ADAPTIVE RESPONSE TO THE ANTIBIOTIC FLORFENICOL PRODUCES "SUPER RESISTANT" STRAINS OF SALMONELLA

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Honors Thesis
***********************
PASS WITH DISTINCTION
Précis

Food-borne bacterial infections usually arise from under-cooked contaminated animal food products or by contact with contaminated surfaces. While these infections are usually short-lived and are relatively harmless beyond the discomfort of diarrheal illness, the long-term consequences can be more severe. For example, a *Salmonella* infection (salmonellosis) can produce joint pain, painful urination, or eye irritation and can last for months or years (CDC, 2010). Some individuals develop chronic arthritis and, if immunologically compromised, may die as a result of the infection. The good news is that antibiotics, in people and livestock alike, can be used to successfully treat infections like salmonellosis. Nevertheless, liberal use, or misuse, of antibiotics has been widely criticized for its possible contribution to selection of antibiotic resistant bacterial strains (Scharwz, S., and Chaslus-Dancla, E., 2001). As bacterial resistance becomes more common and our antibiotic arsenal becomes less potent, our ability to combat infections diminishes. Thus bacterial resistance to antibiotics is of major concern for both veterinary and human medicine and is a chief area of study.

*Salmonella enterica* strain AM04528 was isolated from a human case of salmonellosis in Kansas (1998). This bacterium is resistant to a plethora of antibiotics, including florfenicol, which is used exclusively in veterinary medicine. Resistance to florfenicol also conveys resistance to chloramphenicol, which is used in humans. Most resistant bacteria exhibit a threshold where growth above a certain concentration of an antibiotic is impossible, despite the bacteria’s resistance. This is known as the “minimal inhibitory concentration” (MIC). AM04528 stops growing at a typical MIC concentration of florfenicol. However, we observed that after exposure to lower concentrations of
florfenicol, the bacteria become “pre-conditioned” and are subsequently able to grow in much higher concentrations (> 1 mg/mL). We hypothesized that the increased resistance was a product of up-regulation of the florfenicol resistance gene, floR, and florfenicol’s target, the 23S component of the bacterial ribosome. This study examines the expression of floR under excessive concentrations of florfenicol and searches for secondary modes of resistance to better understand bacterial antibiotic resistance mechanisms.

The specific aims were: 1) to determine if floR is up-regulated proportional to florfenicol concentration; 2) to analyze the expression of 23S ribosomal RNA during exposure to florfenicol; and 3) to determine if other non-efflux resistance mechanisms contribute to resistance.

To address our first aim, we analyzed floR expression using both microarray and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Results from both of these techniques indicate that floR is up-regulated in the presence of florfenicol. The microarray analysis also identified several differentially regulated hypothetical proteins that could play roles in aiding resistance. Using semi-quantitative northern blots we found no significant up-regulation of the 23S ribosomal RNA. Finally, we used the general efflux pump inhibitor Phenyl-Argenine-β-Naphthylamide Dichlorohydride (PAβN) and found no evidence of other non-efflux pump resistance mechanisms in this system.

Collectively, we were able to confirm that up-regulation of floR is consistent with resistance but regulation appeared to be ‘on’ or ‘off.’ Any contribution from secondary non-efflux resistance mechanisms was also ruled out. It remains to be determined if this super resistant phenotype contributes to antibiotic resistance during clinical treatment.
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Introduction

Salmonella enterica can be found in the digestive tracts of all land vertebrates. Pathogenic S. enterica attacks the host gastrointestinal system and, in humans, induces a diarrheal illness commonly referred to as ‘salmonellosis.’ This illness affects an estimated 1.4 million Americans annually, only 40,000 of which are confirmed cases reported to the Centers for Disease Control and Prevention (CDC, 2010). Symptoms of salmonellosis last from four to seven days during which the infected person will experience abdominal cramps and fever accompanied by diarrhea. The condition is usually self-limiting but can have long-term impacts; some Salmonella infections have been known to result in joint pain, eye irritation, and painful urination. These symptoms, collectively known as Reiter's syndrome, may last for several months or years and can also lead to chronic arthritis (CDC, 2010). Salmonellosis can also progress to bacteremia for immunologically compromised patients (Raffatellu et al., 2008). S. enterica infections in humans often originate from contaminated animal products such as poultry, eggs, beef, and milk, but any food, such as vegetables, may become contaminated (CDC, 2010).

