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Analysis of Changes in Genes that lead to Antibiotic Resistance in Klebsiella pneumoniae 43816

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THESIS/DISSERTATION CERTIFICATE OF APPROVAL

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DEDICATION

I would like to thank my parents and family for encouraging me to gain wisdom, be curious, and persevere through obstacles. Their influence cemented me as a person and I hope to honor their memory in all endeavors. To my siblings, extended family, and friends, I appreciate your support and accountability. You all give me grace while still holding me to a standard of excellence.

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Abstract:

Klebsiella pneumoniae is a Gram-negative bacterium responsible for nosocomial infections such as blood stream infections, meningitis, and septicemia. Clinical isolates confirm *K. pneumoniae* is increasingly antibiotic resistant (AR) making treatment more difficult. While bacterial exposure to antimicrobial substances is a known cause of the AR phenotype, there still exists gaps in knowledge about the genetic changes responsible for this fitness change. The laboratory *Klebsiella pneumoniae* 43816 (ATCC 43816) was exposed to sublethal concentrations of Cephalothin for a 14-day period. At the end of the experimental treatment, the adapted population acquired clinical antibiotic resistance along with phenotypic and morphological changes. The goal of this study is to identify and characterize genetic mutations which correlate to increases in antibiotic resistance and morphological changes in the adapted *K. pneumoniae* population. DNA was extracted and sequenced from the adapted bacteria and a population subcultured for 14 days without exposure to antibiotic. Comparison of these sequences to the known ATCC 43816 genome identified eight mutations unique to the adapted culture in seven protein coding regions. Results also found 6 putative promoter regions with mutations in the adapted culture. The data confirm that fixed changes in genotype are correlated with the altered phenotype. Additionally, findings suggest that the development of resistance may involve genes not previously known for β-lactamase function in *Klebsiella pneumoniae* and that the exact mechanism used to overcome antibiotic exposure is not uniform within a treated population.

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Chapter 1: Introduction

Microbial Resistance to Antibiotics

Since the development of antibiotic treatment, bacterial pathogens have been competitively evolving in ways that allow them to survive antibiotic pressure (1–3). Acquisition of the antibiotic resistant (AR) phenotype can occur genetically in two primary ways- through random mutations in the existing bacterial genome and through acceptance and integration of foreign genetic material encoding AR traits. Both methods have caused the current global resistance crisis, but it is the fact that microorganisms mutate in the presence of low concentrations of antibiotic medication that creates difficulty (1, 2). Pathogenic infections which were initially considered mundane in the antibiotic heyday, have reemerged as consistent threats as they become increasingly difficult to treat. Antibiotic resistant forms of common infections is costing the US approximately $5 billion as of 2016 with the cost expected to rise (1). Another assessment in 2020 found that these pathogens are causing over 2 million infections per year and 23,000 deaths (4). A special class of commonly resistant bacteria have gained notoriety as ESKAPE pathogens. The members of this group- *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*- are known for their knack at "escaping" the effects of antimicrobial substances (2, 4, 5).

The misuse and abuse of medications by medical staff, patients, and even the agricultural industry has created a basal level of antibiotic in our environment (6–9). Interestingly, the residual antibiotic concentration isn't high enough to completely eradicate bacterial populations, but it does become a source of survival stress. In proper clinical settings, antibiotics are administered such that the concentration in the affected area reaches a limit referred to as the Minimum Inhibitory Concentration or MIC (10, 11). The MIC of an antibiotic is the concentration at which a bacteria

fails to grow and is dependent upon the compound being tested and the bacteria itself (10, 11). At that level, a normally functioning immune system would be able to clear a pathogen (10). Concentrations below that bacteriostatic limit, referred to as sub-MIC levels, are similar to what has become known as the "selection window" for bacteria (12, 13). An antibiotic concentration within a selection window is high enough to stress a population by killing some bacterial cells but low enough to not completely eradicate that population. It is the concentration at which the occurrence of genomic mutations is highest and can lead to the development of clinical antibiotic resistance in available pathogens (12, 13). Interestingly, the environmentally available antibiotic doesn't always have to be the exact same as those used by clinicians to create resistance to medically prescribed agents. Exposure to chemicals like the surface antiseptic chlorhexidine, and veterinary antibiotics timicosin and florfenicol can create cross-resistance in human pathogens to different classes of medicinal treatments (7, 9).

Resistance Mechanisms Specific to β-Lactam Antibiotics

β-lactam antibiotics are a commonly used treatment by clinicians for a wide range of infectious bacteria. As one of the three largest classes of antibiotics, they have been used to combat bacterial infections for over 60 years (14, 15). β-lactams are antibiotics that disrupt peptidoglycan bonds in the cell walls of Gram-positive and Gram-negative bacteria (16). The ring structure within the antibiotic compounds simulates the D-Ala-Ala substrate used by bacterial enzymes involved in forming and linking the peptidoglycan layer of the cell wall (14). Disabling the penicillin-binding proteins, or PBPs, necessary to link this cell wall component creates instability. The eventual disruption of the cell wall triggers lysis and clearing of the infection in susceptible organisms. β-lactam antibiotics were initially effective against a host of both Gram-positive and

Gram-negative species since they are potent inhibitors of cell-wall synthesis- a process essential for bacterial proliferation (14, 15).

Gram-negative bacteria like *K. pneumoniae* have three methods to overcome the destructive action of this class of antibiotic. The first method involves the enzymatic destruction of β-lactam rings in the antibiotic to prevent their binding with penicillin-binding proteins or PBPs (17). This method deactivates the antibiotic and allows the bacteria to continue synthesizing peptidoglycan for cell wall synthesis. A second pathway to mitigate β-lactam induced lysis is to alter the conformation or collection of penicillin-binding proteins produced by the bacteria (17). Changing PBP conformation or changing the types of PBPs available reduces the likelihood that β-lactams will bind to these proteins and prevent inhibition of cell wall synthesis. Finally, Gram-negative organisms can decrease uptake of the antibiotic by changing the abundance and characteristics of porins (17). Without access into the cell, the antibiotic will not have the opportunity to deactivate necessary PBPs.

Strains of *K. pneumoniae* have been identified utilizing all three of these mechanisms. For example, both nosocomial and hypervirulent strains of the species have modified capsule composition and quantity, the external lipopolysaccharide layer, efflux pumps and porins to prevent or reduce the admittance of antibiotic into the intracellular space (3). Isolates of *K. pneumoniae* with β-lactamase and extended-spectrum β-lactamase (ESBL) genes code for enzymes able to hydrolyze multiple classes of the antibiotic, even the strongest group named carbapenems, rendering them ineffective (14, 15). The bacteria are also known to readily accept plasmids from other species which encode modified PBP enzymes (14, 18). The alterations modify enzyme binding sites such that accessibility for the antibiotic is drastically reduced or obliterated. These adaptations have landed *K. pneumoniae* on a clinical “hot list” of sorts. Medical personnel

and government agencies recognize the rapid rise in resistance among this and other bacteria as costly and eventually detrimental to human health (6, 19).

Laboratory Models of Microbial Adaptation to Antibiotics

Gullberg et. al. (2011) attempted to further understand the selection window hypothesis proposed by Drlicka in 2003 using wild-type *S. typhimurium* and *E. coli* species (20). These organisms were exposed to varying levels of antibiotic prior to a growth competition assay with a counterpart of the same species which was not exposed to antibiotics. The team utilized streptomycin, tetracycline, and ciprofloxacin for antibiotic exposure and competed each strain pair for 80 generations. Regardless of the antibiotic or species there existed a concentration at which the pre-exposed strain's growth was enriched compared to the unexposed bacteria. In Figure 1, a graphical model and equation of comparative fitness rates at sub-MIC antibiotic concentrations and beyond is shown (20). The minimum concentration needed to create this fitness change varied by antibiotic type. Furthermore, the experiment questioned if sub-MIC concentrations of antibiotic could not only affect the ability of the bacteria to survive in the presence of antibiotic but if this could create a resistance phenotype. To do this susceptible bacterial populations were grown in the presence of streptomycin for 700 generations. Moderate resistance to the MIC concentration of antibiotic was observed after 200-400 generations and high resistance within 500-600 generations (20). Their findings are an example of how even low levels of antibiotics, like those found in human tissues after antibiotic use or in agricultural products/land used, can create competitive circumstances that could drive the rise in AR occurrence in bacteria.

Since that experiment, exposure to low concentrations of antibiotics have been linked to changes in resistance phenotypes and genes. In a study using the ESKAPE pathogen *P. aeruginosa*, samples were exposed to sub-MIC concentrations of either ticarcillin or carbenicillin, both β-

lactam antibiotics which inhibit peptidoglycan synthesis (8). The samples were initially exposed on an antibiotic inoculated plate and selected colonies were transferred to broth for resistance analysis. It is important to note that the study measured mutation rate by the ability of the samples to later grow on plates inoculated with rifampicin. It was determined that both ticarcillin and carbenicillin treatments increased the relative proportions of AR colonies compared to the unexposed original strain (8). Later, carbapenem-resistant *K. pneumoniae* (KPC) samples were examined for carbohydrate use and transcription rate changes when exposed to imipenem (21). All the KPC samples used were resistant to imipenem and had known carbapenemase genes at the onset of the trial. Samples grown in the presence of imipenem before the assays preferentially used different carbohydrate metabolites and differentially expressed RNA compared to untreated samples. Most notably the exposed samples upregulated RNA transcripts of genes related to transmembrane transport and hydrocarbon transport/metabolism while downregulating genes involved in translation, ribosome structure, and cell division (21). In contrast, clinical isolates of *P. aeruginosa* showed a lower mutation rate in the *glpT* gene compared to a common lab strain of the species after in vitro exposure to ciprofloxacin (22). However the same study noted six nonsynonymous mutations in the coding regions of known antibiotic-adaptable genes (22).

While these studies have expanded on our body of knowledge concerning sub-MIC exposure and bacterial adaptation there are some key missing pieces. First, these experiments began with strains of bacteria either known to harbor antibiotic resistance genes or shown to be clinically resistant to one form of antibiotic already. Another area for concern is the brief exposure time used before many of these assays. In most cases, the samples were exposed to the sub-MIC concentration of the antibiotic for 24-48 hours before experimentation began. This allowed limited time for the bacteria to evolve a novel genetic change as it may occur in a hospital or environmental

setting with consistent and extended term exposure. Finally, the genetic analyses, if completed, used pre-determined targets or the phenotypes assumed from mutations in predetermined targets (i.e. mutagenesis calculated by growth in rifampicin, mutation rate determined by *glpT* and fosfomycin resistance, sequencing only known antibiotic adaptable genes). The results are definitely informative but not as transferrable to situations in which AR phenotypes evolve over time though sub-lethal exposure.

Klebsiella pneumoniae Pathology and Genome

K. pneumoniae is an ESKAPE pathogen which is not only able to survive in the presence of multiple classes of antibiotics, including resistance to last-resort level medications such as β-lactams and the subclass of carbapenems, but also acquires resistance at a faster rate than many other bacteria in this family (3, 23, 24). According to the 2015 National Action Plan For Combating Antibiotic Resistant Bacteria, resistant *K. pneumoniae* was responsible for over 7,000 patient infections (6). Similar infection rates have been noted globally with many antibiotic-resistant strains of *Klebsiella* species exhibiting resistance to all classes of antibiotics used by medical professionals (3).

In 2015, genome sequencing of over 300 *Klebsiella pneumoniae* isolates confirmed the existence of three subspecies (previously referred to as three phylogroups or serotypes: KPI, KPII, and KPIII) of *K. pneumoniae* that independently evolved mechanisms of resistance and pathogenicity (18). *Klebsiella pneumoniae* 43816 belongs to the KPII subspecies, a phylogroup which was almost exclusively isolated from human hosts. The KPII group was also closely associated with instances of colonization or hospital acquired infection (18). Sequencing of those isolates identified as *K. pneumoniae* indicated the presence of β-lactamase genes in chromosomal regions and in plasmids, which confirmed the evolution of resistance in a bacterial population

through the work of horizontal gene transfer. However, finding resistance genes in chromosomal regions suggests involvement of viral-mediated gene transfer or the bacteria's ability to adapt over time. While the distribution of antimicrobial resistance genes and specific combinations varied by region, isolates from humans on average contained a median of five antimicrobial resistance genes (18). Thus, *Klebsiella pneumoniae* 43816 as a member of the KPII serotype would also share this ability and potentially contain multiple antibiotic resistance genes.

The data obtained in my work uses an adapted strain of ATCC 43816 and will focus on chromosomal genes as opposed to plasmid sequence data. The laboratory strain ATCC 43816 was treated for 14 days with gradually increasing concentrations of cephalothin, a β-lactam class antibiotic (Figure 2). While the concentration of antibiotic administered increased, it never reached the level clinically issued for treatment of infection. Clinical cephalothin concentrations can range from 90-400 μg/mL but only 90% of the dosage is bioavailable when administered and is degraded and cleared by the body within 4-6 hours (25). Therefore, the effective clinical dosage ranges from 9-40 μg/mL. As a control, a sample of *K. pneumoniae* 43816 was also serially passaged for 14 days with similar environmental conditions save for exposure to antibiotics. Both treatments were subcultured every 12 hours, and the concentration of cephalothin for *K. pneumoniae* exposed to antibiotic was increased by 0.5 μg/mL every 24 hours. The concentration of antibiotic in the treated condition increased from an initial 0.5 μg/mL to 7.5 μg/mL by the end of the adaptation period.

