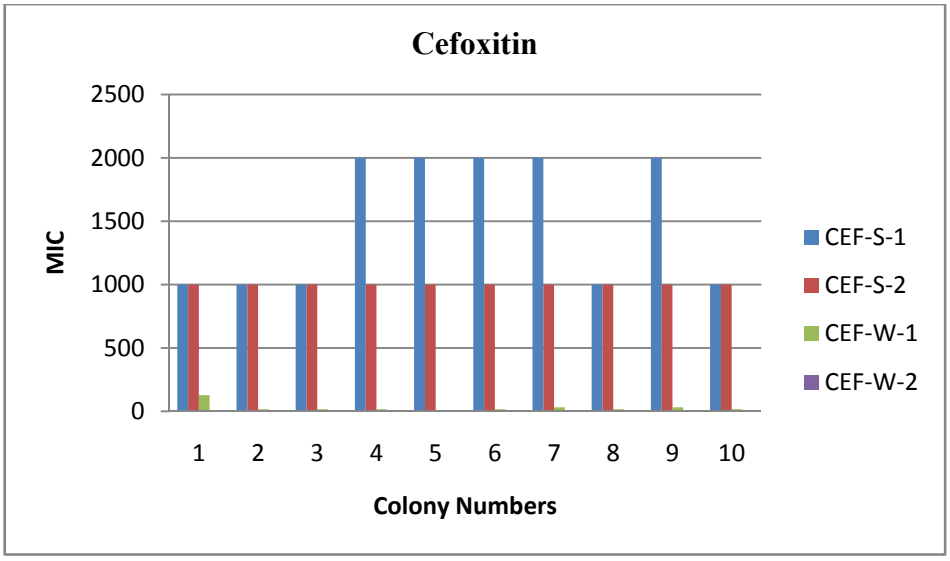

Nalidixic Acid
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
NAL-S-1
NAL-S-2
NAL-W-1
NAL-W-2


Streptomycin
100000
10000
1000
100
10
1
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
STR-S-1
STR-S-2
STR-W-1
STR-W-2


Trimethoprim
MIC
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10
Colony Numbers
TMP-S-1
TMP-S-2
TMP-W-1
TMP-W-2


Ampicillin
MIC
9
8
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
Colony Numbers
AMP-S-1
AMP-S-2
AMP-W-1
AMP-W-2


Amikacin
MIC
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10
Colony Numbers
AMK-S-1
AMK-S-2
AMK-W-1
AMK-W-2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1500
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MIC
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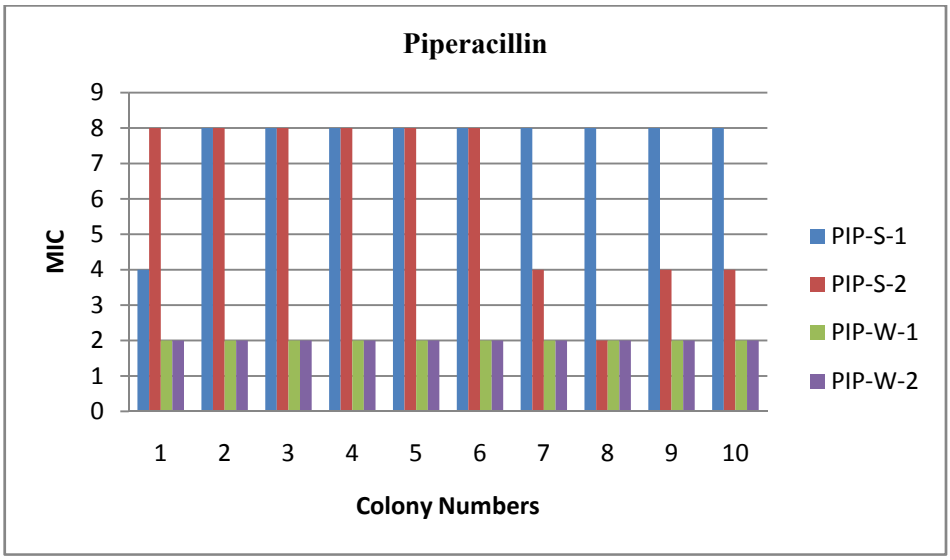
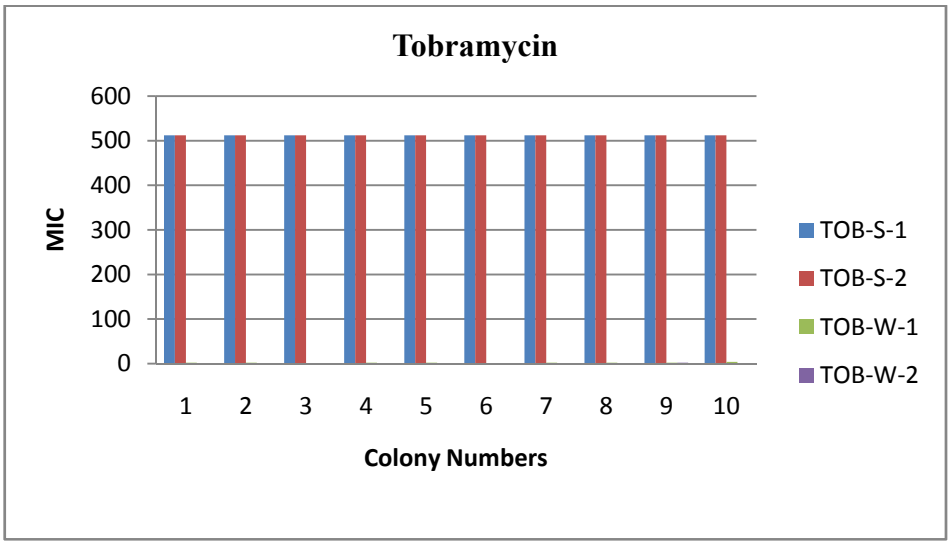
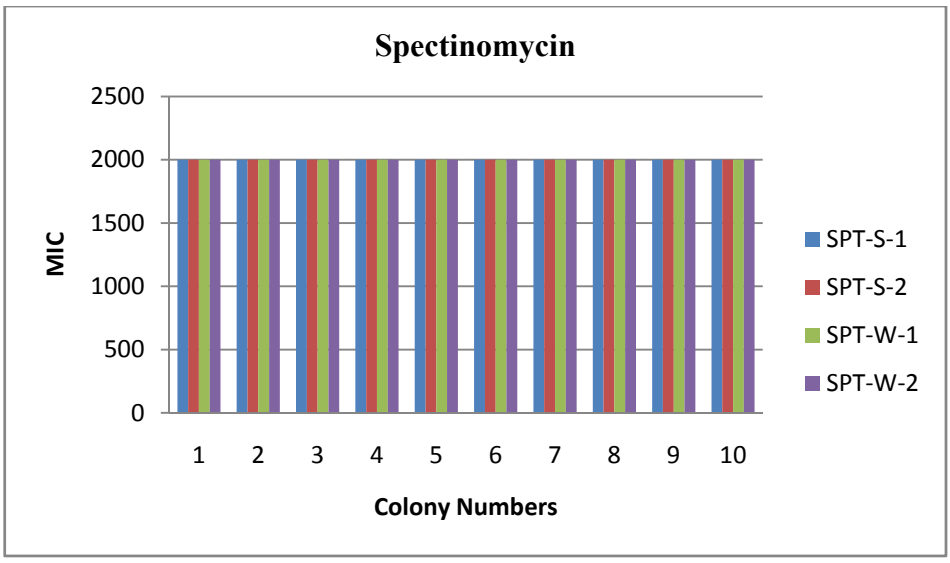
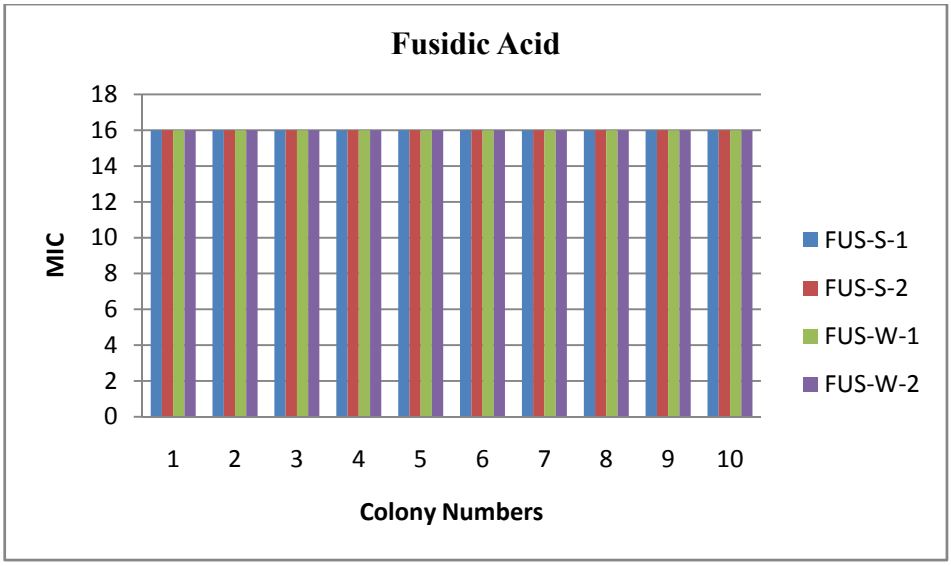
Colony Numbers
CEF-S-1
CEF-S-2
CEF-W-1
CEF-W-2


