Progressive sub-MIC Exposure of *Klebsiella pneumoniae* 43816 to Cephalothin Induces the Evolution of beta-lactam Resistance without Acquisition of beta-lactamase Genes

Running Title: Antibiotic Resistance Evolved via sub-MIC Antibiotic Exposure

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Abstract

Bacterial exposure to antibiotic concentrations below the minimum inhibitory concentration (MIC) may result in a selection window allowing for the rapid evolution of resistance. These sub-MIC concentrations are commonly found in the greater environment. This study aimed to evaluate the adaptive genetic changes in *Klebsiella pneumoniae* 43816 after prolonged but increasing sub-MIC levels of the common antibiotic cephalothin over a fourteen-day period. Over the course of the experiment, antibiotic concentrations increased from 0.5 µg/mL to 7.5 µg/mL. At the end of this extended exposure, the final adapted bacterial culture exhibited clinical resistance to both cephalothin and tetracycline, altered cellular and colony morphology, and a highly mucoid phenotype. Cephalothin resistance exceeded 125 µg/mL without the acquisition of beta-lactamase genes. Whole genome sequencing identified a series of genetic changes that could be mapped over the fourteen-day exposure period to the onset of antibiotic resistance. Specifically, mutations in the *rpoB* subunit of RNA Polymerase, the *tetR/acrR* regulator, and the *wcaJ* sugar transferase each fix at specific timepoints in the exposure regimen where the MIC susceptibility dramatically increases. These mutations indicate that alterations in the secretion of colanic acid and attachment of colonic acid to LPS, may contribute to the resistant phenotype. These data demonstrate that very low, sub-MIC concentrations of antibiotics can have dramatic impacts on the bacterial evolution of resistance. Additionally, this study demonstrates that beta-lactam resistance can be achieved through sequential accumulation of specific mutations without the acquisition of a beta-lactamase gene.

Importance: Bacteria are constantly exposed to low levels of antibiotics in the environment. The impact of this low-level exposure on bacterial evolution is not well understood. In this work, we developed a model to expose *Klebsiella pneumoniae* to progressive, low doses of the antibiotic cephalothin. After a fourteen-day exposure regimen, our culture exhibited full clinical resistance to this antibiotic without the traditional acquisition of inactivating genes. This culture also exhibited resistance to tetracycline, had a highly mucoid appearance, and exhibited altered, elongated cellular morphology. Whole genome sequencing identified a collection of mutations to the bacterial genome that could be mapped to the emergence of the resistant phenotype. This study demonstrates that antibiotic resistance can be achieved in response to low level antibiotic exposure and without the traditional acquisition of resistance genes. Further, this study identifies new genes that may play a role in the evolution of antibiotic resistant bacteria.

Keywords: *Klebsiella pneumoniae*, antibiotic resistance, selection window, capsule, beta-lactams

Introduction

The mis- and overuse of antibiotics by the medical and agricultural industries has created a basal level of antibiotic exposure present in our environment (1–5). Antibiotics may be introduced into the environment via three major pathways. The first is from urine and excretions of people, pets, and livestock. Between 40-90% of administered antibiotics are excreted though urine and feces with the molecule still in its active form (1). Second, antibiotics such as chlorhexidine are used in commercial agriculture and aquaculture (3). These substances can then contaminate nearby lands via wastewater and groundwater seepage (1, 6). Finally, antibiotics can enter the local environment through improper disposal of unused or expired prescriptions (1, 3). While concentrations of antimicrobial substances in the environment will vary based on location and potential sources of contamination, it is generally agreed that the residual environmental antibiotic concentration is not high enough to eradicate native bacterial populations. However, even at low concentrations these compounds become a source of survival stress for bacteria in the soil and water supplies. Concentrations below the bacteriostatic limit, referred to as sub-MIC levels, may result in the creation of a selection window for bacteria (7–9). This window is the concentration at which the occurrence of genomic mutations is highest and can lead to the development of clinical antibiotic resistance in pathogens.

Experiments have shown that sub-MIC antibiotic treatment can alter the resistance profile, nutrient use, protein expression, gene transcription and mutation rates among ESKAPE pathogens (4, 10, 11). However, current studies of sub-MIC exposure and bacterial adaptation have a number of limitations. First, these experiments frequently utilize strains of bacteria either known to harbor antibiotic resistance genes or are otherwise clinically resistant to one specific class of antibiotic. Second, the experimental designs include only brief exposure times to antibiotics. In most studies,

77 the samples were exposed to the antibiotic for only 24-48 hours, providing limited time for the
78 bacteria to evolve novel genetic changes like those seen in a clinical or environmental setting
79 where antibiotic exposure is both consistent and long term. Finally, genetic analyses frequently
80 used pre-determined targets, or the phenotypes assumed from mutations in predetermined targets.
81 While the results of these studies are informative, they are not comparable to situations in which
82 antibiotic resistance phenotypes evolve over time though prolonged sub-lethal antibiotic exposure.

83 In this study, we utilized *Klebsiella pneumoniae* 43816, which does not exhibit clinical
84 resistance to the major antibiotic classes. Bacterial cells were cultured using a progressive
85 exposure method to gradually increasing sub-MIC concentrations of the beta-lactam antibiotic
86 cephalothin over a fourteen day period. At the end of this prolonged exposure period to sub-MIC
87 concentrations the bacteria exhibited clinical resistance to both cephalothin and tetracycline,
88 altered cellular and colony morphology, and a highly mucoid phenotype. Whole genome
89 sequencing identified a series of genetic changes that could be mapped over the fourteen-day
90 exposure period to the onset of antibiotic resistance. Specifically, mutations in the *rpoB* subunit
91 of RNA Polymerase, the *tetR/acrR* regulator, and the *wcaJ* sugar transferase each fix at specific
92 points in the exposure regimen where the MIC susceptibility dramatically increases. These
93 mutations indicate that secretion and export of colanic acid, and its association to
94 lipopolysaccharides, may contribute to the resistant phenotype. These data demonstrate that very
95 low, sub-MIC concentrations of antibiotics can have dramatic impacts on the bacterial evolution
96 of resistance. Additionally, this study demonstrates that antibiotic resistance can be achieved
97 through accumulation of mutations without the traditional acquisitions of beta-lactamase or other
98 drug inactivating genes.

99

Materials and Methods

Bacterial Strains and Progressive Antibiotic Exposure

Klebsiella pneumoniae 43816 (ATCC, Manassas, VA) was used as the starting culture for the progressive antibiotic exposure experiment (Figure 1). All cultures were grown in Luria-Bertani (LB) broth (BD Difco, Franklin Lakes, NJ) at 37°C with shaking at 200 rpm. All antibiotics and reagents are from Thermo Fisher Scientific (Waltham, MA) unless otherwise indicated. *K. pneumoniae* was grown overnight from frozen stocks and 50 µL of this culture was added to 5 mL of fresh LB with 0.5 µg/mL cephalothin added. The concentration of 0.5 µg/mL cephalothin was significantly below the MIC for this organism. Cultures were grown for 12 hours, at which point 50 µL were transferred to 5 ml of fresh LB with the same concentration of cephalothin. After a total of 24 hours exposure to one dose of antibiotics, 50 µL of culture were transferred to a new 5 mL of LB with a higher concentration of cephalothin. Each stepwise exposure increased the dose of cephalothin by 0.5 µg/mL, to a final dose of 7.5 µg/mL cephalothin. An untreated culture of *K. pneumoniae* 43816 was grown in parallel without the addition of antibiotic. Frozen glycerol stocks were made of each culture at each 12-hour transfer point for later analysis.