Antibiotics are often used to combat bacterial infections in animals and in humans. In veterinary medicine, antibiotics are also used 1) to prevent or combat bacterial infections in herds or individuals (prophylaxis), and 2) to promote animal growth (Scharwz, S., and Chaslus-Dancla, E., 2001). Both practices have been criticized for their possible contribution to the selection of antibiotic resistant microbes, making it more difficult to treat important infections such as salmonellosis (Scharwz, S., and Chaslus-Dancla, E., 2001).
Antibiotic resistance is encoded by genes that can be found on the bacterial chromosome or on horizontally transmissible extrachromosomal DNA known as plasmids. Resistance is mediated by various mechanisms, such as enzymatic inactivation or decreased intracellular accumulation of the antibiotic, and protection or alteration of the antibiotic’s target (Butaye et al., 2003). Strain AM04528, a *Salmonella enterica* serovar Newport (*S. Newport*), harbors a plasmid that is roughly 160,000 base pairs (bp) in size (Call et al., 2010). This plasmid, pAM04528, contains a number of antibiotic resistance genes, including *floR*, which encodes an efflux pump that confers resistance to the antibiotics florfencicol (FF) and chloramphenicol. FF was introduced to veterinary medicine as a broad-spectrum antibiotic for clinical use in the mid-1990s. FF is a bacteriostatic antibiotic that binds to the 23S component of the bacterial 50S ribosomal subunit, rendering protein formation impossible (White, et al., 2000). FF is clinically ineffective against bacterial strains that harbor *floR* or similar genes (Schwarz et al., 2004). Efflux mediated resistance, such as that encoded by *floR*, works to decrease intracellular accumulation of the antibiotic by exporting the antibiotic from the cell. This task is completed via an energy-dependent process (Butaye et al., 2003). Without a resistance gene *S. enterica* will not grow in concentrations ≥16 μg/mL FF. While determining the minimum inhibitory concentration of FF, we studied strain AM04528 and found that it will grow in media containing up to 200 μg/mL FF. During these experiments we observed that when AM04528 is grown in higher (> 50 μg/mL and < 200 μg/mL) concentrations of FF, growth may not appear until up to 24 hrs after inoculation. When breakout strains of AM04528 are serially passaged from low to increasingly higher concentrations of FF (up to 1200 μg/mL; known as “super
resistance”), growth occurs but only if this process involves a stepwise increase in concentration. While we have not conducted an exhaustive survey, super resistance to FF only occurs when $floR$ is present.

There are several approaches to exploring the mechanisms involved in AM04528’s resistance to FF. First, gene expression can be analyzed through methods such as reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR), semi-quantitative northern blots, or microarray analysis. Each of these techniques relies on analyzing the relative copy number of RNA transcribed from the gene of interest. Microarray analysis provides a general overview of gene activity throughout the entire organism, as it comparatively evaluates RNA for many genes. First, RNA is extracted from bacteria grown in a condition of interest. This RNA is then converted into single-stranded DNA called cDNA, which is labeled with a fluorophore dye and then hybridized onto a glass slide that includes DNA probes corresponding to the genes of interest. The resulting hybridized slide is then imaged where fluorescent intensity is correlated with the amount of cDNA present. This method is very helpful in identifying multiple genes that are differentially regulated under the same condition.