Piperacillin
9
8
7
6
5
4
3
2
1
0
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
PIP-S-1
PIP-S-2
PIP-W-1
PIP-W-2


Tobramycin
600
500
400
300
200
100
0
MIC
1 2 3 4 5 6 7 8 9 10
Colony Numbers
TOB-S-1
TOB-S-2
TOB-W-1
TOB-W-2


Spectinomycin
2500
2000
1500
1000
500
0
MIC
1
2
3
4
5
6
7
8
9
10
Colony Numbers
SPT-S-1
SPT-S-2
SPT-W-1
SPT-W-2


Fusidic Acid
18
16
14
12
10
8
6
4
2
0
MIC
1
2
3
4
5
6
7
8
9
10
Colony Numbers
FUS-S-1
FUS-S-2
FUS-W-1
FUS-W-2

Appendix B: Whole Genome Sequencing – Mutations

636102	[ybdO] NP_415136.1 putative DNA-binding transcriptional regulator
1273685	[ychO] NP_415738.2 putative invasin
3204885	[ttdA] NP_417533.1 L-tartrate dehydratase, alpha subunit
1644077	[ydfV] NP_416083.1 Qin prophage; putative protein
3856156	[yidJ] NP_418134.1 putative sulfatase/phosphatase
2569257	[eutE] NP_416950.1 aldehyde oxidoreductase, ethanolamine utilization protein
2890985	[ygcN] NP_417246.4 putative oxidoreductase with FAD/NAD(P)-binding domain
504406	[ushA] NP_415013.1 bifunctional UDP-sugar hydrolase/5'-nucleotidase
4044187	[yihG] NP_418299.1 inner membrane protein, Predicted acyltransferas
4400960	[hflK] NP_418595.1 modulator for HflB protease specific for phage lambda cII repressor
1065805	[agp] NP_415522.1 glucose-1-phosphatase/inositol phosphatase
1708344	[rsxG] NP_416148.1 electron transport complex protein required for the reduction of SoxR
1109415	[opgG] NP_415566.1 osmoregulated periplasmic glucan (OPG) biosynthesis periplasmic protein
4451975	[yjfF] NP_418652.2 putative sugar transporter subunit: membrane component of ABC superfamily
2211060	[yehU] NP_416630.1 putative sensory kinase in two-component system with YehT, inner membrane protein
3814791	[yicC] NP_418101.1 conserved protein, UPF0701 family
1617422	[marR] NP_416047.4 DNA-binding transcriptional repressor of multiple antibiotic resistance
3178101	[ygiB] NP_417509.3 conserved protein, UPF0441 family
985568	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
3994178	[yigA] NP_418255.1 conserved protein, DUF484 family
2462895	[mlaA] NP_416848.1 ABC transporter maintaining OM lipid asymmetry, OM lipoprotein component
3450110	[rplD] NP_417778.1 50S ribosomal subunit protein L4
1411301	[ydaQ] NP_415862.4 Rac prophage; conserved protein
4415505	[yjfP] NP_418611.1 acyl CoA esterase
3091191	[yqgE] NP_417423.4 hypothetical protein
4349471	[dcuS] NP_418549.1 sensory histidine kinase in two-component regulatory system with DcuR, regulator of anaerobic fumarate respiration
1842573	[ynjI] NP_416276.4 inner membrane protein
2188932	[yehB] NP_416612.1 putative outer membrane protein
1372108	[ycjO] NP_415827.1 putative sugar transporter subunit: membrane component of ABC superfamily
4369590	[groL] NP_418567.1 Cpn60 chaperonin GroEL, large subunit of GroESL
1417204	[ydaF] NP_415872.2 Rac prophage; putative protein
1933726	[zwf] NP_416366.1 glucose-6-phosphate 1-dehydrogenase
3448680	[rplB] NP_417776.1 50S ribosomal subunit protein L2
578086	[borD] NP_415089.1 DLP12 prophage; predicted lipoprotein
3219786	[ebgR] NP_417546.1 DNA-binding transcriptional repressor
395944	[sbmA] NP_414911.1 microcin B17 transporter
2294807	[ccmB] NP_416704.1 heme exporter subunit

571753	[ybcN] NP_415079.1 DLP12 prophage; putative protein
4477393	[yjgM] YP_026287.1 putative acetyltransferase
3469708	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
4025993	[fadA] YP_026272.1 3-ketoacyl-CoA thiolase (thiolase I)
529391	[selU] NP_415036.1 tRNA 2-selenouridine synthase, selenophosphate-dependent
3103465	[mltC] NP_417438.2 membrane-bound lytic murein transglycosylase C
2337195	[gyrA] NP_416734.1 DNA gyrase (type II topoisomerase), subunit A
2217053	[osmF] NP_416635.1 putative transporter subunit: periplasmic-binding component of ABC superfamily
2587454	[acrD] NP_416965.1 aminoglycoside/multidrug efflux system
1784986	[ppsA] NP_416217.1 phosphoenolpyruvate synthase
3443179	[rpsE] NP_417762.1 30S ribosomal subunit protein S5
1617223	[marR] NP_416047.4 DNA-binding transcriptional repressor of multiple antibiotic resistance
1721019	[ydhK] NP_416162.1 putative efflux protein (PET) component of YdhJK efflux pump
1812602	[katE] YP_025308.1 catalase HPII, heme d-containing
492388	[dnaX] NP_415003.1 DNA polymerase III/DNA elongation factor III, tau and gamma subunits
3393629	[yhdP] YP_026208.1 conserved membrane protein, predicted transporter
404757	[proC] NP_414920.1 pyrroline-5-carboxylate reductase, NAD(P)-binding
3450199	[rplD] NP_417778.1 50S ribosomal subunit protein L4
3938325	[hsrA] NP_418210.1 putative multidrug or homocysteine efflux system
916553	[ybjD] NP_415397.1 conserved protein with nucleoside triphosphate hydrolase domain
3161196	[plsC] NP_417490.1 1-acyl-sn-glycerol-3-phosphate acyltransferase
485003	[acrR] NP_414997.1 DNA-binding transcriptional repressor
3146232	[gpr] NP_417474.1 L-glyceraldehyde 3-phosphate reductase
2211749	[yehU] NP_416630.1 putative sensory kinase in two-component system with YehT, inner membrane protein
1687419	[manA] NP_416130.3 mannose-6-phosphate isomerase
4215787	[aceA] NP_418439.1 isocitrate lyase
1760687	[sufB] NP_416198.2 component of SufBCD Fe-S cluster assembly scaffold
1113310	[msyB] NP_415569.4 multicopy suppressor of secY and secA
2551265	[hemF] NP_416931.1 coproporphyrinogen III oxidase
1445666	[feaB] NP_415903.4 phenylacetaldehyde dehydrogenase
2278304	[rsuA] NP_416688.1 16S rRNA pseudouridine(516) synthase
3472447	[rpsL] NP_417801.1 30S ribosomal subunit protein S12
3997256	[uvrD] NP_418258.1 DNA-dependent ATPase I and helicase II
388323	[hemB] NP_414903.4 5-aminolevulinate dehydratase (porphobilinogen synthase)
912965	[hcp] NP_415394.4 hybrid-cluster [4Fe-2S-2O] protein in anaerobic terminal reductases
2931496	[fucA] NP_417280.1 L-fuculose-1-phosphate aldolase
1553065	[maeA] NP_415996.2 malate dehydrogenase, (decarboxylating, NAD-requiring) (malic enzyme)
4031625	[trkH] YP_026273.1 potassium transporter
1294996	[adhE] NP_415757.1 fused acetaldehyde-CoA dehydrogenase/ iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase
2670868	[hcaD] NP_417037.1 phenylpropionate dioxygenase, ferredoxin reductase subunit
2119476	[wcaJ] NP_416551.1 colanic biosynthesis UDP-glucose lipid carrier transferase
2160107	[mdtD] NP_416581.1 putative arabinose efflux transporter