Bacterial Growth and Morphology

At the 12-hour timepoint in each exposure, the bacteria were transferred to fresh LB with antibiotic. At this point, the time required for the bacterial culture to reach an OD600 of 1.0 was determined using an Eppendorf Biophotometer (Hamburg, Germany). Growth curves were generated for the original, untreated fourteen-day culture and antibiotic adapted strain from frozen stocks. Frozen stocks were grown overnight in LB and then 50 uL were transferred to

fresh LB. 7.5 μg/mL of cephalothin was added to all adapted culture media. Growth was determined at OD_{600} every 30 minutes using the Eppendorf Biophotometer.

Changes in colony morphology were determined by quadrant streaking on LB agar plates. To determine cellular morphology, overnight bacterial cultures were centrifuged at 5,000 rpm for 5 minutes and re-suspended in phosphate-buffered saline (PBS) (pH 8.0). Samples were then diluted in PBS to an optical density of approximately 1.0 OD_{600}. Cells were negative stained with 1% Nigrosin and visualized by brightfield microscopy at 1,000X with an oil immersion lens.

Determination of MIC50

Broth microdilution assays were used to determine the minimum inhibitory concentration (MIC) of cephalothin, tetracycline, and amikacin. 96 well plates were seeded with LB broth containing 10^3 CFU/mL starting concentration. Antibiotics were added in a two- fold serial dilution starting at 500 μg/mL. Cultures were incubated overnight, and growth was measured at OD600 using a Biotek Gen5 plate reader (Winooski, VT). Wells containing only LB and bacteria were used as controls for normal growth. MIC50 was determined as the lowest antibiotic concentration at which 50% of the untreated sample optical density was observed. All samples were tested in triplicate.

Genomic DNA Isolation

Genomic DNA was extracted using a protocol modified from Wright et. al. (12). Briefly, 50 mL of overnight culture was pelleted for 10 minutes at 10,000 rpm at 4°C. The pellet was washed twice with TE25S buffer (25 mM Tris-HCl, 25 mM EDTA, 0.3 M sucrose, pH 8.0) and resuspended in TE25S with 10 mg/mL lysozyme and RNAse. The mixture was incubated for two

hours at 37°C with shaking at 150 rpm. Proteinase K and 10% SDS were added and incorporated by inversion and incubated for 1-2 hours at 50-55°C with periodic inversions. 5M NaCl was added followed by 3.25 mL of CTAB (Cetyl Trimethyl Ammonium Bromide)/NaCl. The solution was mixed by inversion and incubated at 55°C for 10 minutes. A 24:1 chloroform/isoamyl alcohol solution was added and incubated at room temperature with shaking at 100 rpm for 20 minutes. After incubation, the solution was centrifuged at10,000 rpm and 4°C for 15 minutes, and the upper aqueous layers were transferred into fresh tubes. This chloroform treatment was repeated a second time. The upper aqueous layers of both samples were combined and an isopropyl alcohol volume of 0.6 of the collected sample was added and the mixture was gently inverted. After five minutes, the purified 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). Purified DNA was quantified by Qubit (Thermofisher).

Genome Sequencing

Endpoint samples of DNA from *K. pneumoniae* 43816, the fourteen-day antibiotic adapted culture, and the fourteen-day untreated population were analyzed using SMRT Cell Sequencing by the National Center for Genome Resources (www.ncgr.org). Samples were compared against the published reference sequence NZ_CP009208.1 for *K. pneumoniae 43816* (13). Genetic variation between genomes was calculated using a modified form of F_{ST} or analysis of variance referred to as Θ (14). F_{ST} values are evaluated against the null hypothesis that the populations are not genetically unique (14). 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 (https://www.migscenter.com) which utilized an Illumina sequencing technique similar to that used by Baym *et. al*. (15). Any variations were analyzed using a proprietary breseq variant calling algorithm (16).

Capsule Extraction and Characterization

Capsular polysaccharide (CPS) was extracted using the protocol outlined by Domineco *et al*. (17). Briefly, 500 µL of an overnight culture were mixed with 100 µL of 1% Zwittergent 3-14 in 100 mM citric acid, pH 2.0. The mixture was vortexed vigorously, incubated at 50°C for 20 minutes, and centrifuged for 5 minutes at 14,000 rpm. The supernatant was then transferred to a fresh tube, mixed with 1.2 mL of absolute ethanol, and incubated for 90 minutes at 4°C. Precipitate was collected after centrifugation at 14,000 rpm for 10 minutes and dissolved in 200µL DI water.

CPS was quantified following a previously established protocol by Lin *et al.*(18) . Purified CPS was vortexed vigorously with 1.2mL of 12.5mM sodium tetraborate in concentrated sulfuric acid and heated for 5 minutes at 95°C. The samples were cooled before the addition of 20µL of 0.15% m-hydroxydiphenyl; and the absorbance was measured at 540 nm. A standard curve was generated using D-glucuronic acid to determine the concentration of glucuronic acid in the CPS samples. To ensure quantification of CPS from the same number of bacteria, strains were normalized to 10^8 CFUs/mL. Each assay was performed in triplicate from six individual cultures.

The carbohydrate composition of the CPS was characterized by GC-MS as previously detailed in Brunson et al (19). Briefly, CPS was purified from LPS using sodium deoxycholate at

a final concentration of 6 mM as described by Kachlany et al. (20). The CPS was pelleted with cold ethanol, then freeze-dried before being hydrolyzed in 0.5 M HCl at 85°C for 18 h. The hydrolyzed carbohydrates were modified with the Tri-Sil HTP reagent (Thermo Scientific, Wltham MA.), as described by York et al. (21). The modified carbohydrates were dried and resuspended in 1 ml hexane. The carbohydrate suspension in hexane was centrifuged at 1000 x g for 5 minutes and the supernatant was collected for GC-MS analysis.

GC-MS analyses were conducted with a CP-3800 GC (Varian, Palo Alto, CA.) using a Supelco SPB-608 30-m fused silica capillary column, containing a bonded stationary phase (0.25 µm film thickness). The TMS (tri-methyl silyl) conjugated glycans were analyzed using the electron ionization mode with a Saturn 2200 GC/MS (Varian, Palo Alto, CA.). The initial oven temperature was 80 °C, held for 2 min. Then, the temperature was raised by 20°C/min and held at 160°C for 12 min. Finally, the oven temperature was raised by 20°C/min and held at 260°C for 7 min.

Quantification of Lipopolysaccharide:

Whole cell lipopolysaccharide levels were quantified using the Purpald assay (22, 23). Briefly stated, cultures were centrifuged at 5,000 rpm for 5 minutes and re-suspended in PBS. 50 µL of this cell solution was treated with 32 mM sodium periodate solution in a 96-well plate and incubated at room temperature for 25 minutes. After this incubation period, a 136 mM purpald solution in 2N NaOH was added, and the plate was incubated for an additional 20 minutes. Following the completion of this step, 64 mM sodium periodate solution was added, and the plate was incubated for 20 minutes. Absorbance was immediately determined at 540 nm and compared to a standard curve of pure lipopolysaccharide isolated from *K. pneumoniae* (Sigma.

St. Louis MO.). This assay was also used for capsule extracted as described above for the glucuronic acid assay. Results are presented with LPS concentrations normalized to 10^8 CFUs.

Results

Experimental design of progressive sub-MIC exposure to cephalothin

This study aimed to evaluate the adaptive genetic changes in *Klebsiella pneumoniae* 43816 upon prolonged but increasing exposure to sub-MIC levels of the common antibiotic cephalothin. *Klebsiella pneumoniae* 43816 was utilized because *Klebsiella* is known for its genomic plasticity and rapid evolution of antibiotic resistance (24, 25). Additionally, the genome of this strain has been sequenced (13), allowing for tracking of genetic changes over the course of the progressive exposure.