RT-qPCR provides a much more quantitative and precise measure of gene expression. Like microarray analysis, RT-qPCR relies on extracted RNA converted to cDNA. However, differences lie in the analysis of the transcript copy number. First, oligonucleotide primers are designed to amplify a small target segment of the gene. Taking reaction efficiency into account, a smaller amplification product is more reliable than amplifying the entire gene. A product that is too large sacrifices efficiency while a product that is too small forgoes specificity. Thus, a combination of the two is required to
generate a reliable assay. These primers are then used in a polymerase chain reaction (PCR) to amplify the targeted cDNA template. Amplification occurs in cycles that consist of three events: denaturing, annealing, and extension. In denaturing, the temperature of the reaction mixture is raised to 94°C and the double stranded DNA template is separated as the hydrogen bonds between the two strands break. The temperature is then lowered (around 55°C) to allow the primers to anneal to the single stranded template. At 72°C a DNA polymerase binds to the primer and synthesizes a complementary strand of DNA. This cycle is repeated 30 times in an exponential process of amplification (>1×10⁹ copies in a perfect reaction). During each amplification cycle, a fluorescent dye (SsoFast Evagreen, BioRad) intercalates into the double-stranded product. When hit by light of a specific wavelength (excitation), the dye fluoresces (emission) and the resulting fluorescence value is recorded. The fluorescence values are directly proportional to DNA copy number and can be used to calculate the amount of template present at the beginning of the reaction (Sigma-Aldrich, 2008).

The third method, semi-quantitative northern blotting, also looks at gene expression but by examining the RNA. This technique involves running RNA samples through formaldehyde denaturing agarose gels to separate the RNA by size using electrophoresis. The RNA is then transferred to a solid membrane called a ‘blot.’ The blot is then hybridized with a digoxigenin-UTP (DIG) probe designed to anneal to the RNA segment of interest. The probe can be detected in different ways. We used an immunodetection technique that binds an alkaline phosphatase conjugated antibody to the probe (α-DIG) and thus allowed us to visualize with a colorimetric substrate (Roche Applied Science, 2003). Genes expressed at different levels will appear on the blot as
having different densities. Band density is analyzed using computer software, such as ImageJ (Wayne Rasband, 2011). Each of these methods was utilized in examining gene expression of AM04528 while in the presence of FF.

Growth above what was previously the inhibitory concentration of FF is an intriguing and perplexing finding. Understanding the mechanisms behind this increased resistance to FF may serve to help us better understand antibiotic resistance mechanisms and their regulation. This greater understanding offers the possibility of helping us combat antibiotic resistance and develop superior antibiotics. Thus, the aim of this research project is to determine what permits the S. Newport strain AM04528 to gain enhanced resistance to FF.

**Hypothesis**

The objective of this project was to determine the basis for *Salmonella enterica*’s capacity to withstand greater than inhibitory concentrations of FF. We hypothesized that increased resistance arises from alterations in the expression of one or more of the following: the *floR* gene, 23S rRNA (the target of FF, a ribosomal structure necessary for normal cell function), or other non-efflux resistance mechanisms. The specific aims were:

1. Determine if *floR* is up-regulated during exposure to FF.
2. Analyze the expression of the 23S ribosomal RNA during exposure to FF.
3. Determine if other non-efflux resistance mechanisms contribute to resistance.
Methods and Materials

Bacterial Strains and Cultures

Strain AM04528 was originally provided by the Centers for Disease Control and Prevention, originating from a case of human salmonellosis in Kansas. An *E. coli* strain harboring plasmid pAM04528 was generated in house by electroporation (Sambrook, 1989) using GeneHog electrocompetent *E. coli* cells (strain DH10B, Invitrogen). The GeneHog strain was used as a comparison to wild-type AM04528 as a control for chromosomally encoded resistance genes that might be present in AM04528.

Evaluating Florfenicol Resistance

Wild-type AM04528 (WT) and pAM04528 GeneHog (GH) strains, not previously exposed to FF, were incubated with constant shaking at 37°C in Luria Bertani media (LB) for 24 hrs to act as “seeder cultures” for growth curve analysis. All incubation periods, unless otherwise noted, were conducted as described above. For preconditioning in FF 50 µg/mL, seeder cultures were grown (inoculated at 1:1000) in LB with a FF concentration of 50 µg/mL. To precondition in FF 200 µg/mL, preconditioned FF 50 µg/mL strains were grown (inoculated at 1:1000) in LB with a FF concentration of 200 µg/mL. Preconditioning at higher concentrations of FF was not performed, as strains preconditioned at FF 200 µg/mL were ultimately able to grow in up to 1200 µg/mL (a concentration greater than 50 times the levels of FF expected in tissue). Growth at concentrations beyond this was not tested.