1195455	[icd] NP_415654.1 e14 prophage; isocitrate dehydrogenase, specific for NADP+
2360177	[rhmR] NP_416751.1 putative DNA-binding transcriptional regulator for the rhm operon
2133543	[wzc] NP_416564.4 protein-tyrosine kinase
883442	[mdfA] NP_415363.1 multidrug efflux system protein
1386108	[tyrR] NP_415839.1 DNA-binding transcriptional dual regulator, tyrosine-binding
1617374	[marR] NP_416047.4 DNA-binding transcriptional repressor of multiple antibiotic resistance
1186811	[ycfD] NP_415646.4 cupin superfamily protein
861224	[ybiW] NP_415344.1 putative pyruvate formate lyase
2809265	[mprA] NP_417169.1 DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux
4514825	[fecR] NP_418712.1 KpLE2 phage-like element; transmembrane signal transducer for ferric citrate transport
4224788	[metH] NP_418443.1 homocysteine-N5-methyltetrahydrofolate transmethylase, B12-dependent
3776879	[lldP] NP_418060.1 L-lactate permease
2462896	[mlaA] NP_416848.1 ABC transporter maintaining OM lipid asymmetry, OM lipoprotein component
396841	[sbmA] NP_414911.1 microcin B17 transporter
3406003	[panF] NP_417724.4 pantothenate:sodium symporter
2073907	[yeeR] NP_416505.2 CP4-44 prophage; predicted membrane protein
2872282	[cysN] NP_417231.1 sulfate adenylyltransferase, subunit 1
2307723	[ada] NP_416717.1 fused DNA-binding transcriptional dual regulator/ O6-methylguanine-DNA methyltransferase
837437	[ybiI] NP_415324.1 DksA-type zinc finger protein
578815	[ybcV] NP_415090.2 DLP12 prophage; putative protein
1091498	[pgaA] NP_415543.1 biofilm adhesin polysaccharide PGA secretin; OM porin; poly-beta-1,6-N-acetyl-D-glucosamine export protein
911406	[hcp] NP_415394.4 hybrid-cluster [4Fe-2S-2O] protein in anaerobic terminal reductases
3968099	[wzzE] NP_418232.2 Entobacterial Common Antigen (ECA) polysaccharide chain length modulation protein
345354	[yahN] NP_414862.1 amino acid exporter for proline, lysine, glutamate, homoserine
888983	[ybjL] NP_415368.1 putative transporter
1773282	[aroD] NP_416208.1 3-dehydroquinate dehydratase
1955486	[torY] NP_416387.1 TMAO reductase III (TorYZ), cytochrome c-type subunit
2477192	[dsdX] NP_416866.1 D-serine permease
802885	[ybhJ] NP_415292.2 putative hydratase
1406309	[ydaN] NP_415858.1 putative Zn(II) transporter
305436	[ecpD] NP_414824.1 putative receptor
473964	[tesB] NP_414986.1 acyl-CoA thioesterase II
1234632	[fadR] NP_415705.1 DNA-binding transcriptional dual regulator of fatty acid metabolism
2310640	[ompC] NP_416719.1 outer membrane porin protein C
2204671	[yehM] NP_416624.1 hypothetical protein
2643570	[pbpC] NP_417014.1 penicillin-binding protein PBP1C murein transglycosylase; inactive transpeptidase domain
2200139	[yehI] NP_416621.1 hypothetical protein
2073784	[yeeR] NP_416505.2 CP4-44 prophage; predicted membrane protein
482636	[acrB] NP_414995.1 multidrug efflux system protein
793325	[modE] NP_415282.1 DNA-binding transcriptional repressor for the molybdenum transport operon modABC

2097421	[ugd] NP_416532.1 UDP-glucose 6-dehydrogenase
3443181	[rpsE] NP_417762.1 30S ribosomal subunit protein S5
4275919	[soxR] NP_418487.1 DNA-binding transcriptional dual regulator, Fe-S center for redox-sensing
4589958	[tsr] NP_418775.1 methyl-accepting chemotaxis protein I, serine sensor receptor
2809146	[mprA] NP_417169.1 DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux
2865722	[nlpD] NP_417222.1 activator of AmiC murein hydrolase activity, lipoprotein
985631	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
3148003	[yghA] NP_417476.1 putative oxidoreductase
4312125	[yjdP] YP_026281.1 hypothetical protein
3524151	[nudE] NP_417856.1 adenosine nucleotide hydrolase; substrates include Ap3A, Ap2A, ADP-ribose, NADH
1671998	[tqsA] NP_416118.1 pheromone AI-2 transporter
648706	[citF] NP_415148.1 citrate lyase, citrate-ACP transferase (alpha) subunit
986178	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
206222	[dnaE] NP_414726.1 DNA polymerase III alpha subunit
2443462	[yfcA] NP_416830.1 inner membrane protein, UPF0721 family
3534600	[ompR] NP_417864.1 DNA-binding response regulator in two-component regulatory system with EnvZ
1174912	[lolC] NP_415634.1 lipoprotein-releasing system transmembrane protein
468200	[mdlA] NP_414982.1 fused predicted multidrug transporter subunits of ABC superfamily: ATP-binding components
3483722	[yhfA] NP_417815.1 conserved protein, OsmC family
2204640	[yehM] NP_416624.1 hypothetical protein
1347175	[yciW] NP_415803.2 putative oxidoreductase
3322850	[folP] NP_417644.4 7,8-dihydropteroate synthase
3438945	[rpoA] NP_417754.1 RNA polymerase, alpha subunit
3298314	[yhbS] NP_417625.1 putative acyltransferase with acyl-CoA N-acyltransferase domain
985734	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
1181136	[potD] NP_415641.1 polyamine transporter subunit
3038287	[fldB] NP_417371.1 flavodoxin 2
4455000	[mpl] NP_418654.1 UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase
1890900	[yoaA] NP_416322.1 conserved protein with nucleoside triphosphate hydrolase domain
4204941	[purH] NP_418434.1 fused IMP cyclohydrolase/ phosphoribosylaminoimidazolecarboxamide formyltransferase
1663606	[clcB] NP_416109.2 H(+)/Cl(-) exchange transporter
55879	[lptD] NP_414596.1 LPS assembly OM complex LptDE, beta-barrel component
1946940	[aspS] NP_416380.1 aspartyl-tRNA synthetase
3409267	[dusB] NP_417726.1 tRNA-dihydrouridine synthase B
3928520	[ravA] NP_418202.4 fused predicted transcriptional regulator: sigma54 activator protein/conserved protein
1715421	[pdxH] NP_416155.1 pyridoxine 5'-phosphate oxidase
3786341	[yibQ] NP_418071.4 putative polysaccharide deacetylase
2970274	[tas] NP_417311.1 putative oxidoreductase, NADP(H)-dependent aldo-keto reductase; suppresses tyrosine requirement of tyrA14 O6 strain
847046	[glnH] NP_415332.1 glutamine transporter subunit
2722072	[pssA] NP_417080.4 phosphatidylserine synthase (CDP-diacylglycerol-serine O-phosphatidyltransferase)