The experimental design of this exposure protocol is diagramed in Figure 1. Fresh LB cultures were grown at 37C with shaking and an initial cephalothin concentration of 0.5 μg/mL. After the first 12 hours of culture, a sample was transferred to fresh LB with the same antibiotic concentration and allowed to grow for a second 12-hour period. Therefore, the bacteria were exposed to any one dose of antibiotics for 24 hours in total; at which point a sample was transferred to a culture with an increased concentration of antibiotic. At each step, the antibiotic concentration was increased by 0.5 μg/mL, up to a final exposure of 7.5 μg/mL, well below the CLSI standard for antibiotic resistance which is set at 16 μg/mL (26). As a control, an untreated culture, without antibiotics, was grown in parallel. Frozen stocks were made of all cultures at each 12-hour timepoint and saved for later analysis.

Progressive antibiotic exposure alters *K. pneumoniae* growth and cellular and colony morphology

In order to evaluate the impact of such low antibiotic concentrations, we measured the rate of growth of the culture at the 12-hour transfer mark. As seen in Figure 2A, the antibiotic

exposed culture exhibited a greatly reduced growth rate (time to OD 1.0), indicative of a high stress level. The growth rate of the treated culture slowed greatly once antibiotic concentrations exceeded 4.5 μg/mL; while the growth rate of the untreated culture was not significantly affected over the course of the experiment (Figure 2A). The final adapted culture was able to grow rapidly in 7.5 μg/mL cephalothin and the growth rate of this fully adapted culture was not found to be significantly different from either the originating culture or the untreated culture after fourteen days of continuous growth in LB (Figure 2B).

While the growth rates remained similar, the appearance of the antibiotic treated culture was significantly altered after the progressive exposure experiment. The antibiotic adapted culture exhibited a highly mucoid appearance in liquid culture compared to the clonally related untreated group (Figure 3A). While the viscosity changed, the adapted population did not qualify as a hypermucoviscous strain as indicated by a string test (data not shown).

Differences were also observed in cellular and colony morphology. The antibiotic exposed culture resulted in cells with a greatly elongated, filamentous structure (Figures 3B and 3C). Further plating of the final antibiotic adapted culture resulted in two consistent colony morphologies (Figure 3D), designated small and large colonies. When each colony morphology was isolated, grown in liquid culture and then replated, each of these morphologies consistently reproduced only either the small or large phenotype (data not shown). The fact that colony size was maintained after isolation indicated that the morphological change was heritable.

Progressive antibiotic exposure induces clinical resistance to multiple classes of antibiotics

Changes in the minimum inhibitory concentration (MIC) of several different classes of antibiotic were determined by the broth microdilution assay. The concentration of antibiotic

necessary to depress bacterial growth by half, also called the MIC50, was found to be a more sensitive measure in detecting changes in resistance. As seen in Table 1, the fourteen-day growth period of untreated cells did not greatly impact the MIC50 values against any antibiotic tested. At the end of the fourteen-day progressive antibiotic exposure, the adapted culture exhibited extremely high MIC50 values to cephalothin, indicative of full clinical resistance. However, that the bacteria were only directly exposed to 7.5 μg/mL of cephalothin, which is approximately half the traditional MIC cutoff concentration (26).

To more fully evaluate the impact of progressive sub-MIC cephalothin exposure on the bacteria, antibiotics utilizing different cellular targets were also tested for changes in MIC50. Progressive exposure had little impact on the MIC50 value for the aminoglycoside antibiotic amikacin, but it did induce clinical resistance to tetracycline.

Additionally, MIC50s were determined for the two isolated colony morphologies from the final antibiotic adapted culture. Both the small and large morphologies exhibited clinical resistance to cephalothin, with the large colonies exhibiting extreme MICs above that of either the small or the adapted final culture. Both isolates also exhibited elevated MIC50 to tetracycline; but were more sensitive than the mixed final adapted culture. Amikacin MICs were not significantly changed in either phenotype. These data demonstrate that progressive sub-MIC concentrations of a single antibiotic provide sufficient evolutionary pressure to drive the evolution of clinical levels of resistance to multiple classes of antibiotics.

Whole genome sequencing reveals distinctive genetic changes because of progressive antibiotic exposure

To determine the genetic changes responsible for this antibiotic resistant mucoid phenotype, DNA from the fourteen-day untreated and final adapted cultures 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 (Figure 4). 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 final antibiotic adapted cells (Table 2). Notably, none of these were identified as penicillin binding proteins or other cell wall modification genes highly associated with traditional beta-lactam resistance.

The only mutations that were not 100% fixed in the population at the conclusion of the fourteen-day progressive exposure were nucleotide substitutions in the coding region of the SGNH/GDSL hydrolase family protein and a deletion in the promoter of SGNH/GDSL (Table 2). This may be linked to the emergence of the small and large colony variants. Other genetic changes that were fixed by the 14-day endpoint include deletions in the coding regions of the following: N-acetyltransferase, ABC transporter, ATP binding protein, and DNA recombination protein (RmuC). Additionally, a substitution in the coding region of undecaprenyl-phosphate glucose phosphotransferase was discovered. An insertion was found in a globin coding sequence and a large insert in the *tetR/acrR* transcriptional regulator resulted in an early stop codon. The functions of these genes encompassed cellular processes of signal transduction, energy and metabolite use, capsule formation, and nucleic acid proofreading.

In addition to changes in the coding regions of those genes, six unique mutations were identified in promoter regions of five other genes that were fixed in the adapted population (Table 2). Promoter regions were defined as occurring within 150 bp of a protein coding start site. Synonymous mutations between the untreated and adapted cultures were removed as described above. With exception to SGNH/GDSL, all other genetic changes in promoter regions were fully fixed in 100% of the DNA sampled. Not all genes associated with these promoters have been fully characterized in *Klebsiella pneumoniae.* In those cases, the data about the class of each gene and close homologues is presented. Functions of the coding regions encompassed cell metabolism, signal transduction, and mRNA proofreading.

Genome sequencing allows for correlation of genetic changes and increased MICs

To establish connections between phenotypic and genotypic changes over the course of the fourteen-day experiment, DNA was extracted from bacterial stocks of each day of the progressive antibiotic exposure and sequenced by the Microbial Genome Sequencing Center using an Illumina sequencing method and a proprietary statistical algorithm for variant calling. This methodology identified far fewer mutations than the first round of sequencing, in part because it does not analyze changes to non-coding regions of the genome, such as promoters.

Changes to *rpoB*, *tetR/acrR*, *wcaJ*, and *gndA* were identified using both sequencing methodologies (Table 3). The timepoint of 100% fixation of these changes within the bacterial population could then be mapped to the timeline of changes in growth rate and increases in antibiotic MIC50s. As seen in Figure 5, the emergence of clinical tetracycline resistance can be paired with fully fixed alterations in *rpoB*, and large increases in the cephalothin MICs can be correlated with fixed changes to *tetR/acrR*, and *wcaJ.* The large increase in cephalothin MIC associated with *wcaJ* can also be mapped to the decreased growth rate seen in Figure 2A.

Two separate sets of sequence changes in *wcaJ*, and *gndA* were identified using the Illumina methodology (Table 3). The final adapted culture, which is composed of a mix of large and small colony morphologies, identified sequence changes with only 64% genome coverage. When the two morphologies were sequenced separately, a large deletion that impacted both genes was found in only the small colony variant.

Both the large and small colony variants had mutations in the *rpoB* and *tetR/acrR* transcriptional regulator sequences. The mutation in *rpoB* was 100% fixed in both variants. However, the depth of coverage of the *tetR/acrR* transcriptional regulator in the large colony variant was only 52%. The large colony variant also had a distinguishing marginal mutation call in a c*omEC* family protein that was not found in the small colony variant. The small colony variant exhibited an additional SNP in a *yfiR* family protein that was not identified in the large colony variant. All of the mutations in the small colony forming variant were 100% fixed indicating a more homogenous genomic identity than the large colony forming subpopulation.