At the end of the 14-day treatment, the sample exposed to cephalothin successfully grew in the presence of antibiotic whereas the unexposed sample had minimal growth in the same conditions. Cephalothin treatment slowed the growth rate for the adapted population compared to the untreated form, as indicated by the reduced slope in growth curve (0.028 OD units per minute treated, 0.034 OD units per minute untreated) during the exponential phase in Figure 3.

Additionally, the treated and untreated populations exited lag phase sooner than *K. pneumoniae* 43816 (Figure 3). Even though it had never been exposed to clinical doses, this adapted variant exhibited resistance to clinical doses and greater of cephalothin. The antibiotic tolerance was quantified using the minimum concentration of antibiotic required to reduce bacterial growth by 90%. This breakpoint MIC increased in the adapted *K. pneumoniae* population from 4 µg/mL to 125 µg/mL while the sample not exposed to antibiotic still displayed expected phenotypic susceptibility (Table 1). This value is more than triple the concentration necessary to inhibit growth of standard ATCC 43816. When the two cultures were examined, the adapted strain showed a visibly more mucoid phenotype (Figure 4) and was comprised of a small colony producing variant and a large colony producing variant (Figure 5). When plated each size variant reliably produces clones that are in the same size range as the parent colony. Small colony variants also appear more filamentous and are more likely to cluster in chains (Figure 6). While still resistant to clinical levels of cephalothin use, the small colony producing variant is less resistant to Cephalothin treatment than the large colony clones (Table 2).

The present study will focus on a laboratory strain of *K. pneumoniae* which has evolved resistance to the β-lactam antibiotic cephalothin through exposure to incrementally increased, sub-lethal antibiotic dosage levels as described above. The primary goal of this study was to identify and characterize genetic changes in the adapted strain associated with increased antibiotic resistance. Additionally, the small and large-colony variant sequences were compared to identify mutations shared between the two groups which could be an effect of antibiotic exposure. Notable genetic changes could provide insight into the evolutionary pathway of multidrug resistance and provide new targets for therapeutic research.

Chapter 2: Methods

Bacterial Strains and Media

An experimentally treated population of *Klebsiella pneumoniae* was generated to model the acquisition of antibiotic resistance through low-concentration antibiotic exposure. The design utilized *Klebsiella pneumoniae* ATCC 43816 (ATCC, Manassas, VA) and all incubation steps were completed at 37°C with shaking at 180 rpm. As shown in Figure 2, two 50-µL aliquots of *K. pneumoniae 43816* grown in Luria-Bertani (LB) broth (BD Difco, Franklin Lakes, NJ) were dispersed into fresh culture tubes and stressed with a concentration of one µg/mL cephalothin (Fisher Scientific, Waltham, MA) then grown until the cultures reached an OD_{600} of 1.0 read by an Eppendorf Bio photometer (Hamburg, Germany). Four 50 µL aliquots of bacteria from this treatment were subcultured in fresh broth with two samples inoculated by one µg/mL of cephalothin and two samples unexposed to antibiotic. Aliquots from all four populations were subcultured in fresh broth every 12 hours. The cephalothin concentration for the exposed population of *K. pneumoniae*, referred to as "treated" or "adapted", increased by 0.5 µg/mL with each subculture over the course of 14 days. The cephalothin concentration ultimately reached 7.5 µg/mL by the end of the exposure cycle (Figure 2). Samples from each day of treatment were aliquoted to make frozen stock for further use in minimum inhibitory concentration and qPCR assays.

For use in further assays in the project, untreated bacteria samples were grown from the saved frozen stock in LB broth and incubated for 12-18 hours at 37°C at 180 rpm. The treated bacteria samples were also grown from frozen stock in similar conditions with the addition of 7.5

μg/mL cephalothin treatment to maintain the final antibiotic stress to which the population had acclimated.

Growth

Growth curves were generated for both the treated and untreated experimental groups and the parental bacterial strain to assess changes in growth rates over time. Growth curves were generated by inoculating five milliliters of Luria Bertani broth (supplemented with antibiotics when appropriate) with five microliters of overnight culture to determine the effect of cephalothin-exposure on *K. pneumoniae* growth rates. The OD_{600} was determined using an Eppendorf Bio photometer every 30 minutes for five hours.

Genomic DNA Isolation

Genomic DNA from both experimental populations and the parental strain were used for sequence analysis to identify potential mutations compared to the published sequence data for *K.* pneumoniae 43816. DNA extraction steps mirrored those published by Wright et. al. with modifications (26). The procedure utilized 50 mL of overnight culture (inoculated with the appropriate amount of antibiotic) and pelleted by centrifugation for 10 minutes at 10,000 rpm at 4°C. The pellet collected was washed twice with 30 mL of TE25S buffer (25 mM Tris-HCl, 25 mM EDTA, 0.3 M sucrose, pH 8.0). After washing the pellet was resuspended in 25 mL of TE25S with 10 mg/mL lysozyme and 15 μL RNAse (ThermoFisher Scientific, Waltham, MA). The mixture was incubated for one to two hours at 37°C while shaking at 150 rpm. Then 300 μL of proteinase K (ThermoFisher Scientific, Waltham, MA) and 1.5 mL of 10% SDS (ThermoFisher Scientific, Waltham, MA) were added and incorporated by inversion. The sample was then incubated for 1-2 hours at 50-55°C with 3 inversions in the incubation period. After

incubation, 5 mL of 5M NaCl were added and incorporated by inversion followed by 3.25 mL of CTAB(Cetyl Trimethyl Ammonium Bromide)/NaCl. The solution was again mixed by inversion and incubated at 55°C for 10 minutes. The sample was then split into two 50mL Falcon tubes and 20mL of a 24:1 chloroform/isoamyl alcohol solution was added to each tube. The samples were incubated at room temperature and 100 rpm for 20 minutes. After incubation, the solution was centrifuged at 4°C and 10,000 rpm for 15 minutes and the upper aqueous layers were transferred into fresh tubes. The chloroform treatment was repeated a second time. The upper aqueous layers of both samples were isolated and combined. An isopropyl alcohol volume of 0.6 of the collected sample was added and the mixture was gently inverted. This solution incubated for five minutes and the DNA was spooled from the tube onto a sterile Pasteur pipette. The spooled DNA was washed with approximately 5 mL of 70% ethanol and dried before being suspended in 300µL of EB buffer (QIAGEN, Hilden, Germany).

Genome Sequencing

Initial samples of DNA from the *K. pneumoniae* 43816, the 14-day treated population, and 14-day untreated population were analyzed by the National Center for Genome Resources for SMRT Cell Sequencing. The samples were compared against the published reference sequence NZ_CP009208.1 available in GenBank. Genetic variation between genomes was calculated using a modified form of F_{ST} or analysis of variance referred to as Θ (27). F_{ST} values are evaluated against the null hypothesis that the populations are not genetically unique (27). Pacific Biosciences calculated allele frequencies and utilized a proprietary Quiver Algorithm to maximize accuracy in sequence reads using variation between the published genome and prior records.

A secondary round of sequencing detailed the complete sequence of samples from days 1-14 of the adaptation experiment, the small colony forming variant, the large colony forming variant, and the day 14 adapted strain of *K. pneumoniae*. These DNA samples were analyzed by the Microbial Genome Sequencing Center which utilized an Illumina sequencing technique similar to that used by Baym et. al. (28). Any variations were analyzed using a proprietary breseq variant calling algorithm (29).

Minimum Inhibitory Concentration Test

Survivability of both experimental groups and the parental strain in the presence of an antibiotic was assessed using a Minimum Inhibitory Concentration (MIC) assay. Standard *K. pneumoniae 43816* and samples from each day of experimentation were grown in five mL of LB at 37°C at 180 rpm overnight. Overnight cultures were then diluted to reach 10^3 CFUs/mL in LB broth in a 96 well microtiter plate. Bacteria were treated with 500, 250, 125, 62.5, 31.25, 16, 8, 4, 2, 1, and .5 µg/mL of the tested antibiotic: cephalothin, amikacin, or tetracycline (Fisher Scientific, Waltham, MA). Amikacin and Tetracycline were tested due to mutations in genes known to confer resistance in related bacterial species. Wells containing only LB and bacteria were used as controls for normal growth. The absorbance of each well at OD_{600} was measured using a Biotek Gen5 plate reader (Winooski, VT). The minimum inhibitory concentration was determined as the lowest concentration at which growth was significantly inhibited (by at least 90%).

Transcriptional Expression by qPCR

The initial sequence data for the experimental groups identified mutations in coding and potential promoter regions unique to the 14-day treated population. To better understand how

these mutations impacted protein production, the transcription rates for those genes in the 14-day treated population were compared to that of the parental population using qPCR. The QIAGEN RNeasy kit (Hilden, Germany) was used to extract RNA from cultures in the exponential phase with a DNase and lysozyme treatment. Complimentary DNA (cDNA) was reverse transcribed from 1µg of total RNA using random hexamer primer and the Superscript III reverse transcriptase kit (ThermoFisher Scientific, Waltham, MA). Quantitative polymerase chain reaction (qPCR) was performed using the New England Biolabs Luna Universal qPCR kit (Ipswich, MA) on an Eppendorf Mastercycler Realplex 2 (Hamburg, Germany). Data was analyzed using the ΔΔCT method (30). Gene expression was normalized to that of gapA and the adapted strain was normalized to the lab strain 43816. Primers used are listed in Table 3.

Chapter 3: Results

Exposure to Cephalothin Alters *K. pneumoniae* Phenotype and Colony Morphology

At the end of the 14-day experiment there were several morphological differences between the treated and untreated groups. The present study found that the adapted population of *K. pneumoniae* exhibited increased mucoidy in liquid culture compared to the clonally related untreated group (Figure 4). While the viscosity had changed, the adapted population did not qualify as a hypermucoviscous (hmv) strain as indicated by a string test.

Upon plating the untreated group produced uniform colonies of the same size as the *K. pneumoniae* 43816 progenitor. When the adapted population was streaked onto an LB agar plate, two disparate colony morphologies were observed (Figure 5). The plates consistently contained a mixture of larger colonies and smaller colonies which were denoted as “treated large” and “treated small”. Isolation and replating of one colony type produced a plate with uniform colonies of the initial isolate’s size. An isolated large colony sample when propagated in liquid culture and streaked would produce a plate of large colonies and an isolated small colony sample would produce a plate of small colonies. The fact that colony size was maintained after isolation indicated that the morphological change was heritable. A closer look at the small and large colony variants revealed that the small colony forming bacteria were more filamentous in structure and prone to linking with a neighboring individual bacterium (Figure 6).

Cephalothin Treatment Increases Minimum Inhibitory Concentration in a Stepwise Manner and Induces Cross Drug Resistance

A minimum inhibitory concentration assay was used to determine the survival of bacterial populations in various antibiotics. This study primarily highlighted the breakpoint

concentration of antibiotic necessary to depress bacterial growth by half, also called the MIC50, since in a clinical setting this strength would allow an individual's immune system to clear an infection. The original *Klebsiella pneumoniae* 43816 strain demonstrated 50% less growth (MIC50) in a cephalothin concentration of 4 μg/mL (Table 1). After the 14-day treatment period, the untreated population of bacteria maintained the same MIC50 and susceptibility to cephalothin. However, the MIC50 for the adapted strain increased to 125 μg/mL ($p<0.0005$ compared to parental strain). Genome sequence data (see below) indicated mutations in genes implicated in resistance to antibiotic substances with methods of action not akin to β-lactams namely tetracyclines and aminoglycosides. Tetracycline and amikacin were used to check for potential cross-resistance in all bacterial populations. The MIC50 recordings for *Klebsiella pneumoniae* 43816 were 0.5 μg/mL for both antibiotics. The 14-day treatment moderately affected the resistance to amikacin with an untreated population MIC50 of 1 μg/mL and 2 μg/mL ($p<0.05$ and $p<0.0005$ compared to parental strain) for the adapted population. The subcultured populations were more resistant to tetracycline. The untreated population had a doubled MIC50 of 1 μg/mL and the adapted population's MIC50 increased to 16 μg/mL (($p<0.0005$ compared to parental strain).

Our study was also able to map changes in resistance through the adaptation period. Samples from each subcultured day were analyzed by a MIC assay. Figure 7 shows that the adapted strain demonstrated two clear jumps in resistance to cephalothin on day 6 (MIC50 of 31.25 μg/mL) and day 10 (MIC50 of 125 μg/mL) of treatment with periods of stability in-between. Similarly, resistance to tetracycline occurred in two jumps, one on day 2 and the next on day 5.

Whole Group Genome Sequence Analysis of Adapted and Susceptible Populations

DNA from the untreated and adapted population of *K. pneumoniae* 43816 was sequenced by the National Center for Genome Resources using a SMRT Cell methodology. The initial sequence data identified 107 mutations in the control culture and 29 mutations in the genome of the resistant culture. Of the 29 mutations identified in the adapted strain, 15 were nonsynonymous changes that occurred in protein-coding regions of the sequence. To further refine the list of potential targets, any mutations shared with the untreated population of bacteria were removed. Comparing the two genomes highlighted seven protein coding genes which were altered in the resistant sample DNA and none were identified as common β-lactamase genes (Table 4A).