3915559	[atpG] NP_418189.1 F1 sector of membrane-bound ATP synthase, gamma subunit
44460	[fixC] NP_414585.1 putative oxidoreductase with FAD/NAD(P)-binding domain
3876752	[gyrB] YP_026241.1 DNA gyrase, subunit B
1744956	[ydhR] NP_416182.1 putative monooxygenase
3470224	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
1747253	[ydhT] NP_416184.1 hypothetical protein
2918564	[gudX] NP_417268.1 glucarate dehydratase-related protein, substrate unknown
268887	[perR] NP_414788.1 CP4-6 prophage; predicted DNA-binding transcriptional regulator
2186577	[yehB] NP_416612.1 putative outer membrane protein
3081120	[speB] NP_417412.1 agmatinase
445555	[yajR] NP_414961.4 putative transporter
2470630	[yfdN] NP_416858.1 CPS-53 (KpLE1) prophage; putative protein
384852	[tauA] NP_414899.2 taurine transporter subunit
54342	[surA] NP_414595.1 peptidyl-prolyl cis-trans isomerase (PPIase)
703318	[nagE] NP_415205.1 fused N-acetyl glucosamine specific PTS enzyme: IIC, IIB, and IIA components
3469576	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
3160208	[ftsP] NP_417489.1 septal ring component that protects the divisome from stress; multicopy suppressor of ftsI(Ts)
1883390	[yeaW] NP_416316.1 putative 2Fe-2S cluster-containing protein
4413191	[aidB] NP_418608.6 DNA alkylation damage repair protein; flavin-containing DNA binding protein, weak isovaleryl CoA dehydrogenase
3191310	[yqiK] NP_417523.1 PHB family membrane protein, function unknown
2392778	[nuoL] NP_416781.1 NADH:ubiquinone oxidoreductase, membrane subunit L
829057	[ybhG] NP_415316.1 putative membrane fusion protein (MFP) component of efflux pump, membrane anchor
4556529	[iadA] NP_418748.1 isoaspartyl dipeptidase
50273	[folA] NP_414590.1 dihydrofolate reductase
1087859	[pgaB] NP_415542.1 poly-beta-1,6-N-acetyl-D-glucosamine (PGA) N-deacetylase; deacetylase required for biofilm adhesin polysaccharide PGA export;
1195468	[icd] NP_415654.1 e14 prophage; isocitrate dehydrogenase, specific for NADP+
1347477	[yciW] NP_415803.2 putative oxidoreductase
433516	[ribD] NP_414948.1 fused diaminohydroxyphosphoribosylaminopyrimidine deaminase and 5-amino-6-(5-phosphoribosylamino) uracil reductase
2764951	[rnlA] NP_417119.1 CP4-57 prophage; RNase LS
3744558	[yiaO] NP_418036.1 L-dehydroascorbate transporter, periplasmic binding protein for TRAP (TRipartite ATP-independent Periplasmic) family transport>
3867078	[yidR] NP_418144.3 hypothetical protein
2935304	[fucI] NP_417282.1 L-fucose isomerase
952105	[pflB] NP_415423.1 pyruvate formate lyase I
2418438	[yfcF] NP_416804.1 glutathione S-transferase
49884	[folA] NP_414590.1 dihydrofolate reductase
1207007	[stfP] NP_415672.1 e14 prophage; putative protein
1310586	[yciB] NP_415770.1 putative inner membrane protein
693849	[miaB] NP_415194.1 tRNA-i(6)A37 methylthiotransferase

2775583	[ypjF] NP_417133.1 CP4-57 prophage; toxin of the YpjF-YfjZ toxin-antitoxin system
102057	[murC] NP_414633.1 UDP-N-acetylmuramate:L-alanine ligase
1002941	[ycbV] NP_415463.2 putative fimbrial-like adhesin protein
2245623	[lysP] NP_416661.1 lysine transporter
4432824	[cpdB] NP_418634.1 2':3'-cyclic-nucleotide 2'-phosphodiesterase
2070411	[flu] YP_026164.1 CP4-44 prophage; antigen 43 (Ag43) phase-variable biofilm formation autotransporter
2203844	[yehM] NP_416624.1 hypothetical protein
2877629	[ygbT] NP_417235.1 multifunctional endonuclease Cas1, CRISPR adaptation protein; DNA repair enzyme
3080414	[loiP] NP_417411.2 Phe-Phe periplasmic metalloprotease, OM lipoprotein; low salt-inducible; heat shock protein that binds Era
3120256	[glcB] NP_417450.1 malate synthase G
1679783	[ydgC] NP_416124.1 inner membrane protein, GlpM family
1871805	[yeaK] NP_416301.1 hypothetical protein
137377	[cueO] NP_414665.1 multicopper oxidase (laccase)
2687816	[glrK] NP_417051.2 sensor protein kinase regulating glmY sRNA in two-component system with response regulator GlrR
3064489	[ygfI] NP_417396.4 putative DNA-binding transcriptional regulator
4251293	[ubiA] NP_418464.1 p-hydroxybenzoate octaprenyltransferase
3830912	[yicI] NP_418113.1 putative alpha-glucosidase
3125042	[glcD] NP_417453.1 glycolate oxidase subunit, FAD-linked
3448974	[rplB] NP_417776.1 50S ribosomal subunit protein L2
1900832	[manX] NP_416331.1 fused mannose-specific PTS enzymes: IIA component/IIB component
3177594	[tolC] NP_417507.2 transport channel
550209	[ybcF] NP_415054.1 putative carbamate kinase
4131373	[metF] NP_418376.1 5,10-methylenetetrahydrofolate reductase
3136911	[yghU] NP_417463.4 putative S-transferase
1257441	[pth] NP_415722.1 peptidyl-tRNA hydrolase
1187003	[ycfD] NP_415646.4 cupin superfamily protein
1642279	[ydfU] NP_416078.4 Qin prophage; putative protein
4275121	[soxS] NP_418486.1 DNA-binding transcriptional dual regulator
12226	[dnaK] NP_414555.1 chaperone Hsp70, co-chaperone with DnaJ
1973718	[motB] NP_416403.1 protein that enables flagellar motor rotation
2314647	[rcsB] NP_416721.1 DNA-binding response regulator in two-component regulatory system
1428751	[stfR] NP_415890.2 Rac prophage; predicted tail fiber protein
3001451	[xdhB] NP_417343.1 xanthine dehydrogenase, FAD-binding subunit
4275928	[soxR] NP_418487.1 DNA-binding transcriptional dual regulator, Fe-S center for redox-sensing
3348148	[elbB] NP_417676.2 isoprenoid biosynthesis protein with amidotransferase-like domain
2229437	[yohK] NP_416647.1 inner membrane protein, LrgB family
701000	[nagA] NP_415203.1 N-acetylglucosamine-6-phosphate deacetylase
3485248	[yhfK] NP_417817.2 conserved inner membrane protein
1195470	[icd] NP_415654.1 e14 prophage; isocitrate dehydrogenase, specific for NADP+
396573	[sbmA] NP_414911.1 microcin B17 transporter
74244	[thiP] NP_414609.1 fused thiamin transporter subunits of ABC superfamily: membrane components

3249422	[yqjG] NP_417573.1 putative S-transferase
452251	[ampG] NP_414967.1 muropeptide transporter
959520	[ycaL] NP_415429.2 putative peptidase with chaperone function
2836876	[ascG] NP_417194.2 DNA-binding transcriptional repressor
260982	[proA] NP_414778.1 gamma-glutamylphosphate reductase
1792999	[btuC] NP_416226.1 vitamin B12 transporter subunit: membrane component of ABC superfamily
3317785	[argG] NP_417640.1 argininosuccinate synthetase
1250379	[dhaR] NP_415719.2 DNA-binding transcription activator of the dhaKLM operon
3534585	[ompR] NP_417864.1 DNA-binding response regulator in two-component regulatory system with EnvZ
2464658	[intS] NP_416850.1 CPS-53 (KpLE1) prophage; predicted prophage CPS-53 integrase
4470201	[pyrB] NP_418666.1 aspartate carbamoyltransferase, catalytic subunit
1817195	[chbR] NP_416249.1 repressor of chb operon for N,N'-diacetylchitobiose utilization
3318300	[yhbX] NP_417641.4 putative hydrolase, inner membrane
2209775	[yehS] NP_416628.1 conserved protein, DUF1456 family
3310080	[truB] NP_417635.1 tRNA pseudouridine(55) synthase
1431277	[pinR] NP_415892.1 Rac prophage; predicted site-specific recombinase
1354665	[sapA] NP_415810.1 antimicrobial peptide transport ABC transporter periplasmic binding protein
3868548	[dgoT] NP_418146.4 D-galactonate transporter
719983	[ybfK] YP_001165310.1 hypothetical protein
929058	[cydD] NP_415407.1 fused glutathione, cysteine exporter subunits of ABC superfamily: membrane component/ATP-binding component
3032847	[lysS] NP_417366.1 lysine tRNA synthetase, constitutive
107453	[lpxC] NP_414638.1 UDP-3-O-acyl N-acetylglucosamine deacetylase
983426	[ycbL] NP_415447.1 putative metal-binding enzyme
2921998	[yqcC] NP_417272.1 hypothetical protein
210681	[ldcC] NP_414728.1 lysine decarboxylase 2, constitutive
564204	[intD] NP_415069.1 DLP12 prophage; predicted integrase
1060972	[torA] NP_415517.1 trimethylamine N-oxide (TMAO) reductase I, catalytic subunit
3499212	[frlA] NP_417829.2 putative fructoselysine transporter
3443427	[rplR] NP_417763.1 50S ribosomal subunit protein L18
3813953	[rph] YP_001491547.1 defective ribonuclease PH
1723265	[ydhF] YP_025305.1 putative oxidoreductase
3198582	[ygiF] NP_417526.1 putative adenylate cyclase
329967	[betT] NP_414848.1 choline transporter of high affinity
3315876	[rimP] NP_417639.6 ribosome maturation factor for 30S subunits
2913808	[barA] NP_417266.1 hybrid sensory histidine kinase, in two-component regulatory system with UvrY
1107598	[opgC] NP_415565.1 membrane protein required for succinylation of osmoregulated periplasmic glucans (OPGs)
3470425	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
3129698	[yghQ] YP_026192.1 putative inner membrane protein
4136733	[gldA] NP_418380.4 glycerol dehydrogenase, NAD
1683342	[fumC] NP_416128.1 fumarate hydratase (fumarase C),aerobic Class II
587340	[nfrA] NP_415100.1 bacteriophage N4 receptor, outer membrane subunit