Genetic changes in progressive antibiotic adapted cultures are associated with alterations to capsule and LPS

The final adapted culture from this progressive antibiotic exposure experiment exhibited a highly mucoid phenotype indicating that capsule production or composition may be altered. Genome sequencing then identified alterations to *wcaJ*, which is part of the capsule cps operon and initiates production of colanic acid (27). Therefore, we hypothesized that alterations to capsule production and composition may be directly related to the emergence of high concentration cephalothin resistance.

345 To investigate changes in the capsule, extracellular polysaccharide was extracted and
346 quantified by the uronic acid assay. As can be seen in Figure 6A, capsule polysaccharide
347 increases until the fixation of the *tetR/acrR* mutation and emergence of clinical cephalothin
348 resistance. All samples after this point exhibited capsule polysaccharides at levels similar to or
349 below that seen produced by the untreated control culture.

350 Given that the uronic acid assay only detects one sugar moiety within the capsule
351 polysaccharide, other possible components of the capsule were investigated. Extracted capsule
352 from untreated and adapted cultures were found to contain similar levels of sialic acids, which is
353 associated with increased virulence (28) (data not shown). Adapted capsule extracts exhibited
354 reduced total protein content as determined by BCA assay (data not shown); but did have
355 elevated LPS content (Figure 6B). This elevated LPS content was found in both whole cells as
356 well as extracted capsule, indicating high level of outer membrane turnover in the adapted
357 culture. Finally, capsule extract was analyzed by GC/MS to identify new peaks indicative of
358 changes to the sugar composition (Figure 6C). No new peaks were identified indicating that
359 novel carbohydrate changes are not directly associated with the antibiotic resistant phenotype.

360

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Discussion

The goal of this study was to characterize the impact of low concentration antibiotic exposure on the evolution of bacterial resistance. A progressive exposure model was used, in which *Klebsiella pneumoniae* was exposed to slowly increasing sub-MIC concentrations of the antibiotic cephalothin. The final resulting culture had been exposed to a maximum antibiotic concentration of 7.5 μg/mL cephalothin. This final culture exhibited full and extreme clinical resistance to the beta-lactam cephalothin, with MIC values over 125 μg/mL, increased MICs to tetracycline, a highly mucoid appearance, and an elongated cellular morphology. Genome sequencing revealed a series of genetic alterations that could be mapped directly to the emergence of the resistant phenotype. Changes in phenotype and genetic alterations both indicate that alterations in the *tetR* regulator, LPS shedding, and capsule colanic acid synthesis may be directly associated with the resistance phenotype.

The three resistance-correlated mutations occurred in genes *rpoB*, *tetR/acrR*, and *wcaJ*. The increases in resistance occurred in a stepwise manner, similar to the increases in fitness observed in *E. coli* long-term evolution (LTEE) experiments (29–31). LTEE studies also provided estimates for the rapidity of mutation fixation in a constant environment. The *E. coli* population in the LTEE experiments 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 (30, 31). The three resistance-correlated mutations in this present study all fixed within 36-378.9 generations using a generation time estimate from a related *K. pneumoniae* strain of 38-40 minutes (32). While the present study does use incremental changes in antibiotic concentration, it does not counteract that these mutations were acquired rapidly and indicates a fitness benefit to the adapted population.

385 The speed with which these three mutations were acquired also indicates that the sub-
386 MIC concentration of cephalothin in the growth environment provides significant selective
387 pressure. After only 24 hours of exposure, the resistance of the adapted population to tetracycline
388 had the first jump from 1 µg/mL to 8 µg/mL, which correlated with a fixed mutation in *rpoB*.
389 The rapidity of fixation and correlated increase in survivability suggest that this mutation
390 conferred a very high fitness advantage. It is possible that a necessary step in acquiring resistance
391 for this population was mutation in a gene meant to monitor nucleic acid integrity, allowing for
392 further alterations to the genome.

393 The selection window hypothesis holds that sub-MIC level antibiotic exposure should
394 generally increase bacterial mutation rates (7, 8) because higher mutation rates will improve the
395 chances of generating an adaptive genetic change that provides a fitness advantage in conditions
396 of antibiotic stress. Methodologies employed by studies testing this theory have generally
397 identified "mutants" by plating samples of a bacteria exposed to an antibiotic on media
398 inoculated with some other antibiotic substance (4, 9). Mutation rates analyzed using this
399 methodology tend to indicate that lower antibiotic concentrations result in high mutation rates
400 that decline as the concentration of antibiotic nears the MIC (9, 33). However, when whole-
401 genome analysis is incorporated along with mutation accumulation analysis, substitutions and
402 insertions/deletions increased in frequency as exposure to antibiotic increased in *E.coli* (34).
403 Additionally, the rate of mutation in known resistance genes tracked in *P. aeruginosa* isolates
404 exposed to antibiotics and generated conflicting results (11).

405 The present study utilized whole genome sequencing to identify mutations across the
406 genome for the antibiotic-treated and unexposed sample after 14 days of total treatment. The data
407 from SMRT cell sequencing shows a higher number of mutations in the untreated sample

compared to the adapted cohort. As noted by Long et. al., a true mutation accumulation analysis that determines the frequency of genetic polymorphism per generation would require repeatedly passing a bacteria through bottlenecks to mitigate any selective influences (34). The fact that the adapted population in this study was maintained as a whole group when sub-cultured 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 (4, 7–9, 33).

The *rpoB* mutation identified by Illumina sequencing early in the exposure regimen is very close to an alteration detected in the *rmuC* DNA recombination protein in the final sample sequenced using the SMRT cell method. 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.

The protein products of both *rpoB* and *rmuC* interact closely with genomic DNA. RmuC is a regulator which can prevent sequence inversion during replication (35). RmuC has also been identified as a possible multidrug resistance (MDR) gene in other Gram-negative bacterial species (36, 37). However, RpoB is part of the RNA polymerase protein complex and when mutated can inhibit the action of rifamycin in bacteria (38). Mutations in *rpoB* have been linked to rifamycin resistance in *E. coli* (39, 40), and has been identified as a resistance gene in various other classes of bacteria (41). Additionally, the fact that the mutation in a gene tied to nucleic

acid integrity occurs first provides a possible mechanism for further evolution of resistance by increasing the occurrence of replication error mutations in progeny.

TetR regulators are global multi-target transcriptional regulators that affect multiple processes within the cell beyond just efflux pumps (43, 44). Members of this family of regulatory proteins have been show to impact a variety of virulence associated targets including motility, biofilm formation, and osmotic tolerance (42). One member of this family has been directly associated with *ftsZ*, which is known to regulate cell division and bacillus cellular morphology (43). Therefore, it is reasonable for a mutation in a *tetR* type regulator to impact cellular functions and resistance to classes of antibiotics other than tetracycline.

It is interesting that such a large insert and formation of a stop codon would be found in a gene known for resistance to tetracyclines when the adapted population of this study was exposed to a beta-lactam. The development of cross resistance among bacterial populations exposed to one class of antibiotic is not uncommon. Exposure to environmental chemicals such as surface antiseptic chlorhexidine, or veterinary antibiotics tilmicosin and florfenicol have been shown to create cross-resistance in human pathogens to different classes of antibiotics (3, 5).

The genetic alterations associated with the largest increase in beta-lactam resistance were mapped to two adjacent genomic locations. The SMRT method identified an uncharacterized undecaprenyl phosphate-glucose phosphotransferase, which is very close to the mutation found in *wcaJ* by Illumina sequencing (Table 2, Table 3). The second round of sequencing specified that there was a large deletion encompassing the end of the *wcaJ* and beginning of the *gndA* genes which was exclusive to the small colony forming subpopulation.