Table 4 lists mutations of protein coding genes first. The coding region mutations included two nucleotide substitutions in an SGNH/GDSL hydrolase family protein, and a substitution in undecaprenyl-phosphate glucose phosphotransferase. There were deletions in the coding regions for N-acetyltransferase, a sugar ABC transporter ATP binding protein, and DNA recombination protein RmuC. Finally, an insert in the globin coding sequence and a large insert in a TetR/AcrR transcriptional regulator were noted. The only mutations identified in this set that had not fixed in the population (mutations not found in 100% of the sample provided) were those in the SGNH hydrolase coding region. The functions of these genes ranged from signal transduction, energy and metabolite use, capsule formation, and nucleic acid proofreading.

Revisiting the sequence data revealed six unique mutations in potential promoter regions of the adapted population sequence (Table 4B). The list was refined using a similar process as that for the coding region calls. This study considered that promoter regions could occur approximately 150 bp away from the gene of interest, so in reviewing the sequence data any

mutations within 150 bp of a coding region was considered a potential promoter. While the genes associated with these promoters are not fully characterized in *Klebsiella pneumoniae*, there are data about each class of gene represented.

This analysis found mutations in potential promoters for SGNH/GDSL hydrolase, peptide chain release factor 3, a LysR family transcriptional regulator, a type II asparaginase, deoxyribopyrimidine photo-lyase, and cyclic di-GMP phosphodiesterase. The mutation for the SGNH hydrolase promoter was again not fixed and only found in 56% of the sample. All the remaining mutations were ubiquitous. Functions of the coding regions nearby included metabolite use, signal transduction, and mRNA proofreading.

Genome Sequencing by Treatment Day Aligns Adapted Population Mutations and Increases in Antibiotic Tolerance

Frozen stocks were made of the adapted *K. pneumoniae 43816* population on each day of treatment during the evolution experiment. DNA from each day was extracted and sequenced by the Microbial Genome Sequencing Center (MiGS). MiGS identifies polymorphisms using an Illumina sequencing method and a proprietary statistical algorithm for variant calling. The data received for each day identified far fewer mutations than the first round of sequencing (Table 5A). To standardize the new sequencing data, the 14-day adapted population DNA was sequenced by MiGS as well. Ultimately, four mutations in protein-coding regions were identified using this method (Table 5A). The MiGS report did not include sequence polymorphisms in noncoding regions, such as possible promoters, so that analysis could not be done.

Similar to the initial polymorphism data for the adapted strain, the TetR/AcrR transcriptional regulator and *wcaJ*, an undecaprenyl phosphate glucose phosphotransferase, had

an altered sequence compared to that available in GenBank for *K. pneumoniae* 43816 (Table 5). The positions of the mutations are not identical but the type of mutation (insertion and base change from T to G) align with the prior data. The mutation in the TetR/AcrR regulator sequence reaches fixation on day 6 and is a consistently fixed mutation in each sample day afterward. The mutation in *wcaJ* first fixes on day 10, and then the reported percentage of sample with this mutation decreases for the following treatment days to 64% by day 14. Two new marginal mutations were identified in the MiGS data- one affecting the *rpoB* gene and the other affecting the *gndA* gene.

When the DNA sequences are compared for each daily sample, the data show when certain mutations have cemented in the adapted population. One of the earliest mutations fixed in the 14-day treated population on day 1 of treatment was that of *rpoB.* This occurs one day before the first increase in resistance to tetracycline. On day 6 the TetR/AcrR family transcriptional protein mutation moves from a low-frequency marginal call to a fixed mutation with 100% coverage in the adapted strain (Figure 8). On day 10 the same occurs with the *wcaJ* sequence which later decreases in frequency (Figure 8). These two fixation events align with the two MIC jumps for the adapted strain indicating increased resistance to cephalothin (Figure 8).

Transcription of Mutated Genes

Quantitative real-time PCR indicated that extended antibiotic exposure affected the relative transcription of some genes with mutated promotor or coding regions. Figure 9 displays the relative transcription rates of genes with mutated coding regions identified in the first round of sequencing. In that assay, transcription of the TetR/AcrR transcriptional regulator was upregulated almost threefold in the adapted population of bacteria while that of globin was downregulated almost fivefold when compared to the parental *K. pneumoniae* 43816 (Figure 9).

Figure 10 shows the evaluated transcription rate of genes near potential promoters identified in the first round of sequencing. The only promotor mutation which correlated with a statistically significant change in nearby gene transcription was the deoxyribopyrimidine photolyase (Figure 10). The associated gene showed a nearly fourfold reduction in transcription (Figure 10).

Adapted Population Large and Small Colony Variant Sequencing Exposes Genetic Subpopulations in Evolved Group

Inconsistencies in polymorphism coverage in the initial round of sequencing data along with observations of colony size variance and difficulty in qPCR analysis supported the possibility that the adapted population may consist of distinct subpopulations. To test this idea, the large and small colony variants of the 14-day adapted population were separated and their genome sequenced by the Microbial Genome Sequencing Center. The data indicated that the two variants were genetically distinct and may even be comprised of subpopulations themselves (Table 5B and 5C).

Both the large and small colony variants had mutations in the *rpoB* and TetR/AcrR transcriptional regulator sequences (Table 5B and 5C). The mutation in *rpoB* was fixed in both variants. However, the depth of coverage of the TetR/AcrR transcriptional regulator in the large colony variant was 52 (Table 5B). Not all of this sample group developed a mutation to this gene while the same change in the small colony variant had been fixed at 100% coverage.

The large colony variant also had a distinguishing marginal mutation call in a ComEC family protein that was not found in the small colony variant. While the small colony data show a large deletion between the *wcaJ* and gndA genes along with a single nucleotide polymorphism in a YfiR family protein that was not identified in the large colony variant (Table 5C). All of the

mutations in the small colony forming variant were fixed indicating a more homogenous genomic identity than the large-colony forming subpopulation.

Chapter 4: Discussion

An improved understanding of how bacterial pathogens develop antibiotic resistance in response to exposure during growth can improve our ability to prevent and treat resistant forms in a variety of settings. This study attempts to model the process of adaptive evolution by serially subculturing one population of bacteria while gradually increasing cephalothin concentration with each passage. Unlike other evolutionary studies using known resistant strains of bacteria or antibiotic exposure for a shorter period of time, the adapted *K. pneumoniae* population was stressed for a period of 14 days using a subMIC concentration of antibiotic and compared to a clonally related cohort which was also serially passaged for the same amount of time without antibiotic exposure.

Phenotypic Changes and Clinical Impact

This model produced a population with distinct characteristics from the parent species, *Klebsiella pneumoniae 43816*. β-lactam antibiotics function by interrupting the generation and repair of the cell wall for actively growing and dividing cells. Survival in the presence of such an agent could affect the populations appearance and growth patterns. At the end of the treatment period, the adapted population increased in mucoidy and contained distinct cell and colony morphologies (Figure 4 and 5). The adapted population's survivability in cephalothin was expected to increase, however the shift in MIC from 4 μg/mL to 125 μg/mL illustrated that bacterial survival improved exponentially (Table 1). Over the 14-day period, the adapted *K. pneumoniae* developed the ability to survive in a concentration of cephalothin both above that which is used clinically to treat infection and over 10 times above that to which it had been exposed (25). Survivability of the adapted population in two other classes of antibiotic also improved without the sample having ever been exposed to related substances indicating the

development of cross-resistance. The MIC of tetracycline increased from 0.5 µg/mL to 16 µg/mL and that of amikacin increased from 0.5 µg/mL to 2 µg/mL (Table 1). There was a slight increase in MIC for these two additional drugs in the untreated cohort of *K. pneumoniae* but that increase does not compare to that of the adapted population. Current literature recommends a treatment protocol for tetracycline that achieves a maximum antibiotic concentration level (C_{max}) of 2-5 µg/mL and a C_{max} of 8-10 µg/mL of amikacin to prevent potential organ failure (31–33). Based on this procedure, the adapted strain of *K. pneumoniae* developed in this study would be clinically resistant to tetracycline.

Mutations Correlated to Increases in Resistance and Colony Size Variants

The primary aims of this study were to determine mutations unique to the 14-day adapted population of *K. pneumoniae* 43816 which correlated to changes in resistance and to identify mutations unique to the small and large colony variants within the 14-day adapted population. DNA from samples of each treatment day during the adaptation phase of *K. pneumoniae* indicated three mutations that chronologically align with increases in resistance to either tetracycline or cephalothin. The three resistance-correlated mutations occurred in genes coding for *rpoB*, TetR/AcrR transcriptional regulator, and *wcaJ*. The increases in resistance occurred in a stepwise manner, similar to the increases in fitness observed in the *E. coli* long-term evolution (LTEE) studies performed by Richard Lenski and reserachers investigating the populations generated from the study (34–36). Further analyses, including cloning and ablation of genes of interest, are needed to definitively state that the mutations identified in this study induced increases in antibiotic resistance.

The location of the *rpoB* mutation was very close to a prior call in DNA recombination protein RmuC in the sample sequenced using the SMRT cell method. The protein products of

both genes interact closely with genomic DNA. RmuC is a regulator which can prevent sequence inversion during replication (37). The gene has also been identified as possible multidrug resistance (MDR) genes in other Gram-negative bacterial species (38, 39). However, *rpoB* is part of the RNA polymerase protein complex and when mutated can inhibit the action of Rifampicin in bacteria (40). In 1988, *rpoB* mutations were linked to rifamycin and Rifampin resistance in *E. coli* (41, 42). Since that time, mutations in this gene which normally encodes a portion of the RNA polymerase protein complex has been identified as a resistance gene in various classes of bacteria (43). Additionally, the fact that the mutation in a gene tied to nucleic acid integrity occurs first provides a possible mechanism for AR development which requires alterations in the ability of the bacteria to maintain fidelity of its sequence. These mutations could increase the occurrence of replication error mutations in progeny.

The AcrR subset of TetR regulators are one-component signal transduction systems commonly associated with the transcription of efflux pumps in bacterial pathogens (44). TetR regulators can also respond to a variety of ligands and effect different processes within the cell (45). It is interesting that such a large insert would be found in a gene known for resistance to tetracycline antibiotics when the adapted population of this study was exposed to a β-lactam. Although the specific characteristics of this regulator is unknown, it could correspond with a change in the presence of efflux pumps in the adapted population.

The position of the uncharacterized undecaprenyl phosphate-glucose phosphotransferase in the SMRT sequencing report is close to the mutation found in *wcaJ* by Illumina sequencing so they may be the same mutation (Table 4A, Table 5A). In *Klebsiella pneumoniae*, *wcaJ* is part of the capsular cps operon and initiates production of colonic acid (46, 47). Absence of *wcaJ* has been linked to increased resistance to phage treatment, decreased virulence in live murine

models, and increased phagocytosis by macrophages (46). A separate study ablating the same gene found that samples of *Klebsiella pneumoniae* creates a nonmucoid phenotype while increasing biofilm production, altering cell shape, and increasing resistance to polymyxin (47). The same work also confirmed that a lack of the *wcaJ* gene increased phagocytosis and lysis inside of murine macrophages (47). Capsule in *K. pneumoniae* is a known resistance mechanism and virulence factor so the mutation in *wcaJ* is notable. The present data indicate that the adapted strain of bacteria have increased in mucoidy. Further experimentation would need to be done to identify if this mutation or mutations in another capsular gene such as that identified in the sugar ABC transporter ATP binding protein are responsible for the mucoid phenotype.

The LTEE study also provided estimates for the rapidity of fixation of mutations in a constant environment. The population accumulated 20 mutations in the first 10,000 generations of growth with a few rapid mutations that reached fixation in the population within 100 generations (35, 36). The three resistance-correlated mutations in the present study all fixed within 360-378.9 generations. While the present study does use incremental changes in antibiotic concentration, it does not counteract that these mutations were acquired rapidly and indicate a fitness benefit to the adapted population. The speed with which these three mutations were acquired also indicates that the sub-MIC concentration of cephalothin in the growth environment did provide selective pressure. The sample from day one of antibiotic exposure identified fixation of a mutation in *rpoB*. As this time point represents 24 hours of antibiotic treatment, the mutation reached fixation within 36-37.9 generations using a generation time estimate from a related *K. pneumoniae* strain of 38-40 minutes (48). After day one, the resistance of the adapted population to tetracycline had the first jump from 1 μg/mL to 8 μg/mL. The rapidity of fixation and correlated increase in survivability suggest that this mutation conferred a very high fitness

advantage. It is possible that a necessary step in acquiring resistance for this population was the mutation in a gene meant to monitor nucleic acid integrity and possibly allow for further changes to the genome.