4268514	[yjbQ] NP_418480.1 thiamin phosphate synthase
2436526	[flk] NP_416824.1 putative flagella assembly protein
3380750	[degS] NP_417702.1 serine endoprotease, periplasmic
2529722	[cysZ] NP_416908.1 putative inner membrane protein
870059	[gsiB] NP_415351.1 glutathione periplasmic binding protein, ABC superfamily transporter
3858913	[yidL] NP_418136.2 putative transcriptional regulator, AraC family
4395634	[mutL] NP_418591.1 methyl-directed mismatch repair protein
2419381	[folX] NP_416806.1 D-erythro-7,8-dihydroneopterin triphosphate 2'-epimerase and dihydroneopterin aldolase
3876781	[gyrB] YP_026241.1 DNA gyrase, subunit B
1083266	[efeB] NP_415538.1 deferrrochelatase, periplasmic
4141846	[frwB] NP_418385.1 putative enzyme IIB component of PTS
3091888	[yqgF] NP_417424.1 putative Holliday junction resolvase
4123479	[priA] NP_418370.1 Primosome factor n' (replication factor Y)
3913753	[atpC] NP_418187.1 F1 sector of membrane-bound ATP synthase, epsilon subunit
3409497	[fis] NP_417727.1 global DNA-binding transcriptional dual regulator
3389150	[tldD] NP_417711.1 putative peptidase
1820025	[osmE] NP_416253.1 DNA-binding transcriptional activator
4466494	[mgtA] NP_418663.1 magnesium transporter
1776596	[ydiP] NP_416211.1 putative DNA-binding transcriptional regulator
2337184	[gyrA] NP_416734.1 DNA gyrase (type II topoisomerase), subunit A
3876758	[gyrB] YP_026241.1 DNA gyrase, subunit B
138888	[gcd] NP_414666.1 glucose dehydrogenase
2636621	[bamB] NP_417007.1 lipoprotein required for OM biogenesis, in BamABCDE complex
3699608	[yhjV] NP_417996.1 putative transporter
3809326	[rpmG] NP_418093.1 50S ribosomal subunit protein L33
965164	[ycaI] NP_415433.4 inner membrane protein, ComEC family of competence proteins
612445	[fes] NP_415117.1 enterobactin/ferric enterobactin esterase
1615230	[ydeA] NP_416045.1 arabinose efflux transporter, arabinose-inducible
1248080	[dhaM] NP_415716.4 fused predicted dihydroxyacetone-specific PTS enzymes: HPr component/EI component
2340916	[yfaL] NP_416736.1 adhesin
49903	[folA] NP_414590.1 dihydrofolate reductase
1447346	[tynA] NP_415904.3 tyramine oxidase, copper-requiring
4611572	[yjjV] YP_026291.2 putative DNase
2122831	[wcaI] NP_416554.1 putative glycosyl transferase
2606930	[hyfG] NP_416982.1 hydrogenase 4, subunit
3629733	[yhiJ] NP_417945.1 hypothetical protein
2768099	[yfjR] NP_417123.1 CP4-57 prophage; predicted DNA-binding transcriptional regulator
1207012	[stfP] NP_415672.1 e14 prophage; putative protein
872573	[yliE] NP_415354.1 putative membrane-anchored cyclic-di-GMP phosphodiesterase
807017	[ybhB] NP_415294.1 kinase inhibitor homolog, UPF0098 family
358523	[cynT] NP_414873.1 carbonic anhydrase
2316952	[rcsC] NP_416722.2 hybrid sensory kinase in two-component regulatory system with RcsB and YojN

986119	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
3560067	[glpD] NP_417884.1 sn-glycerol-3-phosphate dehydrogenase, aerobic, FAD/NAD(P)-binding
2901374	[ygcE] NP_417256.1 putative kinase
1188391	[phoQ] NP_415647.1 sensory histidine kinase in two-component regulatory system with PhoP
98614	[ftsW] NP_414631.1 lipid II flippase; integral membrane protein involved in stabilizing FstZ ring during cell division
1116242	[yceA] NP_415573.1 putative rhodanese-related sulfurtransferase
1783059	[ppsA] NP_416217.1 phosphoenolpyruvate synthase
255063	[pepD] NP_414772.1 aminoacyl-histidine dipeptidase (peptidase D)
4489113	[idnR] NP_418685.1 DNA-binding transcriptional repressor, 5-gluconate-binding
287866	[yagI] NP_414806.1 CP4-6 prophage; predicted DNA-binding transcriptional regulator
1679516	[folM] NP_416123.1 dihydromonapterin reductase, NADPH-dependent; dihydrofolate reductase isozyme
293443	[yagM] NP_414813.1 CP4-6 prophage; putative protein
985125	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
4451774	[yjfF] NP_418652.2 putative sugar transporter subunit: membrane component of ABC superfamily
1506749	[ydcP] NP_415952.2 putative peptidase
3678090	[kdgK] NP_417983.2 ketodeoxygluconokinase
3304283	[deaD] NP_417631.2 ATP-dependent RNA helicase
4634900	[creC] NP_418816.1 sensory histidine kinase in two-component regulatory system with CreB or PhoB, regulator of the CreBC regulon
2466906	[gtrB] NP_416852.1 CPS-53 (KpLE1) prophage; bactoprenol glucosyl transferase
1012805	[pqiB] NP_415471.1 paraquat-inducible protein B
1687656	[manA] NP_416130.3 mannose-6-phosphate isomerase
4337036	[adiA] NP_418541.2 arginine decarboxylase
2661303	[trmJ] NP_417027.1 tRNA mC32,mU32 2'-O-methyltransferase, SAM-dependent
3472319	[rpsL] NP_417801.1 30S ribosomal subunit protein S12
1273620	[ychO] NP_415738.2 putative invasin
2049192	[yeeJ] NP_416485.4 putative adhesin
801628	[ybhI] NP_415291.1 putative transporter
613272	[ybdZ] YP_588441.1 stimulator of EntF adenylation activity, MbtH-like
3558717	[glpG] YP_026220.1 rhomboid intramembrane serine protease
971091	[ycbJ] NP_415439.1 hypothetical protein
1453804	[paaC] NP_415908.1 ring 1,2-phenylacetyl-CoA epoxidase subunit
3450113	[rplD] NP_417778.1 50S ribosomal subunit protein L4
3338959	[yrbG] NP_417663.1 putative calcium/sodium:proton antiporter
2470663	[yfdN] NP_416858.1 CPS-53 (KpLE1) prophage; putative protein
3214292	[yqjH] NP_417541.1 putative siderophore interacting protein
2126296	[gmd] NP_416557.1 GDP-D-mannose dehydratase, NAD(P)-binding
3534319	[ompR] NP_417864.1 DNA-binding response regulator in two-component regulatory system with EnvZ
2431182	[accD] NP_416819.1 acetyl-CoA carboxylase, beta (carboxyltransferase) subunit
4600411	[yjjP] NP_418784.4 inner membrane protein, H-NS-repressed, DUF1212 family
3813952	[rph] YP_001491547.1 defective ribonuclease PH
3919572	[atpB] NP_418194.1 F0 sector of membrane-bound ATP synthase, subunit a