In *Klebsiella pneumoniae*, *wcaJ* is part of the capsular cps operon and initiates production of colanic acid (27, 44). Absence of *wcaJ* has been linked to increased resistance to phage treatment, decreased virulence in live murine models, and increased phagocytosis by macrophages (27). Studies ablating *wcaJ* in *Klebsiella pneumoniae* result in a nonmucoid phenotype while increasing biofilm production and increasing resistance to polymyxin (44).

Our observation of altered cellular morphology may be related to envelope stress and remodeling. Beta-lactam exposure has been previously documented to induce the formation of filamentous bacterial cells (45). Additionally, Kessler *et. al* demonstrated altered cell shape in *wcaJ* knockouts that exhibited accumulation of periplasmic colanic acid precursors and activation of the Rcs osmotic stress response pathway (46).

Colanic acid polymers can be covalently linked to lipopolysaccharides (47). We observed increased LPS content in our extracted capsule polysaccharides, indicating that the *wcaJ* mutation may trigger increased membrane turnover. The ability to synthesize LPS O-antigen sugars has been documented to directly impact colanic acid synthesis (48). It is therefore reasonable to hypothesize that alterations in *wcaJ*, may likewise impact LPS synthesis, modification, and turnover. Studies of more general osmotic stress indicate the possibility of a shift from O-antigen attachment to colanic acid attachment (47).

Together, these data indicate that low level beta-lactam exposure initiates a cascade of modifications to the outer envelope and capsule polysaccharides that warrants further investigation. Studies examining single knockouts of either *tetR* or *wcaJ* did not find associated changes in beta-lactam MICs (42)(46). Mutations in *rpoB* have been associated with beta-lactam resistance, but not resistance to multiple classes of antibiotics (49). It is therefore highly likely that a combination of multiple mutations is required to achieve a multi-drug resistant phenotype.

Our final adapted culture represented a heterogeneous mixture of two colony morphologies, which was reflected in the genome sequence analysis. 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 increased sensitivity to polymyxin. Similarly, *gndA* is a gene within the cps locus responsible for the K2 serotype and capsule formation of *K. pneumoniae* (50, 51). It therefore seems likely that the mucoid phenotype seen in the fourteen day adapted population whole-group mixture is due to the large colony subpopulation only. This is supported by the fact that the small colony subpopulation has a reduced resistance to cephalothin compared to the large colonies which has an intact *wcaJ* sequence.

In summary, the progressive antibiotic exposure experiment resulted in a mixture of bacteria exhibiting multiple genomic changes. The multiple isolates from this experiment resulted in bacteria that were resistant, rather than tolerant of the antibiotic, as they exhibited normal metabolic activity and growth in the presence of the antibiotic (52). While both the large and small variants isolates exhibited elevated MICs to cephalothin, the mixture of these isolates demonstrated a synergistic protective effect. This mixture is reflective of what would occur in the environment, where individual cells may independently evolve, persist, or assist in the survival of nearby cells. The bacteria in this study achieved clinical resistance without the traditional acquisition of a beta-lactamase gene. These compensatory mutations, and combinations of them, warrant further investigation as they may accelerate and enhance resistance associated with traditional horizontal gene transfer.

Funding and Acknowledgements

This research was supported by funding from UNF to T.N.E., including a Dean's Council Fellowship Award, a Research Enhancement Plan Award, and the Transformational Learning Opportunity Program. We thank Frank Smith for assistance with bioinformatics software, and all members of the Ellis lab for thoughtful input and comments.

503 **Figure Legends:**

504 **Figure 1: Experimental design**. *Klebsiella pneumoniae* 43816 was exposed to increasing
505 concentrations of cephalothin in LB media over a continuous 14-day period. Each single dose
506 exposure lasted 24 hours and included a sub-culture into fresh media with antibiotics at the 12-
507 hour mark. Frozen bacterial stocks were made every 12 hours for subsequent analysis. A control
508 culture was also grown and sub-cultured in LB media without antibiotics for 14 days.

509

510 **Figure 2: Changes in growth properties as a result of low dose antibiotic exposure**. A)
511 Measurement of time required for adapted and untreated *K. pneumoniae* 43816 sub-culture to
512 reach an OD600 of 1.0 after 12-hour exposure to new antibiotic dose. B) Growth curves of
513 original *K. pneumoniae* 43816, 14-day untreated, and 14-days adapted cultures.

514

515 **Figure 3: Changes in cell, liquid culture, and colony morphology as a result of antibiotic**
516 **exposure.** A) Broth culture appearance of original stock and adapted culture after 14-day
517 antibiotic exposure. Negative stain (1000x) of cell morphology of untreated culture (B) and
518 adapted culture (C) showing elongated cell morphology. D) 14 day adapted culture on LB agar
519 culture showing two different colony morphologies.

520

521 **Table 1: Changes in minimum inhibitory concentration (MIC) of *K. pneumoniae* 43816**
522 **cultures after progressive low dose exposures**. Broth microdilution assays (n=5) determined
523 the MIC 50% of three different antibiotics for endpoint cultures and endpoint colony
524 morphology isolates. * indicates values that exceed the CLSI breakpoint for clinical resistance
525 (26).

Figure 4: Genomic changes triggered by low dose cephalothin exposure. Venn diagram of non-synonymous SNPs, insertions or deletions identified by SMRT Cell genomic sequencing of cultures growth for 14 days with or without low dose antibiotic exposure.

Table 2: Non-synonymous single nucleotide polymorphisms and genomic changes identified in 14 Day adapted culture. SMRT Cell genomic sequencing was used for whole genome sequencing. The genome sequence of *K. pneumoniae 43816* was used as the reference genome.

Table 3: SNPs and genomic changes identified in large and small colony subpopulations of 14 day adapted culture. Illumina genomic sequencing was used for sequencing of colony subpopulations. The genome sequence of *K. pneumoniae 43816* was used as the reference genome.

Figure 5: MIC breakpoints map to onset of clinical resistance and fixation of genomic changes. Broth microdilution assays (n=3) determined MIC breakpoints of cultures over 14-day progressive exposure. Arrows indicate jumps above clinical resistance breakpoint and fixation of specific genetic changes as identified by Illumina whole genome sequencing.

Figure 6: Onset of clinical resistance coincides with changes in quantity and composition of capsule and LPS on outer bacterial surface. A) Total capsule production quantified by glucuronic acid over the course of 14-day progressive experiment. B) LPS content of whole cell or extracted bacterial capsule determined by the Purpald Assay. C) GC-MS analysis of the carbohydrate composition of capsules extracted from untreated and adapted cultures.

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684

Table 1: 50% Minimum Inhibitory Concentration (MIC50) of *K. pneumoniae* 43816 cultures after progressive low dose Exposure

Antibiotic	*K. pneumoniae* 43816	Day 14 Untreated	Day 14 Adapted	Adapted Large Colony	Adapted Small Colony
Tetracycline	0.5 µg/mL	1 µg/mL	16 µg/mL*	8 µg/mL*	8 µg/mL*
Cephalothin	4 µg/mL	4 µg/mL	125 µg/mL*	500 µg/mL*	125 µg/mL*
Amikacin	0.5 µg/mL	1 µg/mL	2 µg/mL	0.5 µg/mL	0.5 µg/mL

* Indicates MICs above the CLSI Cutoff for clinical resistance

Table 2:Non-synonymous Single Nucleotide Polymorphisms and Genomic Changes Identified in 14 Day Adapted Culture