On day 6 a mutation in an uncharacterized TetR/AcrR family transcriptional regulator is fixed, the same day that the first jump in survivability in cephalothin occurred (Figure 7). The insert in this gene is first reported at a frequency of 25 % on day 1 of treatment. Given that the average generation time for *Klebsiella pneumoniae* species ranges from 38-40 minutes and that a marginal presence of the mutation on day 1 indicates the presence of the mutation at the beginning of the experiment, this mutation becomes fixed in approximately 216-227.4 generations (48). While this is still a relatively fast acquisition, it is nowhere near as fast as the mutation in *rpoB*. However, the increase in antibiotic survivability associated with the date this mutation fixed was higher than that of *rpoB* (Fig 8). The TetR/AcrR mutation may be associated with some other fitness cost that impeded fixation.

The third mutation correlated with an increase in antibiotic survivability was in the *wcaJ* gene and appears as a fixed mutation on day 10. The MiGS data does not show any marginal call of this mutation in prior samples and the percent of the population maintaining this mutation dwindles thereafter to 64% in the 14-day whole group DNA sample. Since there was no prior marginal call for the mutation, it is possible that it's presence in the adapted population began after sample collection on day 9. This would allow for 24 hours of growth to the fixation point giving a fixation time of 36-37.9 generations. This mutation was associated with the highest increase in antibiotic resistance (Fig 8). The increase in survivability coupled with the rapidity of fixation indicate that this may be the most fit mutation of the three. A confounding factor to consider is that the presence of the mutation in later sample days declines and this mutation is

found to only be completely fixed after 14days in the small colony-forming variant. Future work should assess if the fixation call was due to sampling error or an overabundance of small-colony forming variant individuals at day 10.

The data collected also identified a unique genomic structure for the small and large colony forming variants of the 14-day adapted population. Only 52% of large colony forming subpopulation DNA contained the insert in TetR/AcrR family transcriptional regulator while the insert was fixed throughout the small colony subpopulation. This mutation may be more advantageous to the small colony variant population due to their smaller size. Additionally, the deletion spanning between *wcaJ* and *gndA* only occurred in the small colony forming population. As noted above, ablation of *wcaJ* in *K. pneumoniae* is linked to a decrease in species mucoidy and antibiotic resistance. Similarly, *gndA* is a gene within the cps locus responsible for the K2 serotype and capsule formation of *K. pneumoniae* (49, 50). It seems likely that the enlarged pellicle seen in the 14-day adapted population whole-group mixture is due to the large colony variant only. Furthermore, the small colony subpopulation has a reduced resistance to cephalothin compared to the large colony forming variant which has an intact *wcaJ* sequence. The absence of the sequence differentially impacts resistance to a β-lactam which could be explained by the two different classes of drugs. The prior study which ablated the capsule gene *wcaJ*, tested resistance to polymyxin- a drug which acts by targeting LPS (47). Cephalothin targets internal functions of gram-negative prokaryotes so the presence of a capsule barrier would be beneficial. It could be that the small-colony forming subpopulation mutated independently to rely more on the mutation in the TetR/AcrR regulator than capsule. Further experimentation could focus on elucidating the downstream targets of this regulator to better understand the resistance mechanism.

Other Notable Mutations

Alterations in gene transcription between *Klebsiella pneumoniae* 43816 and the 14-day adapted population also indicate three genes of interest. The potential function of TetR/AcrR transcriptional regulator is discussed above. The increased transcription in the adapted population could be due to a common signaling pathway used in response to stress caused by tetracyclines in better understood organisms. Transcription of globin is decreased. Globins are used to sequester and transport oxygen. These findings could suggest that the mutated population has an altered metabolic profile which doesn't require as much oxygen. Finally, the transcription of deoxyribopyrimidine photolyase is also decreased. Ultraviolet radiation can create a distinct form of DNA damage in the form of cyclobutene pyrimidine dimers which interfere with DNA replication (51). Deoxyribopyrimidine photolyases can repair this damage and prevent potential replication errors (52, 53). Reduced production of this gene removes another mechanism the cell can prevent errors during DNA duplication. This may also serve the purpose of permitting more mutations and more variation in the presence of antibiotic stress.

Assessing the adapted population's survivability in amikacin came as a result of findings in the first round of whole genome sequencing. In that report, a mutation was identified in an uncharacterized n-acetyltransferase (NAT) coding region. In general, the different classes of NATs act to transfer the acetyl group from acetyl-CoA to a target (54). Early identification of this protein as a marker of resistance used the human genome. Human NATs, especially those classified as arylamine NATs, are responsible for the effective activation of different antibiotics (54). Arylamine NATs are also a known plasmid-mediated resistance gene in *K. pneumoniae* and inhibit the effects of aminoglycoside class antibiotics (55, 56). This same subset of NATs have been associated with bacterial resistance to antibiotics in other Gram negative bacteria (57). The

mutated protein can prevent aminoglycosidic compounds from interacting with bacterial lysosomes and prevent damage to the cell. The data in Table 4A shows a one nucleotide deletion unique to the antibiotic resistant DNA sample in an N-acetyltransferase gene.

NATs have been identified throughout the genome of *Klebsiella pneumoniae* subspecies acting as part of toxin-antitoxin systems, elements of transposons, or as arylamine NATs (58–60). The data in Table 1 show an increase in resistance to amikacin, a commonly used aminoglycoside, in both the untreated and adapted populations of *Klebsiella pneumoniae* 43816. The increased MIC for the untreated population is minimal but the population exposed to cephalothin for 14 days had an MIC four times higher than the parent strain. This data can confirm that arylamine NAT-mediated resistance does not have to be transmitted on a plasmid. In the present study, random mutation of the genomic region itself could have altered the protein in a way that enhanced aminoglycoside tolerance.

Another gene of interest was the SGNH/GDSL hydrolase family protein. Initial sequence results found 2 mutations in the coding region for this gene and one potential promoter mutation in the adapted population (Figure 4A and 4B). The coverage of the coding region and promoter mutations was near 50% and could be due to a distinction between the small and large colony forming subpopulations in the 14-day treated sample. The SGNH superfamily derives its name from a shared serine-glycine-asparagine-histidine amino acid sequence in which serine is incorporated into the enzyme's active site (61). The GDSL subfamily of the SGNH hydrolase group is further specified by the inclusion of a glycine-aspartine-serine-leucine motif near the active site (61). The SGNH protein family is a unique group with broad specificity and the ability to cleave multiple esters and lipids (61). The active site of this enzyme can accept a range of substrates and is known to undergo conformational changes based on available substrates (61).

While this group has been extensively described in terms of structure and composition, much work is yet to be done to determine in vivo functionality.

Comparing SMRT Cell and Illumina Sequencing Results

The sequence data obtained add to the growing set of literature regarding types of genomic resistance genes, the effect of sub-MIC antibiotic exposure to mutation rates, and the efficacy and sensitivity of different forms of whole genome sequencing. SMRT Cell sequencing of the whole adapted population and untreated population of *K. pneumoniae* identified eight nonsynonymous mutations in coding regions of seven genes and six potential promoter mutations unique to the adapted population (Table 4A and B). A second round of Illumina sequencing of the adapted whole-group identified only four unique mutations (Table 5A). Both methods referenced the samples to the given NCBI sequence for *Klebsiella pneumoniae* 43816 (Reference number NZ_CP009208.1) but the sequencing and statistical methodology differed. Illumina sequencing is a high-throughput method which sequences amplified DNA fragments (62). The method is known to be fast and inexpensive, which proved to be true in the present study as well (62). Illumina's low error rate has allowed it to be used successfully identify single-nucleotide polymorphisms in an effort to track pathogenic outbreaks (62). SMRT cell sequencing is a long-read sequencing technique which involves circularizing sample DNA with a hairpin structure that is loaded into a cell that houses a stationary DNA polymerase (62, 63). Even though larger segments of DNA can be processed at one time, the potential for error in a single read can be as high as 14% requiring the use of multiple reads to validate base calls (62). It is a more expensive sequencing option but has the benefit of avoiding amplification bias in base calls as amplification isn't necessary for the process, it can handle many of the genomic structures common to AR elements like transposons and extended repeats, and is preferred for *de*

novo sequencing of new references (62–64). Recent work has suggested that both long-read methods, like SMRT cell sequencing, and short-read methods, like Illumina sequencing, be combined for a hybrid approach for the most accurate formation of novel sequences especially those of the *Enterobacteriaceae* family (64). The data from both rounds of sequencing found that the SMRT cell method identified more polymorphisms in the adapted population genome and was able to identify mutations in noncoding regions unlike the Illumina method. Additionally, the evolution of a resistant phenotype can require cooperative mutations adding difficulty to the present study's ability to directly trace the effects of one mutation to a quantifiable change in antibiotic survival (65). Both the SMRT cell and Illumina sequence methods should be used on all of the DNA samples to create a better library of potential mutations.

Both sequencing methods noted mutations in genes related to signal transduction, regulation of transcription, DNA integrity, and capsule formation. The mutations identified do not all match between both sequencing methods, nor are the calls for similar genes found in the exact same position or using the same base changes (Table 4 A and B, Table 5 A-C). This could reflect differences in methodology, genome construction in the two methods, and/or inherent error. However, it is more likely that the inconsistencies in the two sequencing methods results from the sample of DNA provided for analysis. The frozen stocks created of the adapted *K. pneumoniae* and the frozen stocks for each treatment day consisted of a subset of a population of bacteria that were grown in a stress condition. A sample of that stock was then regrown and allowed to proliferate then lysed to generate DNA for analysis. The process of sampling and growing from a heterogenous population allows for a degree of variance in the individual bacterium that make up the sample population. Regardless of methodology, each report showed a wide range in affected gene function, many of which are not classically considered AR genes in

Klebsiella pneumoniae. The results from both forms of sequencing were absent of known β-lactamase genes and penicillin binding proteins. The adapted population of *K. pneumoniae* generated by long-term antibiotic exposure did not have evidence of one of the most common bacterial β-lactam defense mechanisms. Any genetic analysis other than a whole-sequence format would likely have found no mutations limiting the ability to gain insight into how antibiotic exposure influences genetic evolution.

Mutation Number, Rate, and Genetic Heterogeneity

The selection window hypothesis holds that sub-MIC level antibiotic exposure should generally increase bacterial mutation rates (12, 13). The idea is that higher mutation rates improve the chances of generating an adaptive genetic change that will help the organisms survive antibiotic stress. Methodologies employed by studies testing this theory have generally identified "mutants" by plating samples of a bacteria exposed to an antibiotic on media inoculated with some other antibiotic substance (8, 20). The number of successful colonies grown is compared to that of a sample placed on an un-inoculated media (8, 20). In these cases, the mutation rate reported is better understood as "the *in vitro* frequency at which detectable mutants arise" (65). Regardless, mutation rate analyses using that methodology tend to indicate that at lower antibiotic concentrations the mutation rate is high and this rate declines as the concentration of antibiotic nears the MIC (20, 65). Contrastingly, when whole-genome analysis is incorporated along with mutation accumulation analysis, substitutions and insertions/deletions increased in frequency as exposure to norfloxacin increased in *E.coli* (66). Additionally, the rate of mutation in known resistance genes was tracked in *P. aeruginosa* isolates exposed to antibiotics and generated conflicting results (22).

The present study utilized whole genome sequencing to identify mutations across the entire *K. pneumoniae* genome for the antibiotic-treated and unexposed sample after 14 days of total treatment. The data from SMRT cell sequencing shows a higher number of mutations in the untreated sample compared to the adapted cohort. It is interesting to note that the number of genetic changes at fixation and below that level outnumber the adapted population. As noted by Long et. al., a true mutation accumulation analysis which could be interpreted as a frequency of genetic polymorphism per generation would require repeatedly passing a bacteria through bottlenecks to mitigate any selective influences (66). The fact that the adapted population in this study was maintained as a whole group when subcultured may affect the relative number of mutations identified by whole genome sequencing. Another factor at work is that the treatment concentration of cephalothin did surpass the initial MIC of *K. pneumoniae* 43816 for over half of the adaptation period (Table 1). Both the selection window hypothesis and adaptive studies of bacterial mutation would suggest that such a high concentration would decrease mutation rates compared to an unexposed cohort and explain the low number of identified polymorphisms (8, 12, 13, 20, 65).

The depth of coverage results from both rounds of sequencing also indicate a certain level of genetic heterogeneity in the adapted population of *K. pneumoniae*. Many genes can generate the phenotype of antibiotic resistance therefore the individual genotypes from resistant samples are not always identical (65). The presence of unfixed mutations among the adapted population in genes such as the SGNH/GDSL hydrolase protein, *wcaJ*, and *gndA* in the whole group analyses and TetR/AcrR transcriptional regulator in the large colony-forming variant are understandable. Functional investigations of these genes in *K. pneumoniae* would better elucidate how or if these mutations affect the organism's survival in antibiotic.

Conclusions

The present study confirms findings in other pathogens regarding low level antibiotic exposure over time. The adapted *K. pneumoniae* population showed that sub-MIC exposure can generate to cross-resistance. The adapted population here developed an increased resistance to drugs in two different antibiotic classes after treatment. The phenotypic findings correlate to identified mutations in known resistance genes for other bacteria- n-acetyltransferase and TetR/AcrR transcriptional regulator.

Secondly, the development of resistance by the adapted population in this model confirm findings that *K. pneumoniae* has resistance genes inherent to its own genome and doesn't require acquisition of foreign DNA to develop an AR phenotype (18). The treatment methodology did not expose either the untreated or treated populations to genetic material from an outside source. The populations were subcultured separately and any mutations that arose, fixed or not, had to have been developed in the DNA replication process. This highlights the need to better understand bacterial pathogen genomes to better predict, prevent, and treat resistant forms.