2143501	[yegE] NP_416571.1 putative diguanylate cyclase, GGDEF domain signaling protein
2353576	[glpC] NP_416746.1 anaerobic sn-glycerol-3-phosphate dehydrogenase, C subunit, 4Fe-4S iron-sulfur cluster
1131118	[flgD] NP_415593.1 flagellar hook assembly protein
4377058	[frdD] NP_418575.1 fumarate reductase (anaerobic), membrane anchor subunit
13782	[dnaK] NP_414555.1 chaperone Hsp70, co-chaperone with DnaJ
1862103	[yeaD] NP_416294.4 hypothetical protein
3471188	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
801642	[ybhI] NP_415291.1 putative transporter
3322744	[folP] NP_417644.4 7,8-dihydropteroate synthase
1903785	[mntP] NP_416335.4 putative Mn(2+) efflux pump, mntR-regulated
4443749	[tamB] NP_418642.1 translocation and assembly module for autotransporter export, inner membrane subunit
4380036	[frdA] NP_418578.1 fumarate reductase (anaerobic) catalytic and NAD/flavoprotein subunit
2666415	[hcaR] NP_417032.1 DNA-binding transcriptional activator of 3-phenylpropionic acid catabolism
4100472	[kdgT] NP_418345.2 2-keto-3-deoxy-D-gluconate transporter
3409403	[fis] NP_417727.1 global DNA-binding transcriptional dual regulator
4090795	[frvA] NP_418336.1 putative enzyme IIA component of PTS
1282377	[narG] NP_415742.1 nitrate reductase 1, alpha subunit
1562385	[dosP] NP_416006.4 oxygen sensor, c-di-GMP phosphodiesterase, heme-regulated; cold- and stationary phase-induced bioflim regulator
377334	[frmB] NP_414889.1 S-formylglutathione hydrolase
1710022	[nth] NP_416150.1 DNA glycosylase and apyrimidinic (AP) lyase (endonuclease III)
3534074	[ompR] NP_417864.1 DNA-binding response regulator in two-component regulatory system with EnvZ
850503	[ybiP] NP_415336.1 putative hydrolase, inner membrane
2123896	[wcaH] NP_416555.2 GDP-mannose mannosyl hydrolase
3932751	[rbsA] NP_418205.1 fused D-ribose transporter subunits of ABC superfamily: ATP-binding components
3203536	[ttdR] NP_417532.1 transcriptional activator of ttdABT
2809160	[mprA] NP_417169.1 DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux
449164	[cyoB] NP_414965.1 cytochrome o ubiquinol oxidase subunit I
714985	[ybfP] NP_415215.1 lipoprotein
361229	[lacY] NP_414877.1 lactose permease
2262723	[setB] NP_416675.1 lactose/glucose efflux system
4388777	[rsgA] NP_418585.4 ribosome small subunit-dependent GTPase A
2273314	[yejE] NP_416684.1 microcin C transporter YejABEF, permease subunit; ABC family
537487	[ybbW] NP_415044.4 putative allantoin transporter
1959288	[argS] NP_416390.1 arginyl-tRNA synthetase
529386	[selU] NP_415036.1 tRNA 2-selenouridine synthase, selenophosphate-dependent
1677229	[ydgH] NP_416121.1 hypothetical protein
3472446	[rpsL] NP_417801.1 30S ribosomal subunit protein S12
332568	[yahA] NP_414849.1 c-di-GMP-specific phosphodiesterase
3915554	[atpG] NP_418189.1 F1 sector of membrane-bound ATP synthase, gamma subunit
4064029	[yihP] NP_418313.4 putative transporter

4632681	[rob] NP_418813.1 right oriC-binding transcriptional activator, AraC family
411349	[araJ] NP_414930.3 arabinose-inducible predicted transporter, MFS family
3916825	[atpA] NP_418190.1 F1 sector of membrane-bound ATP synthase, alpha subunit
995757	[ssuA] NP_415456.4 aliphatic sulfonate binding protein, SsuABC ABC transporter
481922	[acrB] NP_414995.1 multidrug efflux system protein
73799	[thiP] NP_414609.1 fused thiamin transporter subunits of ABC superfamily: membrane components
4031406	[trkH] YP_026273.1 potassium transporter
1078658	[putP] NP_415535.1 proline:sodium symporter
2350765	[glpA] NP_416744.1 sn-glycerol-3-phosphate dehydrogenase (anaerobic), large subunit, FAD/NAD(P)-binding
2806082	[proX] NP_417165.1 glycine betaine transporter subunit
770728	[cydA] NP_415261.2 cytochrome d terminal oxidase, subunit I
2587372	[acrD] NP_416965.1 aminoglycoside/multidrug efflux system
2789076	[lhgO] NP_417146.2 L-2-hydroxyglutarate oxidase
503474	[fsr] NP_415012.1 putative fosmidomycin efflux system
3542001	[yhgA] NP_417870.1 putative transposase
450606	[cyoA] NP_414966.1 cytochrome o ubiquinol oxidase subunit II
4297367	[fdhF] NP_418503.1 formate dehydrogenase-H, selenopolypeptide subunit
3443175	[rpsE] NP_417762.1 30S ribosomal subunit protein S5
1335816	[acnA] NP_415792.1 aconitate hydratase 1
4417622	[ulaG] NP_418613.2 L-ascorbate 6-phosphate lactonase
328784	[betT] NP_414848.1 choline transporter of high affinity
1195500	[icd] NP_415654.1 e14 prophage; isocitrate dehydrogenase, specific for NADP+
813892	[uvrB] NP_415300.1 excinulease of nucleotide excision repair, DNA damage recognition component
1679046	[folM] NP_416123.1 dihydromonapterin reductase, NADPH-dependent; dihydrofolate reductase isozyme
3498207	[frlA] NP_417829.2 putative fructoselysine transporter
4186886	[rpoC] NP_418415.1 RNA polymerase, beta prime subunit
3078890	[tktA] YP_026188.1 transketolase 1, thiamin-binding
3467132	[chiA] NP_417797.1 periplasmic endochitinase
3031189	[idi] NP_417365.1 isopentenyl diphosphate isomerase
2167265	[yegS] NP_416590.1 phosphatidylglycerol kinase, metal-dependent
3915549	[atpG] NP_418189.1 F1 sector of membrane-bound ATP synthase, gamma subunit
2419513	[folX] NP_416806.1 D-erythro-7,8-dihydroneopterin triphosphate 2'-epimerase and dihydroneopterin aldolase
606258	[ybdK] NP_415113.1 weak gamma-glutamyl:cysteine ligase
4243669	[malE] NP_418458.1 maltose transporter subunit
745387	[nei] NP_415242.1 endonuclease VIII/ 5-formyluracil/5-hydroxymethyluracil DNA glycosylase
1406960	[ydaN] NP_415858.1 putative Zn(II) transporter
3920072	[atpB] NP_418194.1 F0 sector of membrane-bound ATP synthase, subunit a
1035439	[hyaD] NP_415494.1 hydrogenase 1 maturation protease
2112946	[wcaM] NP_416547.1 colanic acid biosynthesis protein
258831	[phoE] NP_414776.1 outer membrane phosphoporin protein E
4072916	[yihW] NP_418320.2 putative DNA-binding transcriptional regulator