Position	Change in Sequence	Depth of Coverage	Gene/Promoter	Gene Family
10279	G→C	43	Gene	SGNH/GDSL hydrolase
10285	G→C	43	Gene	SGNH/GDSL hydrolase
10412	T Deletion	56	Promoter	SGNH/GDSL hydrolase
412527	G Deletion	100	Gene	N-acetyltransferase
1125537	Insertion T	100	Gene	Globin
2008934	Insertion TTTCGCTA	100	Gene	TetR/AcrR Transcription regulator
2063994	C Deletion	100	Gene	ABC Transporter
3225592	C Deletion	100	Gene	RmuC DNA Recombination
5188116	T → G	100	Gene	UDP Phosphate Glucose Phosphotransferse
2657272	G Deletion	100	Promoter	Peptide Chain Release Factor 3
1078904	C Deletion	100	Promoter	LysR Transcriptional Regulator
1294495	G Deletion	100	Promoter	Type II Aspariginase
1823692	A Insertion	100	Promoter	Pyrimidine Photo-lyase
5050494	A Insertion	100	Promoter	Cyclic Di-GMP Phosphodiesterase

Table 3: SNPs and Genomic Changes Identified in Large and Small Colony Variants of Adapted Culture

Colony Variant	Position	Original	New	Coverage (Small/Large)	Gene
Small and Large	2,008,948	.	Insert TATTTCGC	100/52	TetR/AcrR family transcriptional regulator
Small and Large	3,191,984	A	T	100/100	*rpoB*/DA-directed RNA polymerase subunit beta
Large	1,548,640	Unspecified	Unspecified	15	ComEC family protein
Small	4,676,685	T	G	100	YfiR family protein
Small	5,187,773	Deletion 2,103 bp.	.	100	*wcaJ* and *gndA*
Final adapted mixture	5,187,772	T	G	64	*wcaJ U*ndecaprenyl phosphate-gluocse phosphotransferase
Final adapted mixture	5,189,876	unspecified	unspecified	64	*gndA* NADP-dependent phosphogluconate dehydrogenase

Figure 1:

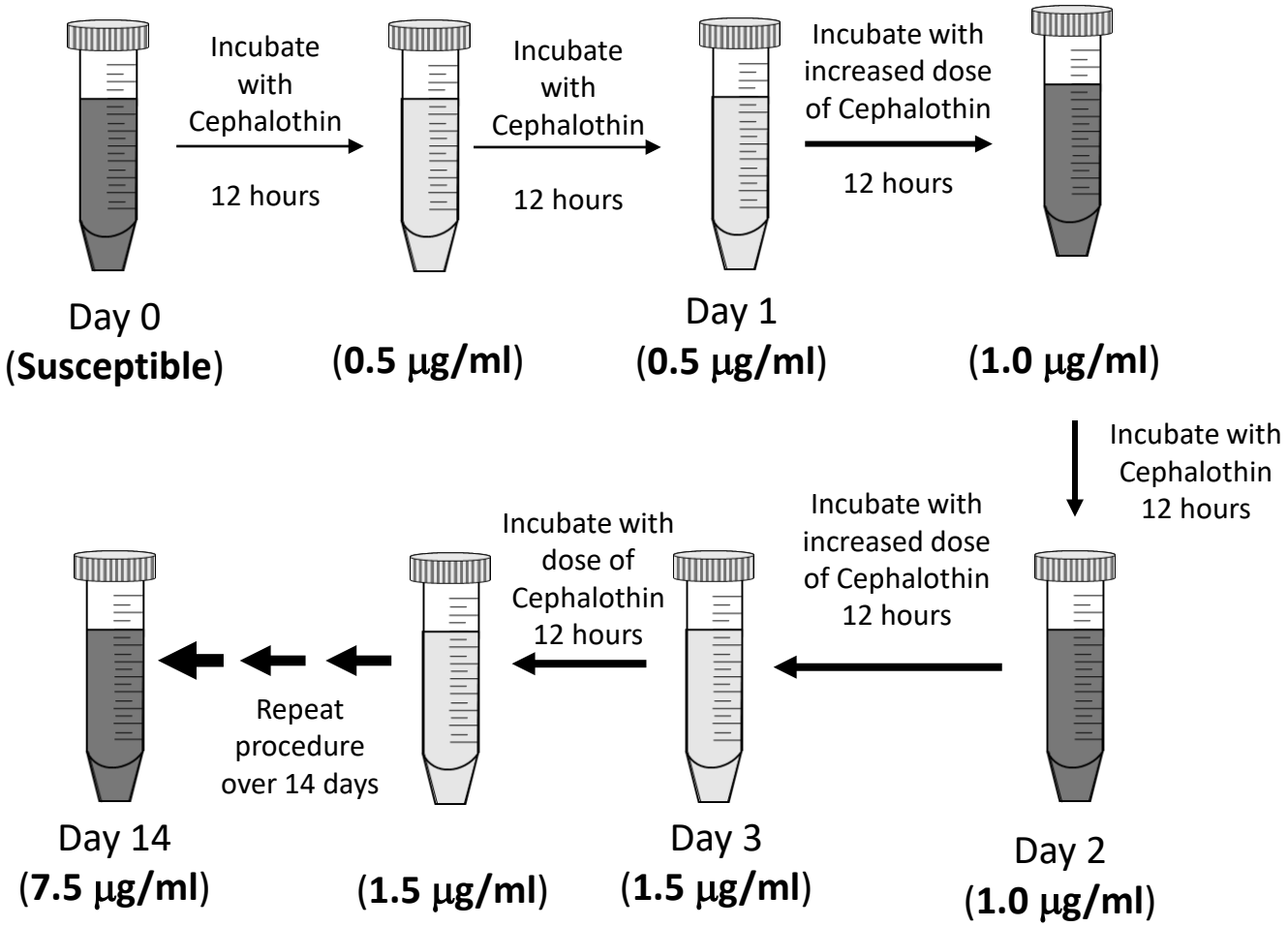
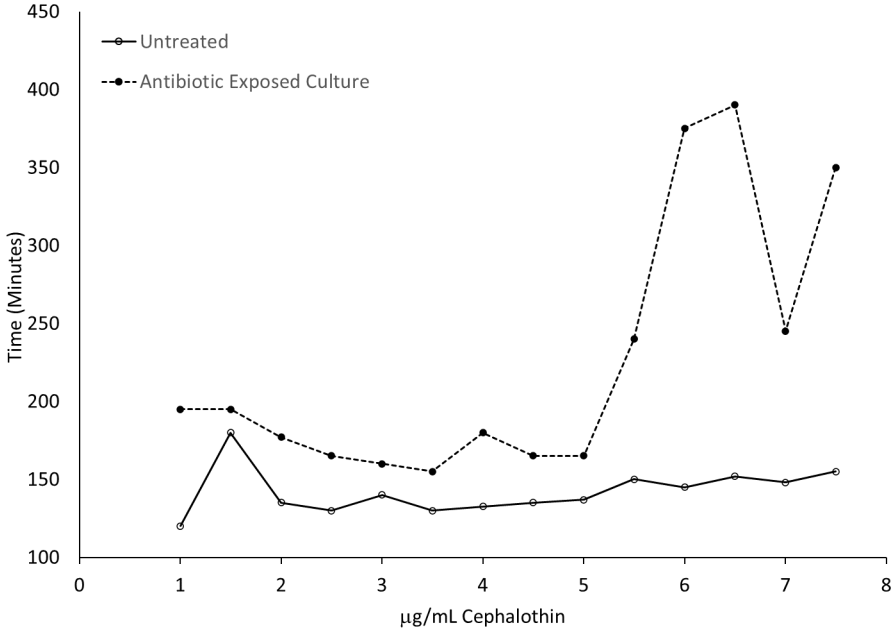

Incubate with Cephalothin 12 hours
Incubate with Cephalothin 12 hours
Incubate with increased dose of Cephalothin 12 hours
Incubate with Cephalothin 12 hours
Incubate with increased dose of Cephalothin 12 hours
Incubate with dose of Cephalothin 12 hours
Repeat procedure over 14 days
Day 0 (Susceptible)
(0.5 μg/ml)
Day 1 (0.5 μg/ml)
(1.0 μg/ml)
Day 2 (1.0 μg/ml)
Day 3 (1.5 μg/ml)
(1.5 μg/ml)
Day 14 (7.5 μg/ml)

Figure 2:

A.