Finally, two mutations can be correlated to recorded increases in MIC for the adapted strain- *rpoB* on day 6, and *wcaJ* on day 10 of treatment. While *wcaJ* is a known virulence factor and resistance gene for *K. pneumoniae*, *rpoB* is not. This mutation also became fixed on a day with the largest increase in MIC. Without sequencing the whole-genome of these samples, it is very likely that the mutation would have been overlooked. The majority of antibiotic adaptation analyses focus on known or suspected AR genes. The present data indicate that a more thorough query into all genes and potential promoters could shed more light into how antibiotic adaptation occurs.

Tables and Figures

Figure 1: Bacterial growth rates as a function of antibiotic exposure from Gullberg et. al. 2011. A) red line represents resistant strain and the blue line represents the susceptible strain. The green zone is the range of antibiotic concentrations below the selection window, orange and red zones are mutant selective. B) Relative exponential growth rates for susceptible (open circle) and resistant (closed circle) strains.

Figure 2: Experimental Design used to develop antibiotic resistant population of *K. pneumoniae* 43816

Figure 3: Growth of ATCC 43816, sample subcultured for 14 days without antibiotic, and sample exposed to Cephalothin for 14 days.

Figure 4: Aqueous culture with capsule from 3 samples of *K. pneumoniae* 43816. Sample titled *Klebsiella pneumoniae* 43816 uses the laboratory isolate, sample titled untreated is from the population of ATCC 43816 serially passaged without antibiotic and the sample on the right was treated with Cephalothin for 14 days.

Figure 5: Diluted plate sample of adapted *K. Pneumoniae* 43816 with large and small colony variants demarcated.

Figure 6: Enlarged gram stain of adapted *K.pneumoniae* 43816 cultures. The image is on the left is from the large colony variant and the image on the right is from the small colony variant.

Figure 7: Graph of MIC50 breakpoints for the adapted strain in response to Cephalothin treatment. Data for Cephalothin MIC50 is represented by the blue line and data for Tetracycline MIC50 is represented by the orange line. Each dot on the map denotes a sample from one treatment day.

Figure 8: Graph of MIC50 breakpoints for the adapted strain in response to Cephalothin treatment with fixed mutations noted. Data for Cephalothin MIC50 is represented by the blue line and data for Tetracycline MIC50 is represented by the orange line. Mutations fixed in the adapted strain are marked by day of fixation.

Figure 9: Transcriptional expression of genes with mutated coding regions assessed by qPCR and analyzed using the ΔΔCt method. Genes are normalized to *gapA* and extracts from the treated population (14T) are compared to lab strain *K. pneumoniae* 43816 (Day 0) *$p<0.01$

Figure 10: Transcriptional expression of genes with mutated promotor regions assessed by qPCR and analyzed using the ΔΔCt method. Genes are normalized to *gapA* and extracts from the treated population (14T) are compared to lab strain *K. pneumoniae* 43816 (Day 0) *$p<0.01$

Table 1: Minimum inhibitory concentrations calculated for untreated culture grown for 14 days and culture treated with low-level antibiotic for 14 days. Concentration determined from average of 3 data points for each culture. * $p<0.05$; ** $p<0.0005$

Table 2: Minimum inhibitory concentrations to Cephalothin for adapted population colony size variants. Concentration determined from average of 3 data points for each culture.

Table 3: List of primers used for qPCR.

Table 4: List of identified nucleotide changes for AR *K. pneumoniae 43816* culture using SMRT Cell sequencing. These changes are not found in the susceptible counterpart. **A** contains unique changes in protein coding regions, **B** contains the unique changes in potential promoter regions.

Table 5: List of identified nucleotide changes for AR *K. pneumoniae 43816* culture using MiGS Illumina sequencing. These changes are not found in the susceptible counterpart. **A** contains unique changes in protein coding regions, **B** lists mutations identified in the large colony forming variant of the adapted population, and **C** lists the mutations identified in the small colony forming variant of the adapted population.

Figure 1:

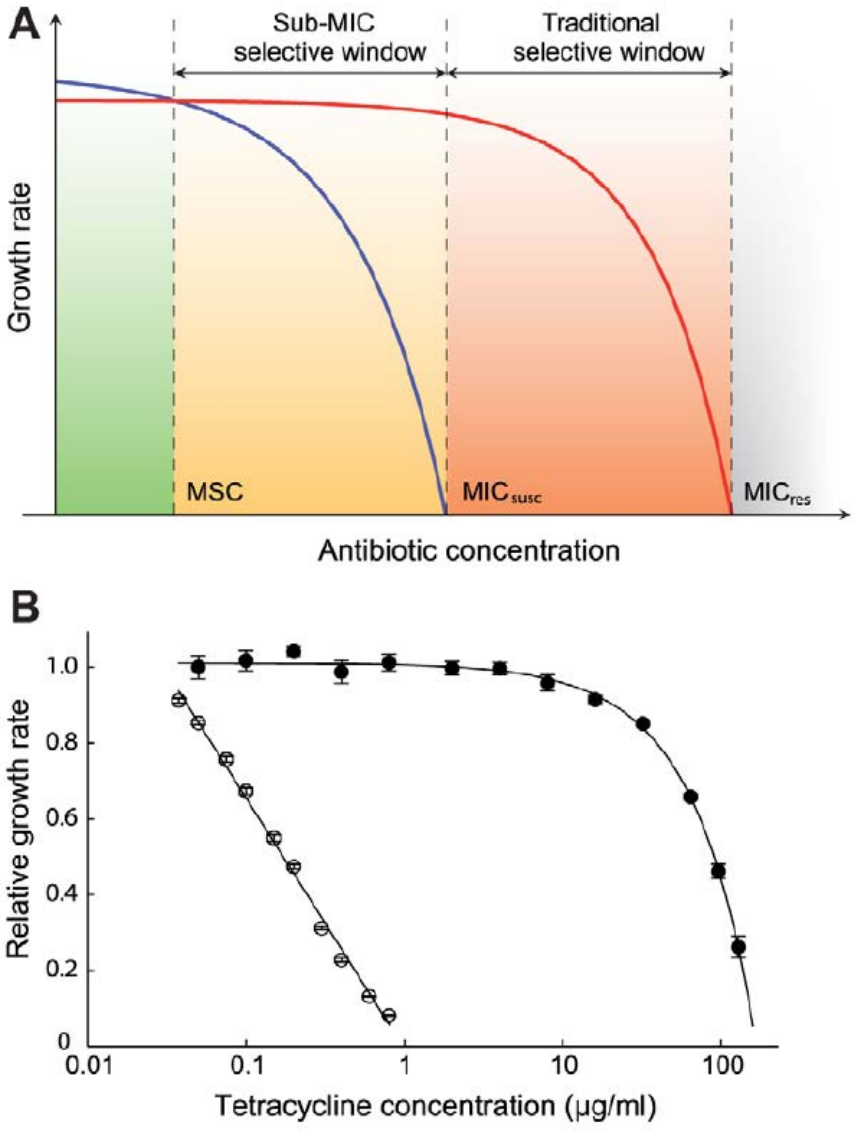

A
Sub-MIC
selective window
Traditional
selective window
Growth rate
MSC
MIC susc
MIC res
Antibiotic concentration
B
Relative growth rate
1.0
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
Tetracycline concentration (µg/ml)

Figure 2:

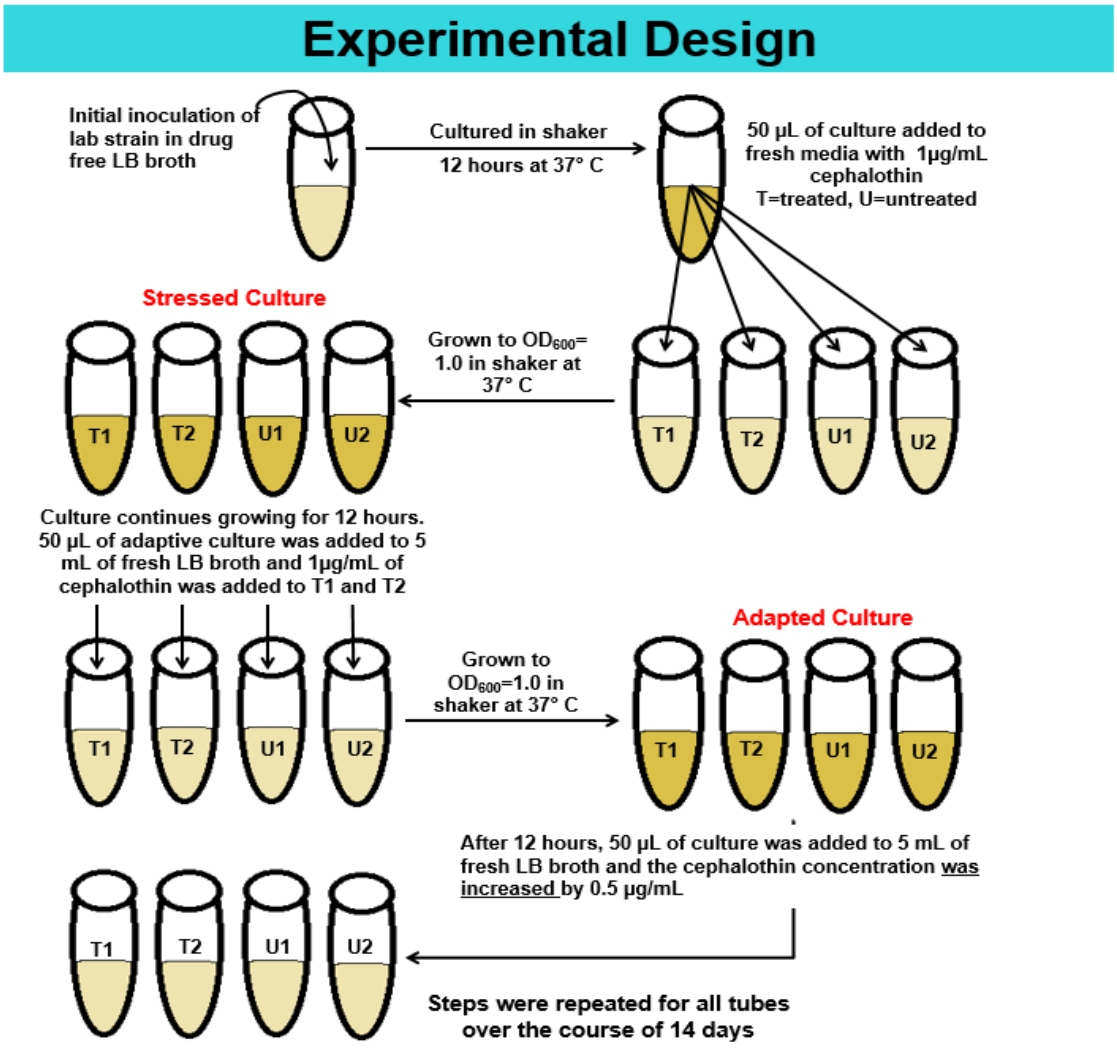

Experimental Design
Initial inoculation of lab strain in drug free LB broth
Cultured in shaker
12 hours at 37° C
50 µL of culture added to fresh media with 1µg/mL cephalothin
T=treated, U=untreated
Stressed Culture
Grown to OD600= 1.0 in shaker at 37° C
T1
T2
U1
U2
Culture continues growing for 12 hours. 50 µL of adaptive culture was added to 5 mL of fresh LB broth and 1µg/mL of cephalothin was added to T1 and T2
Adapted Culture
Grown to OD600=1.0 in shaker at 37° C
After 12 hours, 50 µL of culture was added to 5 mL of fresh LB broth and the cephalothin concentration was increased by 0.5 µg/mL
Steps were repeated for all tubes over the course of 14 days

Figure 3:

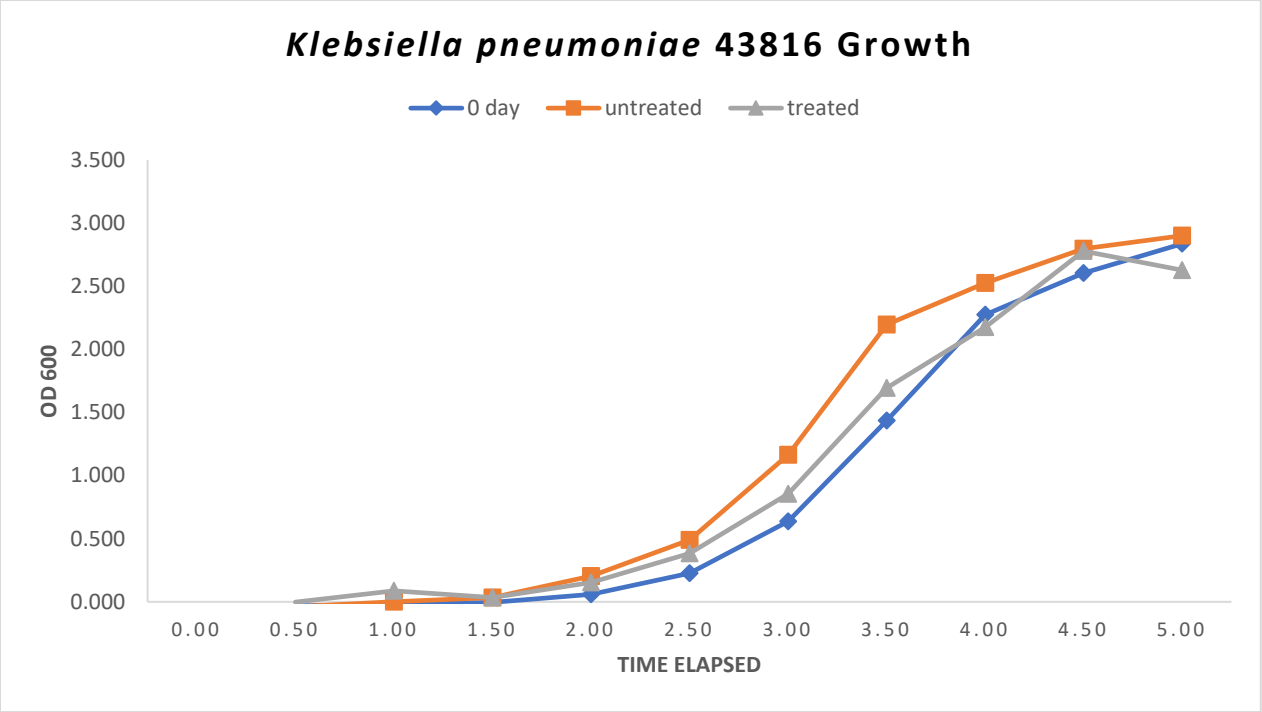
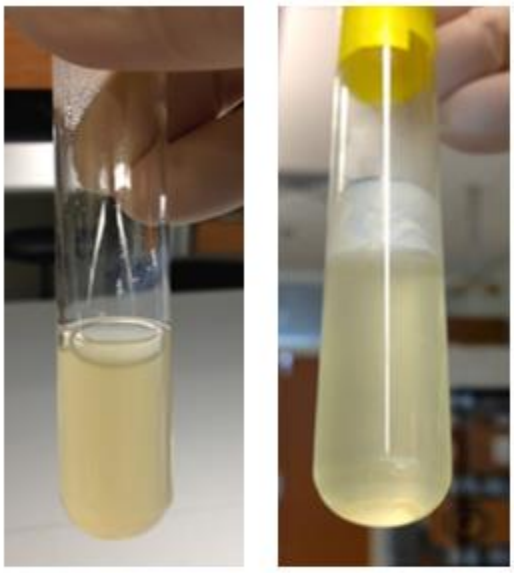

Klebsiella pneumoniae 43816 Growth
0 day
untreated
treated
OD 600
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
TIME ELAPSED

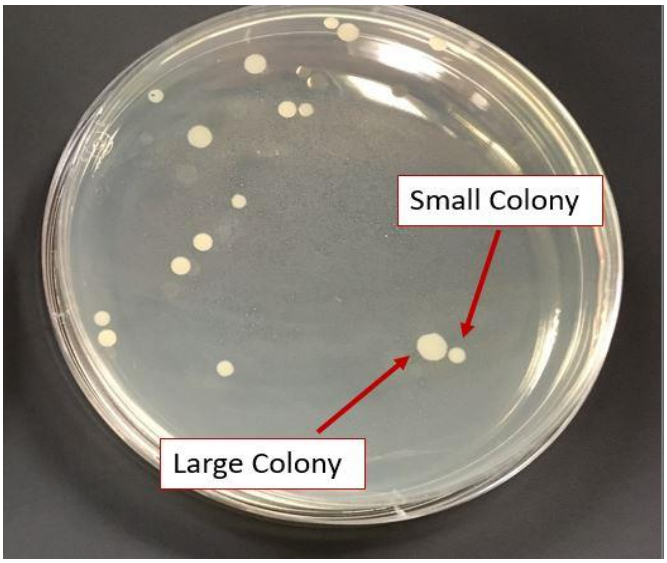
Figure 4:



Untreated Culture

Treated Culture

Figure 5:



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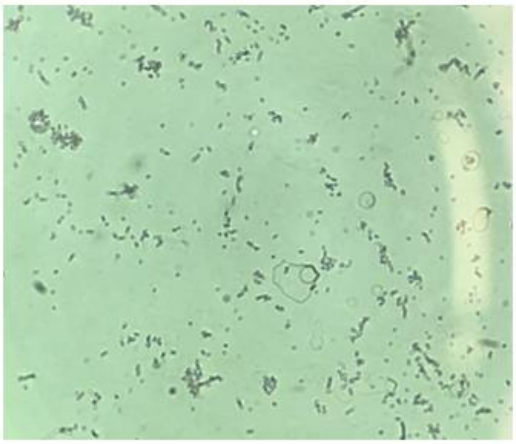
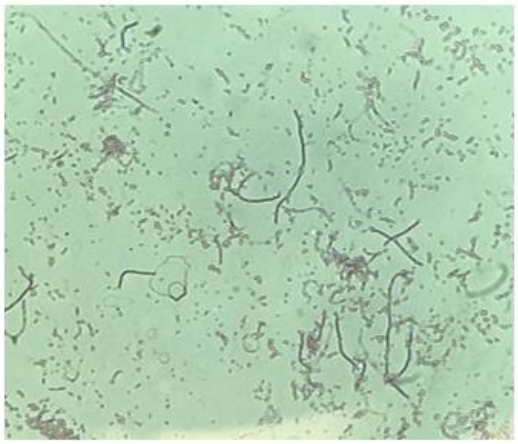


Large colonies 14TB1



Small colonies 14TB1

Figure 7:

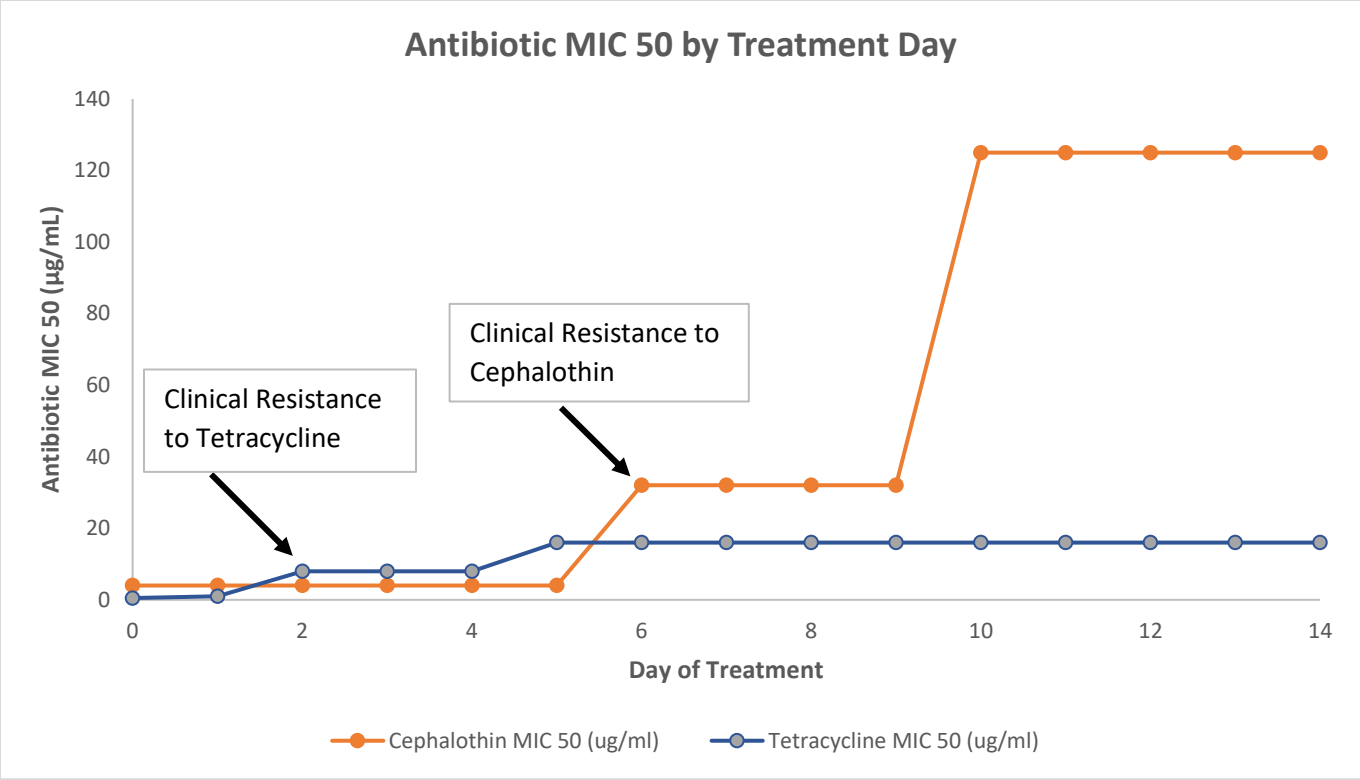

Antibiotic MIC 50 by Treatment Day
Antibiotic MIC 50 (µg/mL)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Day of Treatment
Clinical Resistance to Tetracycline
Clinical Resistance to Cephalothin
Cephalothin MIC 50 (ug/ml)
Tetracycline MIC 50 (ug/ml)

Figure 8:

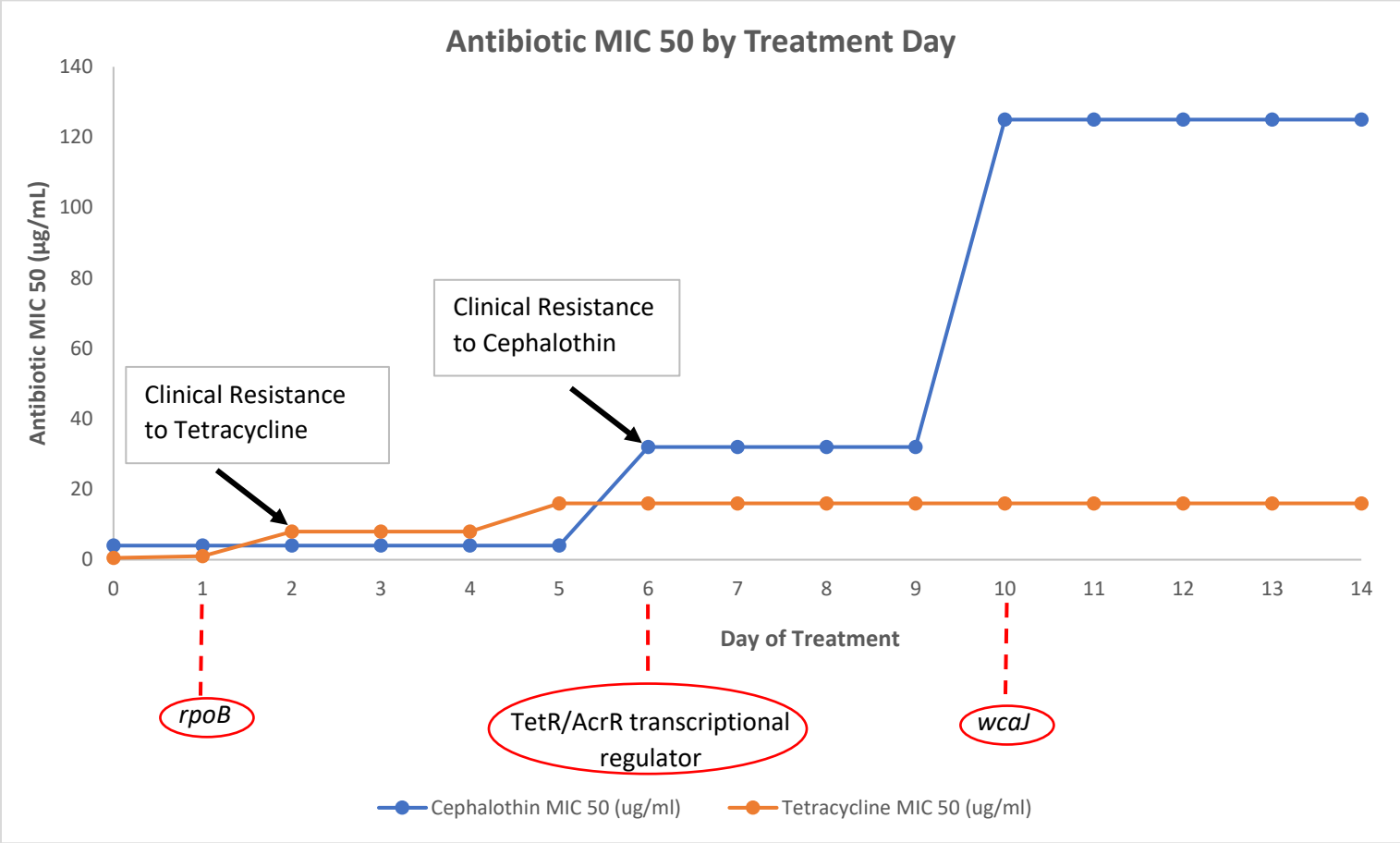

Antibiotic MIC 50 by Treatment Day
Antibiotic MIC 50 (µg/mL)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Day of Treatment
Clinical Resistance to Tetracycline
Clinical Resistance to Cephalothin
rpoB
TetR/AcrR transcriptional regulator
wcaJ
Cephalothin MIC 50 (ug/ml)
Tetracycline MIC 50 (ug/ml)