1898961	[yoaE] NP_416330.1 fused predicted membrane protein/conserved protein
2809115	[mprA] NP_417169.1 DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux
2610279	[hyfR] NP_416986.4 DNA-binding transcriptional activator, formate sensing
3462354	[gspL] NP_417792.2 general secretory pathway component, cryptic
3534564	[ompR] NP_417864.1 DNA-binding response regulator in two-component regulatory system with EnvZ
107236	[lpxC] NP_414638.1 UDP-3-O-acyl N-acetylglucosamine deacetylase
2279913	[yejH] NP_416689.1 putative ATP-dependent DNA or RNA helicase
4429600	[ytfE] NP_418630.1 iron-sulfur cluster repair protein RIC
2335231	[gyrA] NP_416734.1 DNA gyrase (type II topoisomerase), subunit A
1555940	[ddpF] NP_416000.1 D,D-dipeptide permease system, ATP-binding component
1585175	[ydeQ] NP_416019.1 putative fimbrial-like adhesin protein
3720775	[glyS] NP_418016.1 glycine tRNA synthetase, beta subunit
1011375	[pqiA] NP_415470.1 paraquat-inducible membrane protein A
49899	[folA] NP_414590.1 dihydrofolate reductase
2094391	[hisF] NP_416529.1 imidazole glycerol phosphate synthase, catalytic subunit with HisH
2457798	[fadI] NP_416844.1 beta-ketoacyl-CoA thiolase, anaerobic, subunit
2230720	[cdd] NP_416648.1 cytidine/deoxycytidine deaminase
394141	[yaiV] NP_414909.2 putative DNA-binding transcriptional regulator
134549	[yacL] NP_414661.2 hypothetical protein
4003289	[pldA] NP_418265.1 outer membrane phospholipase A
1923333	[holE] NP_416356.1 DNA polymerase III, theta subunit
4365606	[aspA] NP_418562.4 aspartate ammonia-lyase
1084229	[phoH] NP_415539.1 conserved protein with nucleoside triphosphate hydrolase domain
3472510	[rpsL] NP_417801.1 30S ribosomal subunit protein S12
985570	[ompF] NP_415449.1 outer membrane porin 1a (Ia;b;F)
2960959	[ygdB] NP_417301.4 conserved protein, DUF2509 family
976244	[mukB] NP_415444.1 chromosome condensin MukBEF, ATPase and DNA-binding subunit
829692	[ybiH] NP_415317.4 putative DNA-binding transcriptional regulator
541401	[glxK] NP_415047.1 glycerate kinase II
2646542	[yfhM] NP_417015.1 hypothetical protein
1939532	[yebA] NP_416370.2 putative peptidase
1174851	[lolC] NP_415634.1 lipoprotein-releasing system transmembrane protein
92536	[ftsI] NP_414626.1 transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)
3409263	[dusB] NP_417726.1 tRNA-dihydrouridine synthase B
3036024	[recJ] NP_417368.1 ssDNA exonuclease, 5' --> 3'-specific
4275853	[soxR] NP_418487.1 DNA-binding transcriptional dual regulator, Fe-S center for redox-sensing
154556	[htrE] NP_414681.1 putative outer membrane usher protein
13794	[dnaK] NP_414555.1 chaperone Hsp70, co-chaperone with DnaJ
2399892	[nuoE] NP_416788.1 NADH:ubiquinone oxidoreductase, chain E
745740	[nei] NP_415242.1 endonuclease VIII/ 5-formyluracil/5-hydroxymethyluracil DNA glycosylase
397655	[yaiW] NP_414912.1 putative lipoprotein required for swarming phenotype
485556	[acrR] NP_414997.1 DNA-binding transcriptional repressor

142091	[can] NP_414668.1 carbonic anhydrase
2154403	[mdtB] NP_416579.1 multidrug efflux system, subunit B
3989990	[cyaA] NP_418250.1 adenylate cyclase
1184515	[potA] NP_415644.1 polyamine transporter subunit
1201235	[ymfJ] NP_415662.2 e14 prophage; putative protein
3150978	[metC] NP_417481.1 cystathionine beta-lyase, PLP-dependent
2389078	[nuoN] NP_416779.2 NADH:ubiquinone oxidoreductase, membrane subunit N
474353	[tesB] NP_414986.1 acyl-CoA thioesterase II
3921667	[rsmG] NP_418196.1 16S rRNA m(7)G527 methyltransferase, SAM-dependent; glucose-inhibited cell-division protein
210256	[ldcC] NP_414728.1 lysine decarboxylase 2, constitutive
3450112	[rplD] NP_417778.1 50S ribosomal subunit protein L4
4220277	[arpA] NP_418441.1 ankyrin repeat protein
3613466	[nikB] NP_417934.1 nickel transporter subunit
485076	[acrR] NP_414997.1 DNA-binding transcriptional repressor
2350107	[glpT] NP_416743.1 sn-glycerol-3-phosphate transporter
2239959	[yeiB] NP_416657.1 putative inner membrane protein
1893073	[pabB] NP_416326.1 aminodeoxychorismate synthase, subunit I
1241402	[ldcA] NP_415710.1 murein tetrapeptide carboxypeptidase; LD-carboxypeptidase A
1561985	[dosP] NP_416006.4 oxygen sensor, c-di-GMP phosphodiesterase, heme-regulated; cold- and stationary phase-induced bioflim regulator
2641863	[rlmN] NP_417012.1 dual specificity 23S rRNA m(2)A2503, tRNA m(2)A37 methyltransferase, SAM-dependent
1905255	[cspC] NP_416337.1 stress protein, member of the CspA-family
4300211	[mdtO] NP_418505.2 membrane translocase (MDR) of MdtNOP efflux pump, PET family
2872959	[cysN] NP_417231.1 sulfate adenylyltransferase, subunit 1
2444787	[aroC] NP_416832.1 chorismate synthase
1498815	[tehA] NP_415946.1 potassium-tellurite ethidium and proflavin transporter
3620862	[rhsB] YP_026224.1 rhsB element core protein RshB
3913929	[atpC] NP_418187.1 F1 sector of membrane-bound ATP synthase, epsilon subunit
848953	[rhtA] NP_415334.1 threonine and homoserine efflux system
1420210	[ydaV] NP_415878.1 Rac prophage; predicted DNA replication protein
2022771	[dsrB] NP_416462.1 hypothetical protein
2297193	[napH] NP_416708.1 ferredoxin-type protein essential for electron transfer from ubiquinol to periplasmic nitrate reductase (NapAB)
1656421	[ynfE] NP_416104.1 putative selenate reductase, periplasmic
1707842	[rsxD] NP_416147.1 electron transport complex protein required for the reduction of SoxR; predicted membrane protein
4105296	[fieF] NP_418350.1 ferrous iron and zinc transporter
268636	[perR] NP_414788.1 CP4-6 prophage; predicted DNA-binding transcriptional regulator
2961948	[ppdA] NP_417303.1 hypothetical protein
3469504	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
75199	[thiB] NP_414610.1 thiamin transporter subunit
2173807	[gatZ] NP_416598.1 D-tagatose 1,6-bisphosphate aldolase 2, subunit

774556	[tolQ] NP_415265.1 membrane spanning protein in TolA-TolQ-TolR complex
4500355	[insG] NP_418698.1 IS4 transposase
92376	[ftsI] NP_414626.1 transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)
3415561	[acrF] NP_417732.1 multidrug efflux system protein
1679652	[folM] NP_416123.1 dihydromonapterin reductase, NADPH-dependent; dihydrofolate reductase isozyme
2637716	[hisS] NP_417009.1 histidyl tRNA synthetase
2236744	[mglA] NP_416654.1 fused methyl-galactoside transporter subunits of ABC superfamily: ATP-binding components
4102440	[cpxA] NP_418347.1 sensory histidine kinase in two-component regulatory system with CpxR
1169484	[ycfS] NP_415631.1 L,D-transpeptidase linking Lpp to murein
3469903	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
3533176	[envZ] NP_417863.1 sensory histidine kinase in two-component regulatory system with OmpR
2742061	[yfiB] NP_417096.1 putative positive effector of YfiN activity, OM lipoprotein
2960869	[ygdB] NP_417301.4 conserved protein, DUF2509 family
890551	[nfsA] NP_415372.1 nitroreductase A, NADPH-dependent, FMN-dependent
1759220	[sufD] NP_416196.1 component of SufBCD Fe-S cluster assembly scaffold
1838249	[ynjE] NP_416271.4 IscS specificity factor for molybdenum cofactor biosynthesis; probable alternate MoaD transpersulfidase; weak thiosulfate:cyan>
3753132	[aldB] NP_418045.4 aldehyde dehydrogenase B
1931980	[edd] NP_416365.1 6-phosphogluconate dehydratase
2650803	[sseA] NP_417016.4 3-mercaptopyruvate sulfurtransferase
3075037	[yggP] YP_026187.1 putative dehydrogenase
933793	[ftsK] NP_415410.1 DNA translocase at septal ring sorting daughter chromsomes
953278	[focA] NP_415424.1 formate channel
2404394	[lrhA] NP_416792.1 DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes
1898621	[yoaE] NP_416330.1 fused predicted membrane protein/conserved protein