Untreated
Antibiotic Exposed Culture
Time (Minutes)
µg/mL Cephalothin
450
400
350
300
250
200
150
100
0
1
2
3
4
5
6
7
8

B.

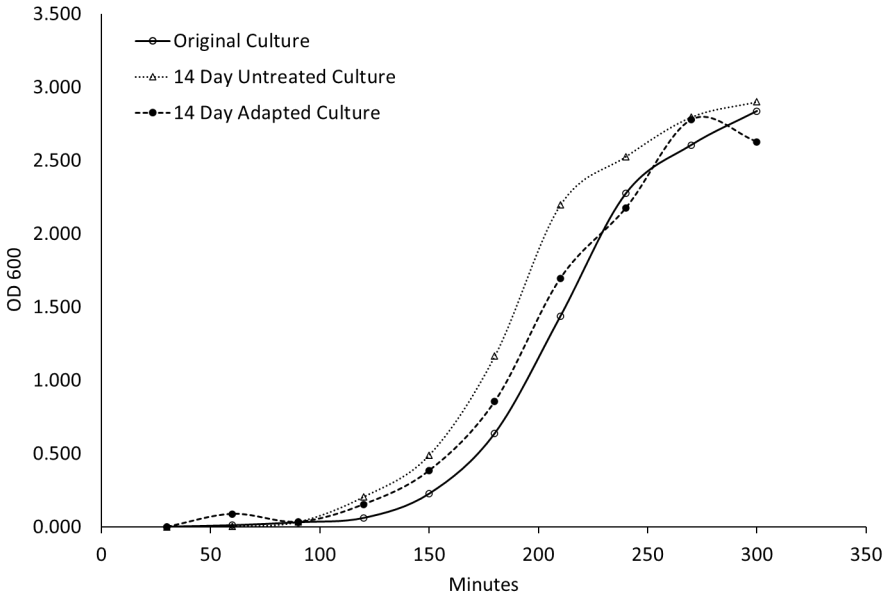
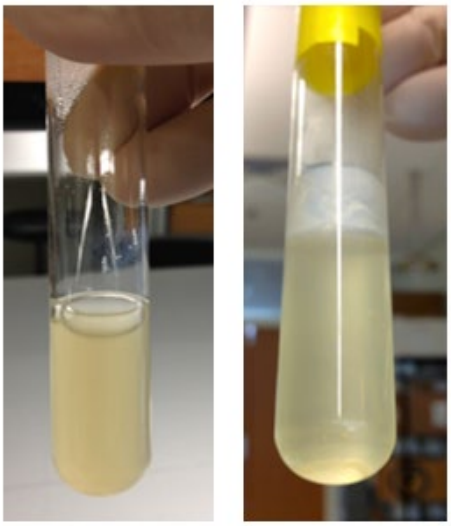

Original Culture
14 Day Untreated Culture
14 Day Adapted Culture
OD 600
Minutes
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0
50
100
150
200
250
300
350

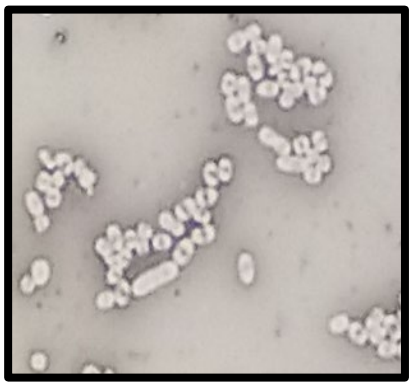
Figure 3:

A.

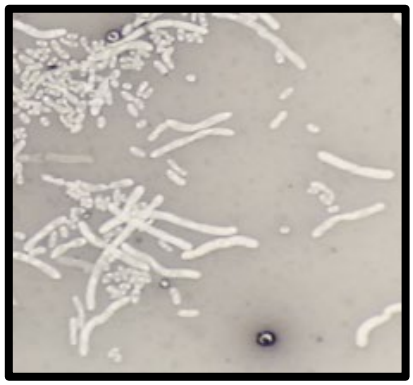


Untreated Culture **Adapted Culture**

B.



C.



D.

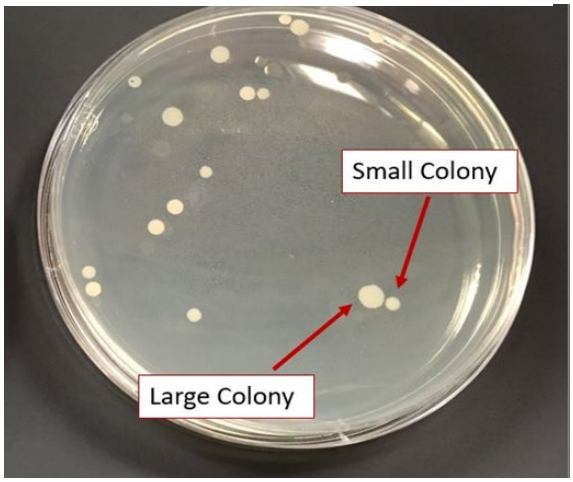


Figure 4:

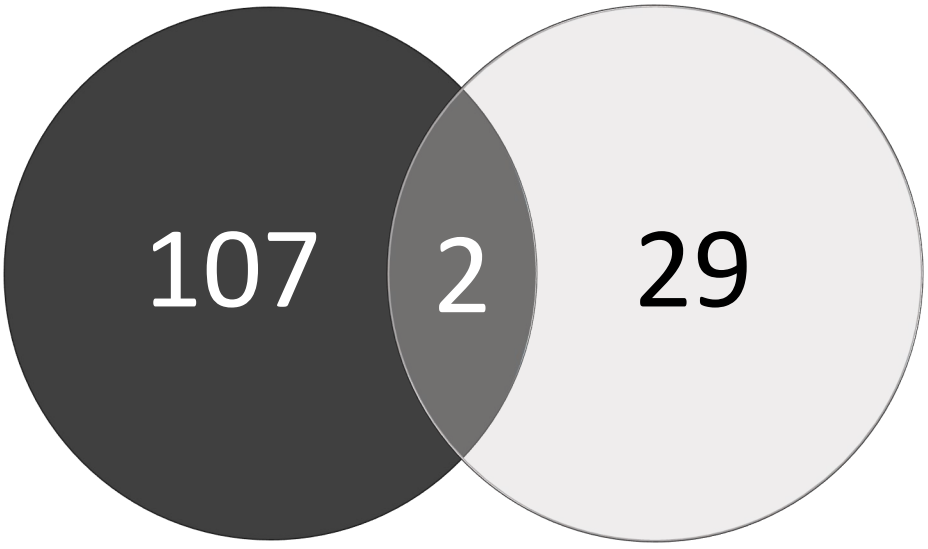

107
2
29
Cultured 14 days
No antibiotics added
Cultured 14 days
Progressive Cephalothin exposure

Figure 5:

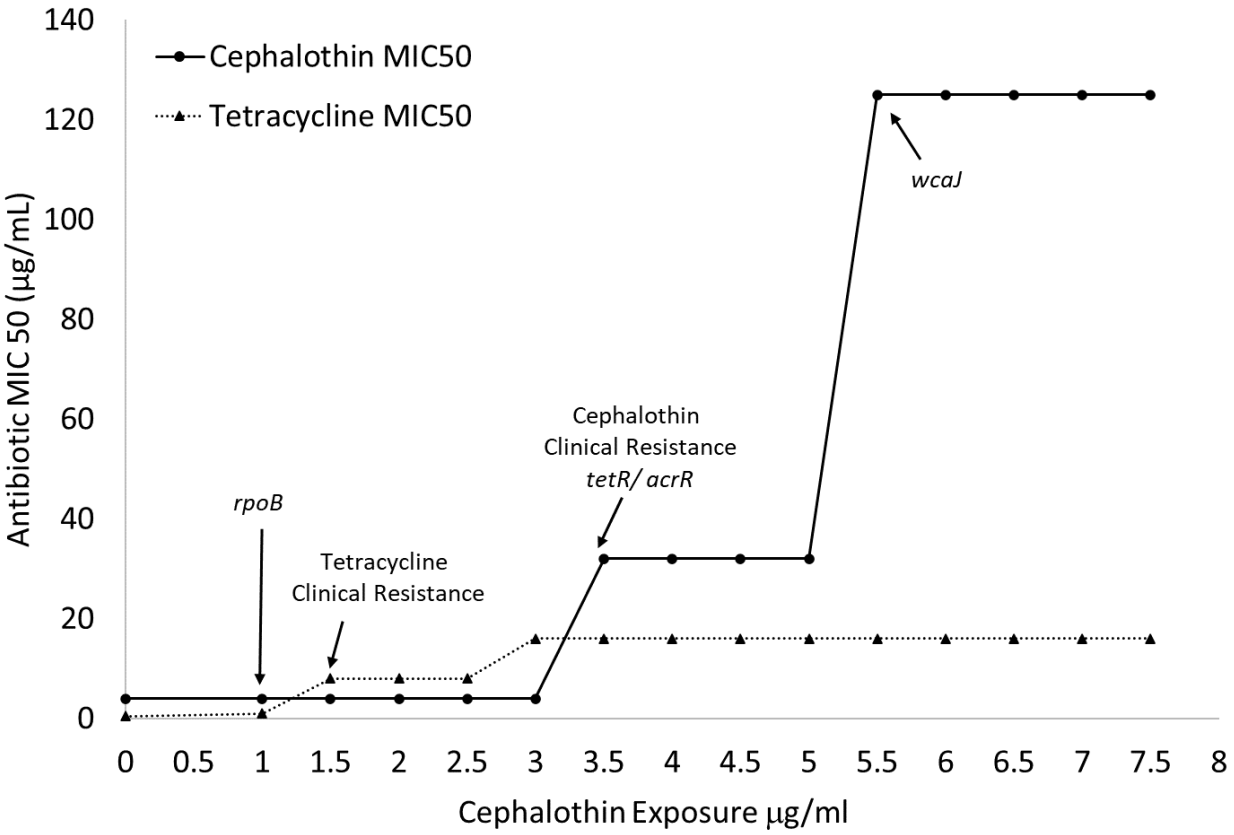
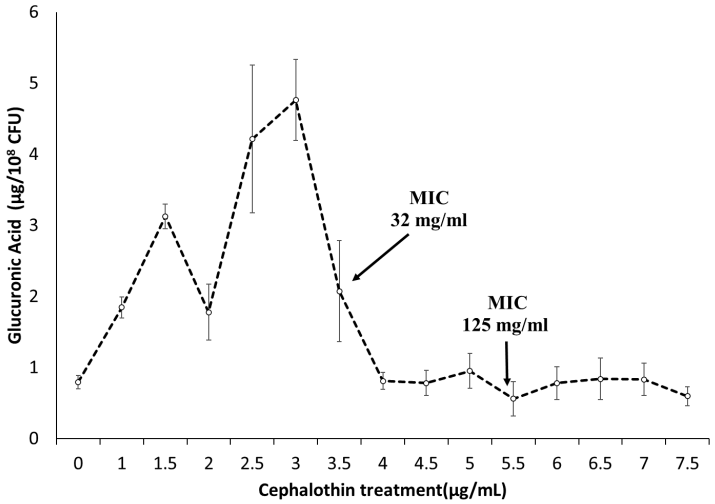
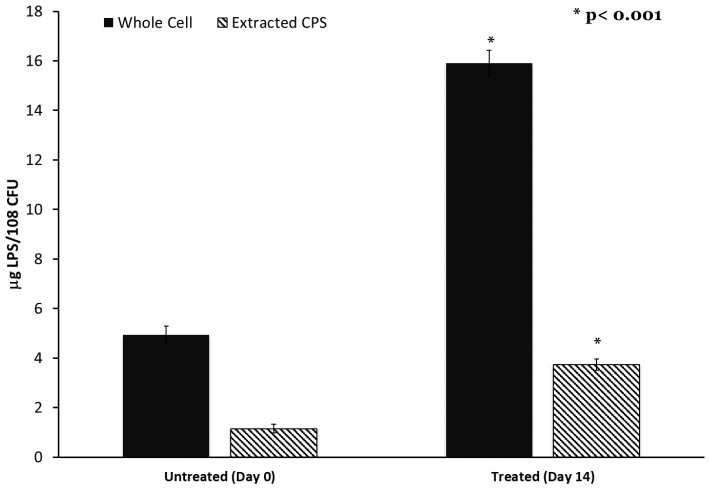

Cephalothin MIC50
Tetracycline MIC50
Antibiotic MIC 50 (µg/mL)
140
120
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
Cephalothin Exposure µg/ml
rpoB
Tetracycline
Clinical Resistance
Cephalothin
Clinical Resistance
tetR/ acrR
wcaJ

Figure 6:

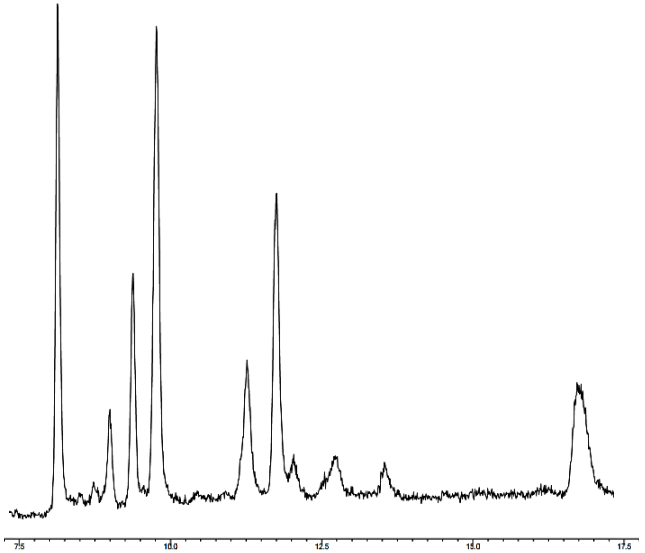
A.



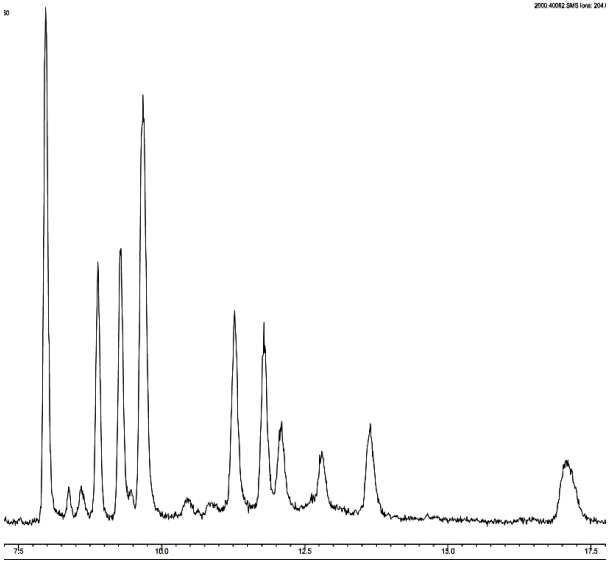
B.



C.



Untreated



14 day Adapted Culture