Figure 9

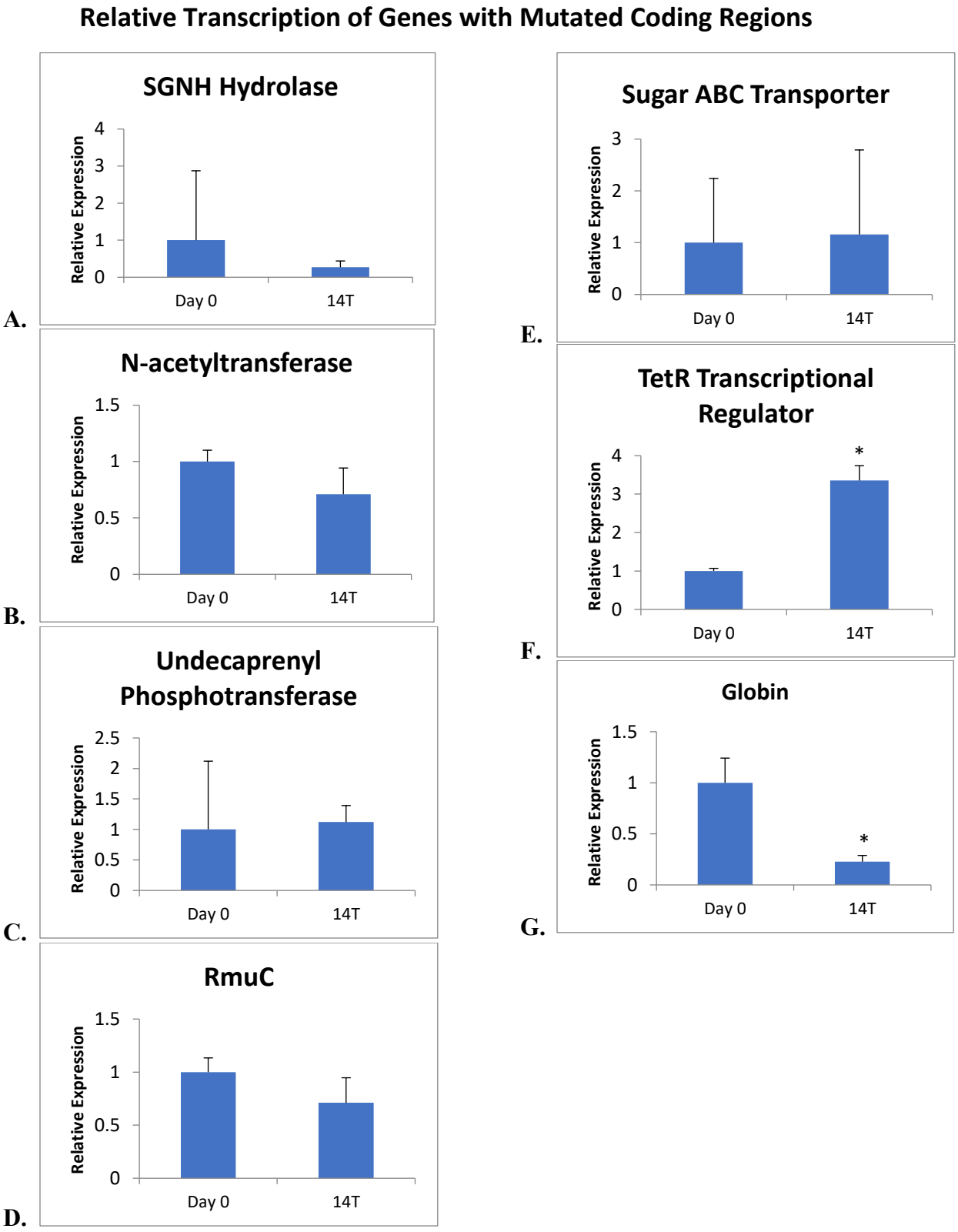

Relative Transcription of Genes with Mutated Coding Regions
A.
SGNH Hydrolase
Relative Expression
4
3
2
1
0
Day 0
14T
B.
N-acetyltransferase
Relative Expression
1.5
1
0.5
0
Day 0
14T
C.
Undecaprenyl Phosphotransferase
Relative Expression
2.5
2
1.5
1
0.5
0
Day 0
14T
D.
RmuC
Relative Expression
1.5
1
0.5
0
Day 0
14T
E.
Sugar ABC Transporter
Relative Expression
3
2
1
0
Day 0
14T
F.
TetR Transcriptional Regulator
Relative Expression
4
3
2
1
0
*
Day 0
14T
G.
Globin
Relative Expression
1.5
1
0.5
0
*
Day 0
14T

Figure 10

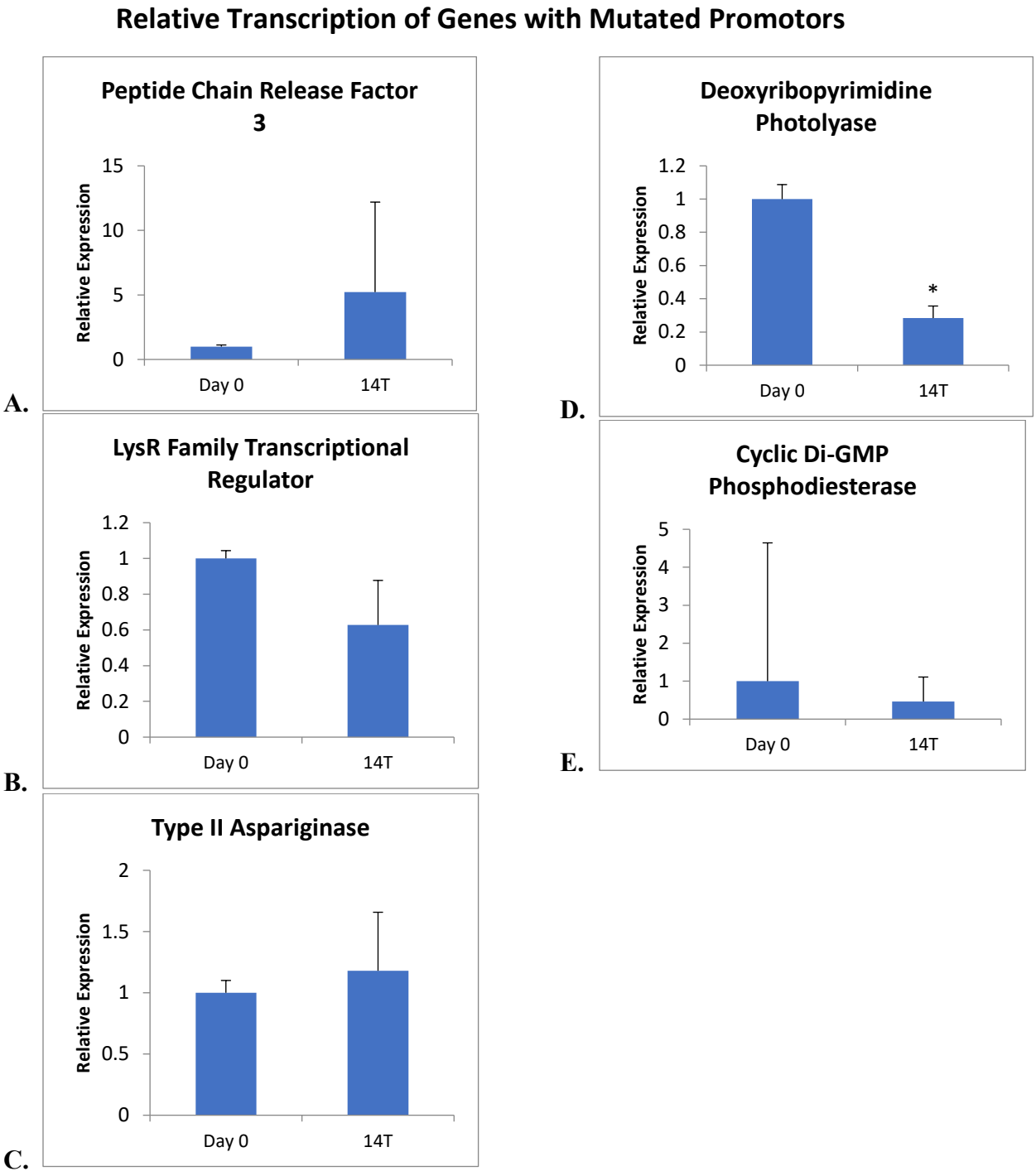

Relative Transcription of Genes with Mutated Promotors
A.
Peptide Chain Release Factor 3
Relative Expression
15
10
5
0
Day 0
14T
B.
LysR Family Transcriptional Regulator
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
14T
C.
Type II Aspariginase
Relative Expression
2
1.5
1
0.5
0
Day 0
14T
D.
Deoxyribopyrimidine Photolyase
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Day 0
14T
E.
Cyclic Di-GMP Phosphodiesterase
Relative Expression
5
4
3
2
1
0
Day 0
14T

Table 1:

50% Minimum Inhibitory Concentration (MIC50) of Antibiotic

Antibiotic	***K. pneumoniae* 43816**	**Day 14 Untreated**	**Day 14 Treated**
Tetracycline	0.5 µg/mL	1 µg/mL*	16 µg/mL**
Cephalothin	4 µg/mL	4 µg/mL	125 µg/mL**
Amikacin	0.5 µg/mL	1 µg/mL*	2 µg/mL**

Table 2:

Minimum Inhibitory Concentration of Cephalothin

	MIC 50	Breakpoint
Large Colony Variant	500 µg/mL	>500 µg/mL
Small Colony Variant	125 µg/mL	500 µg/mL

Table 3: qPCR Primers

Gene	Forward Primer	Reverse Primer
SGNH/GDSL hydrolase family protein	GGAGCTATCCGGTTTAGCTATTT	GACAGCAACGTCGCCTATAA
N-acetyltransferase	CCCAGGTTCATTATCGGTGTATC	GGCTTAACCACTGGGAGAATTA
globin	CGCTGTGCGATGAAGAGAT	TGACCTGCGGTCTGATAGT
TetR/R family transcriptional regulator	GCTCGTCCAAAGAGTGAAGATA	GTGGCGAAATAGCGAAACAG
sugar ABC transporter ATP-binding protein	GGTCGGCTCATCGAGAATTAAG	CGCCGACGATGGATCAAATTA
DNA recombination protein RmuC	GTCGGGCTAACCAGCATAAT	GTGCTGATGTTTATTCCCGTTG
undecaprenyl-phosphate glucose phosphotransferase	GTGCTGGCTTTGTTGTTACTC	CAAACTGATTCCTGCTGGTAAAG
Peptide Chain Release Factor 3	CGGTCTGATACAGGTAGGTTTC	TGCTGGATGAAGTCGAGAAC
LysR Family Transcriptional Regulator	CGGCATCCCTACCTTGAAC	GCCGGTATTATGAGGAGTGTAAA
Type II Aspariginase	CGGTTCCGGCGTAGATAATAC	CTGCCGAAGGTGGTGATT
Deoxyribodipyrimidine Photo-Lyase	GGCGTTCATTGAATTCGTACTG	TACCGCTGTGGGTAGAAGA
Cyclic Di-GMP Phosphodiesterase	TGATCGTCAGCACGTAATAGC	CCGCTGAAGGTCTATCTGTATG

Table 4:

A.

Adapted Population Coding Region Changes				
Position	**Original**	**New**	**Coverage**	**Gene Changed**
10279	G	C	43	SGNH/GDSL hydrolase family protein
10285	G	C	43	SGNH/GDSL hydrolase family protein
412527	G	Deletion	100	N-acetyltransferase
1125537	.	Insert T	100	globin
2008934	.	Insert TTTCGCTA	100	TetR/AcrR family transcriptional regulator
2063994	C	Deletion	100	sugar ABC transporter ATP-binding protein;
3225592	C	Deletion	100	DNA recombination protein RmuC
5188116	T	G	100	undecaprenyl-phosphate glucose phosphotransferase

B.

Adapted Population Promoter Changes				
Position	**Original**	**New**	**Coverage**	**Gene Changed**
10412	T	Deletion	56	2 bases before reading frame for SGNH hydrolase
2657272	G	Deletion	100	78 bases before peptide chain release factor 3
1078904	C	Deletion	100	11 before LysR family transcriptional regulator
1294495	G	Deletion	100	81 bases before type II aspariginase
1823692	.	Insert A	100	127 before deoxyribodipyrimidine photo-lyase
5050494	.	Insert A	100	111 before cyclic di-GMP phosphodiesterase

Table 5:

A.

MiGS Identified Adapted Population Changes				
Position	**Original**	**New**	**Coverage**	**Gene Changed**
2,008,948	.	Insert TATTTCGC	100	TetR/AcrR family transcriptional regulator
3,191,984	A	T	100	*rpoB*/DNA-directed RNA polymerase subunit beta
5,187,772	T	G	64	*wcaJ*/undecaprenyl phosphate-glucose phosphotransferase
5,189,876	unspecified	unspecified	64	*gndA*/NADP-dependent phosphogluconate dehydrogenase

B.