2495951	[alaC] NP_416880.1 valine-pyruvate aminotransferase 3
948708	[ycaN] NP_415420.1 putative DNA-binding transcriptional regulator
3469722	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
3409306	[fis] NP_417727.1 global DNA-binding transcriptional dual regulator
3410103	[yhdJ] NP_417728.4 DNA adenine methyltransferase, SAM-dependent
1110939	[opgH] NP_415567.1 membrane glycosyltransferase
3409254	[dusB] NP_417726.1 tRNA-dihydrouridine synthase B
1599053	[lsrR] NP_416029.1 lsr operon transcriptional repressor
3471160	[fusA] NP_417799.1 protein chain elongation factor EF-G, GTP-binding
2153637	[mdtB] NP_416579.1 multidrug efflux system, subunit B
109508	[secA] NP_414640.1 preprotein translocase subunit, ATPase
49910	[folA] NP_414590.1 dihydrofolate reductase
2075231	[cbeA] NP_416508.1 CP4-44 prophage; cytoskeleton bundling-enhancing factor A; CbtA antitoxin
3443741	[rplF] NP_417764.1 50S ribosomal subunit protein L6
337435	[yahF] NP_414854.1 putative acyl-CoA synthetase with NAD(P)-binding domain and

	succinyl-CoA synthetase domain
3472312	[rpsL] NP_417801.1 30S ribosomal subunit protein S12
3745930	[lyxK] NP_418037.1 L-xylulose kinase
1137330	[flgJ] NP_415599.1 muramidase
806945	[ybhB] NP_415294.1 kinase inhibitor homolog, UPF0098 family
1915668	[yebT] NP_416348.2 hypothetical protein
3447952	[rplV] NP_417774.1 50S ribosomal subunit protein L22
2591685	[ypfH] NP_416968.2 putative hydrolase
2445967	[prmB] NP_416833.4 N5-glutamine methyltransferase
4063212	[yihO] YP_026275.1 putative transporter
129443	[yacH] NP_414659.1 hypothetical protein
961696	[rpsA] NP_415431.1 30S ribosomal subunit protein S1
1995253	[yecC] NP_416427.1 putative transporter subunit: ATP-binding component of ABC superfamily
3644052	[rlmJ] NP_417956.1 23S rRNA m(6)A2030 methyltransferase, SAM-dependent
3883850	[yidC] NP_418161.1 membrane protein insertase
1946152	[yebC] NP_416378.1 conserved protein, UPF0082 family
382780	[yaiP] NP_414897.1 putative glucosyltransferase
2245341	[lysP] NP_416661.1 lysine transporter
483101	[acrB] NP_414995.1 multidrug efflux system protein
3162004	[parC] NP_417491.1 DNA topoisomerase IV, subunit A
2393480	[nuoJ] NP_416783.1 NADH:ubiquinone oxidoreductase, membrane subunit J
481611	[acrB] NP_414995.1 multidrug efflux system protein
4597783	[yjjA] NP_418780.2 hypothetical protein
149083	[panB] NP_414676.1 3-methyl-2-oxobutanoate hydroxymethyltransferase
4052409	[glnG] NP_418304.1 fused DNA-binding response regulator in two-component regulatory system with GlnL: response regulator/sigma54 interaction prot>
1885529	[rnd] NP_416318.1 ribonuclease D
309406	[ecpA] NP_414827.1 cryptic Mat fimbrillin gene
3897779	[yieL] NP_418175.3 putative xylanase
1195443	[icd] NP_415654.1 e14 prophage; isocitrate dehydrogenase, specific for NADP+
3533169	[envZ] NP_417863.1 sensory histidine kinase in two-component regulatory system with OmpR
2531213	[cysK] NP_416909.1 cysteine synthase A, O-acetylserine sulfhydrolase A subunit
1071165	[rutC] NP_415530.1 putative aminoacrylate deaminase, reactive intermediate detoxification
3835938	[setC] NP_418115.1 putative arabinose efflux transporter
890788	[nfsA] NP_415372.1 nitroreductase A, NADPH-dependent, FMN-dependent
708293	[chiP] NP_415207.1 chitoporin, uptake of chitosugars
2883688	[ygcB] NP_417241.1 R-loop helicase-annealase Cas3 needed for Cascade anti-viral activity
2644248	[pbpC] NP_417014.1 penicillin-binding protein PBP1C murein transglycosylase; inactive transpeptidase domain
897129	[potI] NP_415378.1 putrescine transporter subunit: membrane component of ABC superfamily

Appendix C: Whole Genome Sequencing - Amplification Results

Position	Length	Genes	Functions
318621- 329114	10493	betA	Choline dehydrogenase,osmotic adaptation
		betB	Betaine aldehyde dehydrogenase,osmotic adaptation
		betI	Transcriptional repressor of bet genes
389982- 400516	10584	insF1 & insE1	İnsertion sequence, phage ,transposon related
		sbmA	Microcin B17 transporter
		ddlA	Enzyme, murein sacculus peptidoglycan
429408- 435825	6417	tsx	Transport of small molecules, nucleoside channel
		ribD	Biosynthesis of cofactors,carriers: riboflavin
		ribE	Riboflavin synthase, beta chain
		nusB	RNA Synthesis, modification; transcription antitermination protein
449835- 455514	5679	cyoA	Cytochrome o ubiquinol oxidase
		ampG	Regulates beta lactamase synthesis
479421- 486928	7507	acrB	Multidrug efflux system
		acrA	Multidrug efflux system

		acrR	DNA binding transcriptional repressor
557747- 566702	8955	sfmH	fimbrial-like adhesin protein
		intD	Phage or prophage related

Table C.1 All genes in the amplified regions of spiramycin strong selection strain-2

Position	Length	Genes	Functions
317334- 337838	20504	betA	Choline dehydrogenase,osmotic adaptation
		betB	Betaine aldehyde dehydrogenase,osmotic adaptation
		betI	Transcriptional repressor of bet genes
		betT	Transport of small molecules
360880- 366178	5298	lacY	Electrochemical potential driven transporters
		Lac Z	Beta-D-galactosidase, degredat,on of small molecules
419295--436789	~16000	proY	Proline permease transport protein
		malZ	Maltodextri n glucosidase; degredation of small molecules :carbon compounds
		queA	Queuosine biosynthesis; tRNA

			ribosyltransferase isomerase
		tgt	tRNA-guanine- transglycosylase; tRNA modification
		secD & secF	Peptide secretion,transport
		tsx	Transport of small molecules, nucleoside channel
432679- 433782	1103	ribD	Biosynthesis of cofactors, carriers: riboflavin
433871- 434341	470	ribE	Riboflavin synthase, beta chain
434361-434780	419	nusB	RNA Synthesis, modification; transcription antitermination protein
434858- 435835	977	thiL	Biosynthesis of cofactors, carriers:thiamin Thiamin-monophosphate kinase
435813-436331	518	pgpA	phosphatidylglycerophosphatase
447529-455694	8165	cyoB	Cytochrome o ubiquinol oxidase subunit I
456002- 466261	10259	Cyo A	Cytochrome o ubiquinol oxidase subunit I I
		ampG	Regulates beta lactamase synthesis
		tig clpX	Cell division factor Degredation of proteins,peptides

		hupB	DNA binding transcriptional regulator
456002-466261	10259	fadM	Long-chain acyl-coA thioesterase III Fatty acid degredation
		queC	Queuosine biosynthesis
478002-489731	11729	ylaC	İnner membrane protein
		hha	Protein-translation and modification Haemolysin expression modulating protein
		tomB	Hha toxicity attenuator
		acrB	Multidrug efflux system
		acrA	Multidrug efflux system
		acrR	DNA binding transcriptional repressor
		mscK	Mechanosensitivity channel protein
502700-503920		fsr	Fosmidomycin efflux system (putative)
531675- 532157		allA	Ureidoglycolate hydrolase
535810- 536688		glxR	Tartronate semialdehyde reductase
558920-561523	2603	sfmD	Outer membrane protein ,export function (putative)

561559-562542	983	sfmH	İnvolved in fimbrial assembly Putative fimbrial-like adhesion protein
562553-563068	515	sfmF	putative fimbrial-like adhesin protein

Table C.2 All genes in the amplified regions of amikacin weak selection strain-1

Position	**Length**	**Genes**	**Functions**
311848-323033	11185	insE1 & insF1	İnsertion sequence IS3A
		ykgB	İnner membrane protein
390681-397833	7152	ins E1 & insF1	İnsertion sequence IS3B
		sbmA	Microcin B17 transporter , drug/analog sensitivity
560749-566449	5700	sfmH	Fimbrial like adhesin protein İnvolved in fimbrial assembly
		sfmF	putative fimbrial-like adhesin protein
569504-574870	5366	ybcL	Phage or prophage related
		ylcH	Phage or prophage related
		ybcN	Phage or prophage related

		ninE	Phage or prophage related
		ybcO	Phage or prophage related
572594-572956		rusA	Degredation of DNA Phage or prophage related
		ylcG	Phage or prophage related
		quuD	Q-like transcriptional regulator, DLP12 prophage
579044-584871	5827	ybcW	Phage or prophage related
		nohD	Bacteriophage DNA packaging protein Phage or prophage related
		tfaD	Phage or prophage related
		appY	DNA binding global transcriptional activator, DLP12 prophage
		ompT	Outer membrane protein 3b

Table C.3 All genes in the amplified regions of ciprofloxacin weak selection strain-2