Large Colony Adapted Population Changes				
Position	**Original**	**New**	**Coverage**	**Gene Changed**
3,191,984	A	T	100	*rpoB*/DA-directed RNA polymerase subunit beta
1,548,640	unspecified	unspecified	15	ComEC family protein
2,008,948	.	Insert TATTTCGC	52	TetR/AcrR family transcriptional regulator

C.

Small Colony Adapted Population Changes				
Position	**Original**	**New**	**Coverage**	**Gene Changed**
2,008,948	.	Insert TATTTCGC	100	TetR/AcrR family transcriptional regulator
3,191,984	A	T	100	*rpoB*/DA-directed RNA polymerase subunit beta
4,676,685	T	G	100	YfiR family protein
5,187,773	Deletion of 2,103 bp		100	*wcaJ* and *gndA*

References

1. Pendleton JN, Gorman SP, Gilmore BF. 2013. Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther 11:297–308.
2. Santajit S, Indrawattana N. 2016. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. Biomed Res Int 2016.
3. Paczosa MK, Mecsas J. 2016. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev 80:629–661.
4. Ma YX, Wang CY, Li YY, Li J, Wan QQ, Chen JH, Tay FR, Niu LN. 2020. Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections. Adv Sci 7:1901872-undefined.
5. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. 2019. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. Front Microbiol 10:539-undefined.
6. 2015. National Action Plan to Combat Antibiotic-Resistant Bacteria.
7. Kampf G. 2016. Acquired resistance to chlorhexidine – is it time to establish an 'antiseptic stewardship' initiative? J Hosp Infect. W.B. Saunders Ltd.
8. Nair CG, Chao C, Ryall B, Williams HD. 2013. Sub-lethal concentrations of antibiotics increase mutation frequency in the cystic fibrosis pathogen Pseudomonas aeruginosa. Lett Appl Microbiol 56:149–154.
9. Singh AK, Bhunia AK. 2019. Animal-Use Antibiotics Induce Cross-Resistance in Bacterial Pathogens to Human Therapeutic Antibiotics. Curr Microbiol 76:1112–1117.
10. Gallagher J, MacDougall C. 2018. Antibiotics SimplifiedFourth edition. Jones & Bartlett Learning, Burlington MA.
11. Wilson BA, Winkler M, Ho BT. 2019. Bacterial Pathogenesis: A Molecular ApproachFourth. ASM Press, Washington, D.C.
12. Drlica K. 2003. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother 52:11–17.
13. Drlica K, Zhao X. 2007. Mutant Selection Window Hypothesis Updated. Clin Infect Dis 44:681–688.
14. Worthington RJ, Melander C. 2013. Overcoming resistance to β-Lactam antibiotics. J Org Chem 78:4207–4213.
15. Poole K. 2004. Review Resistance to b b-lactam antibiotics. C Cell Mol Life Sci 61:2200–2223.
16. Mascaretti OA. 2003. Bacteria versus Antibacterial Agents. American Society of Microbiology.
17. Rice LB. 2012. Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones. Mayo Clin Proc 87:198–208.

18. Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, Brisse S, Cao H, Wilksch J, Gorrie C, Schultz MB, Edwards DJ, Nguyen K Van, Nguyen TV, Dao TT, Mensink M, Minh V Le, Nhu NTK, Schultsz C, Kuntaman K, Newton PN, Moore CE, Strugnell RA, Thomson NR. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health. Proc Natl Acad Sci U S A 112:E3574-81.

19. Jiang W, Yang W, Zhao X, Wang N, Ren H. 2020. Klebsiella pneumoniae presents antimicrobial drug resistance for β-lactam through the ESBL/PBP signaling pathway. Exp Ther Med 19:2449–2456.

20. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 7:e1002158-undefined.

21. Low YM, Chong CW, Yap IKS, Chai LC, Clarke SC, Ponnampalavanar S, Abdul Jabar K, Md Yusof MY, Teh CSJ. 2018. Elucidating the survival and response of carbapenem resistant Klebsiella pneumoniae after exposure to imipenem at sub-lethal concentrations. Pathog Glob Health 112:378–386.

22. Migliorini LB, Brüggemann H, De Sales RO, Koga PCM, De Souza AV, Martino MDV, Galhardo RS, Severino P. 2019. Mutagenesis induced by sub-lethal doses of ciprofloxacin: Genotypic and Phenotypic Differences between the Pseudomonas aeruginosa Strain PA14 and Clinical Isolates. Front Microbiol 10:1553-undefined.

23. Landman D, Bratu S, Kochar S, Panwar M, Trehan M, Doymaz M, Quale J. 2007. Evolution of antimicrobial resistance among Pseudomonas aeruginosa, Acinetobacter baumannii and Klebsiella pneumoniae in Brooklyn, NY. J Antimicrob Chemother 60:78–82.

24. Neuhauser M, Weinstein R, Rzdman R. 2003. Antibiotic resistance among gram-negative bacilli in us intensive care units: Implications for fluoroquinolone use. JAMA 289:885–888.

25. PubChem. 2004. PubChem Compound Summary for CID 6024, Cephalothin. Natl Cent Biotechnol Inf.

26. Wright MH, Adelskov J, Greene AC. 2017. Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation. J Microbiol Biol Educ 18.

27. Martin AP. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol 68:3673–82.

28. Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony RK. 2015. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One 10.

29. Barrick JE. 2016. breseq Manual — breseq 0.35.2rc1 documentation.

30. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45-undefined.

31. Grayson ML, Cosgrove SE, Crowe S, Hope W, McCarthy JS, Mills J, Mouton JW, Paterson DL. 2017. Kucers' The Use of Antibiotics: A Clinical Review of Antibacterial, Antifungal, Antiparasitic, and Antiviral Drugs, Seventh EditionKucers' The Use of Antibiotics. CRC Press.

32. Shutter MC, Akhondi H. 2020. Tetracycline. StatPearls [Internet].

33. White BP, Lomaestro B, Pai MP. 2015. Optimizing the initial amikacin dosage in adults. Antimicrob Agents Chemother 59:7094–7096.

34. Lenski RE. 2017. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. ISME J. Nature Publishing Group.

35. Good BH, McDonald MJ, Barrick JE, Lenski RE, Desai MM. 2017. The dynamics of molecular evolution over 60,000 generations. Nature 551:45–50.

36. Kleinman A. 2019. Fixation and Adaptation in the Lenski E. coli Long Term Evolution Experiment. Biomed J Sci Tech Res 20.

37. Slupska MM, Chiang JH, Luther WM, Stewart JL, Amii L, Conrad A, Miller JH. 2000. Genes involved in the determination of the rate of inversions at short inverted repeats. Genes Cells 5:425–37.

38. Moskowitz SM, Ernst RK, Miller SI. 2004. PmrAB, a Two-Component Regulatory System of Pseudomonas aeruginosa that Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A. J Bacteriol 186:575–579.

39. Van Dyk TK, DeRose EJ, Gonye GE. 2001. LuxArray, a high-density, genomewide transcription analysis of Escherichia coli using bioluminescent reporter strains. J Bacteriol 183:5496–505.

40. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell 104:901–912.

41. Jin DJ, Gross CA. 1988. Mapping and sequencing of mutations in the Escherichia colirpoB gene that lead to rifampicin resistance. J Mol Biol 202:45–58.

42. Severinov K, Soushko M, Goldfarb A, Nikiforov V. 1994. RifR mutations in the beginning of the Escherichia coli rpoB gene. MGG Mol Gen Genet 244:120–126.

43. Alifano P, Palumbo C, Pasanisi D, Talà A. 2015. Rifampicin-resistance, rpoB polymorphism and RNA polymerase genetic engineering. J Biotechnol 202:60–77.

44. Colclough AL, Scadden J, Blair JMA. 2019. TetR-family transcription factors in Gram-negative bacteria: Conservation, variation and implications for efflux-mediated antimicrobial resistance. BMC Genomics 20:731.

45. Cuthbertson L, Nodwell JR. 2013. The TetR Family of Regulators. Microbiol Mol Biol Rev 77:440–475.

46. Cai R, Wang G, Le S, Wu M, Cheng M, Guo Z, Ji Y, Xi H, Zhao C, Wang X, Xue Y, Wang Z, Zhang H, Fu Y, Sun C, Feng X, Lei L, Yang Y, ur Rahman S, Liu X, Han W, Gu

J. 2019. Three Capsular Polysaccharide Synthesis-Related Glucosyltransferases, GT-1, GT-2 and WcaJ, Are Associated With Virulence and Phage Sensitivity of Klebsiella pneumoniae. Front Microbiol 10:1189.

47. Pal S, Verma J, Mallick S, Rastogi SK, Kumar A, Ghosh AS. 2019. Absence of the glycosyltransferase wcaj in klebsiella pneumoniae atcc13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. Microbiol (United Kingdom) 165:891–904.

48. Regué M, Hita B, Piqué N, Izquierdo L, Merino S, Fresno S, Benedí VJ, Tomás JM. 2004. A Gene, uge, Is Essential for Klebsiella pneumoniae Virulence. Infect Immun 72:54–61.

49. Nelson K, Selander RK. 1994. Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (gnd) in enteric bacteria. Proc Natl Acad Sci U S A 91:10227–10231.

50. Arakawa Y, Wacharotayankun R, Nagatsuka T, Ito H, Kato N, Ohta M. 1995. Genomic organization of the Klebsiella pneumoniae cps region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain chedid. J Bacteriol 177:1788–1796.

51. Sancar A. 1994. Structure and Function of DNA Photolyase. Biochemistry 33:2–9.

52. Kavakli IH, Baris I, Tardu M, Gül Ş, Öner H, Çal S, Bulut S, Yarparvar D, Berkel Ç, Ustaoğlu P, Aydın C. 2017. The Photolyase/Cryptochrome Family of Proteins as DNA Repair Enzymes and Transcriptional Repressors. Photochem Photobiol 93:93–103.

53. Ozturk N. 2017. Phylogenetic and Functional Classification of the Photolyase/Cryptochrome Family. Photochem Photobiol 93:104–111.

54. Taylor JB (John B, Triggle DJ. 2007. Comprehensive medicinal chemistry II. Elsevier.

55. Ramirez MS, Nikolaidis N, Tolmasky ME. 2013. Rise and dissemination of aminoglycoside resistance: the aac(6′)-Ib paradigm. Front Microbiol 4:121.

56. Chiem K, Fuentes BA, Lin DL, Tran T, Jackson A, Ramirez MS, Tolmasky ME. 2015. Inhibition of aminoglycoside 6'-N-acetyltransferase type Ib-mediated amikacin resistance in Klebsiella pneumoniae by zinc and copper pyrithione. Antimicrob Agents Chemother 59:5851–3.

57. Rather PN, Munayyer H, Mann PA, Hare RS, Miller GH, Shawt KJ. 1992. Genetic Analysis of Bacterial Acetyltransferases: Identification of Amino Acids Determining the Specificities of the Aminoglycoside 6'-N-Acetyltransferase lb and Ila Proteins. J Bacteriol 174:3196–3203.

58. Qian H, Yao Q, Tai C, Deng Z, Gan J, Ou H-Y. 2018. Identification and characterization of acetyltransferase-type toxin-antitoxin locus in *Klebsiella pneumoniae*. Mol Microbiol 108:336–349.

59. Nobuta K, Tolmasky ME, Crosa LM, Crosa JH. 1988. Sequencing and expression of the 6'-N-acetyltransferase gene of transposon Tn1331 from Klebsiella pneumoniae. J Bacteriol 170:3769–3773.

60. Hui C-S, Kuo H-M, Yu C-S, Li T-M. 2004. Evidence for arylamine N-acetyltransferase activity in Klebsiella pneumoniae. J Microbiol Immunol Infect 37:208–15.

61. Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. 2004. GDSL family of serine esterases/lipases. Prog Lipid Res 43:534–552.

62. Quainoo S, Coolen JPM, Sacha A. F. T. van Hijum C, Martijn A. Huynen c WJGM, Willem van Schaik E, Wertheim HFL. 2017. Whole-Genome Sequencing of Bacterial Pathogens : the Future of Nosocomial Outbreak Analysis. Clin Microbiol Rev 30:1015–1064.

63. Amarasinghe SL, Su S, Dong X, Zappia L, Ritchie ME, Gouil Q. 2020. Opportunities and challenges in long-read sequencing data analysis. Genome Biol 21:1–16.

64. De Maio N, Shaw LP, Hubbard A, George S, Sanderson ND, Swann J, Wick R, Oun MA, Stubberfield E, Hoosdally SJ, Crook DW, Peto TEA, Sheppard AE, Bailey MJ, Read DS, Anjum MF, Sarah Walker A, Stoesser N. 2019. Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. Microb Genomics 5:e000294-undefined.

65. Martinez JL, Baquero F. 2000. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 44:1771–1777.

66. Long H, Miller SF, Strauss C, Zhao C, Cheng L, Ye Z, Griffin K, Te R, Lee H, Chen CC, Lynch M. 2016. Antibiotic treatment enhances the genome-wide mutation rate of target cells. Proc Natl Acad Sci U S A 113:E2498–E2505.