Cell growth in the presence or absence of FF was measured using optical density at an absorbance of 480-520 nm and was measured using a Bioscreen C spectrophotometer (Growth Curves Ltd.). Both WT and GH strains were grown in the
absence of antibiotic to determine normal uninhibited growth (turbidity). LB with various concentrations of FF was inoculated 1:1000 with fresh cultures (with or without preconditioning). Aliquots (200 μL) of these mixtures were pipetted into Bioscreen 100-well plates. The Bioscreen was then run for a period of 48 to 72 hrs at 37°C, shaking for 5 sec immediately before recording absorbance. Growth-curve data was graphed using Microsoft Excel (Microsoft Corp., Redmond, WA).

**Evaluating floR Expression**

We examined the expression of the floR gene using real-time PCR (also referred to as RT-qPCR). RNA was extracted from these strains, after growth in varying concentrations of FF, using a Qiagen RNeasy Mini Kit (Qiagen). RNA was extracted from three biological replicates at the end of log phase (based on time, not OD). The RNA extracted here was used for each experiment involving RNA use (microarray, RT-qPCR, and semi-quantitative northern blots) to minimize experimental variance. RNA was converted to cDNA with SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was conducted using SSoFast EvaGreen Supermix (Bio-Rad) and an iCycler iQ thermocycler (Bio-Rad). Data was analyzed using REST2009 software (Qiagen and M. W. PFaffl, 2009). Expression data was normalized using the rpoD housekeeping gene (appendix A lists primers).

A promoter study was conducted to analyze the activity of the floR promoter. The promoter region had previously been mapped by Arcangioli et al., 1999. Wild-type AM04528 harboring a vector with Green Fluorescent Protein (GFP, Chalfie et al., 1994) driven by the floR promoter was exposed to various concentrations of FF. Exposure to FF results in transcription and translation of GFP, resulting in increasing levels of
fluorescence, which should correlate with increased activity of the floR promoter and presumably higher floR expression. This experimental design was achieved by cloning the promoter of floR and the coding region of GFP (appendix A). PCR products were digested using BamHI (New England Biolabs) and then ligated together using T4 ligase (Invitrogen). The ligation product was then TOPO TA cloned into the plasmid vector pCR2.1 (Invitrogen). Wild-type AM04528 was transformed with the floR-GFP construct using electroporation methods (the resulting strain is referred to as WT+GFP). Overnight cultures of WT and WT+GFP were grown in defined minimal media (appendix B) with 50 μg/mL of FF and 50 μg/mL of kanamycin, respectively (kanamycin served a selective agent for WT+GFP). Five mL aliquots of minimal media and FF were then inoculated from the overnight cultures and were grown with constant shaking at 37°C. Minimal media was chosen over LB as LB's natural fluorescence saturated the fluorescence reading. Aliquots (200 μL) were taken from the five mL experimental cultures at each time point and loaded into 96-well plates for OD and fluorescence measurements. A Pharos FX Plus Molecular Imager (Bio-Rad) set to an absorbance of 488 nm recorded fluorescence values. Optical density was measured in tandem with fluorescence at an absorbance of 595 nm (using BioTek ELx808 Absorbance Microplate Reader). WT in minimal media only was normalized to its respective OD_{595} and then subtracted (as background fluorescence) from WT+GFP normalized to its respective OD_{595}. This double normalization accounted for culture cell density and natural bacterial fluorescence.

**Evaluating 23S Ribosomal RNA Expression**

Changes in the expression of the 23S rRNA copy number, after growth with varying concentrations of FF, were quantified using semi-quantitative northern blots.
RNA was extracted using methods previously detailed. A TurboBlotter rapid downward transfer system (Whatman) was used for transfer of RNA from formaldehyde denaturing gel matrices onto a nylon membrane. The resultant blot was probed with 23S rRNA and rpoD probes labeled with DIG-dUTP for detection purposes. The probes were generated and labeled in-house using a Roche Applied Science PCR DIG probe synthesis kit (appendix A). The blots were analyzed using image J software (Wayne Rasband, 2011). Band density of 23S was normalized using the expression level of rpoD.

**Evaluating the Contribution of Non-Efflux Mechanisms**

To determine if non-efflux mechanisms contributed to FF resistance, we used an efflux pump-blocking compound. If a super-resistant strain remains resistant to FF (a minimum inhibitory concentration of greater than 100 µg/mL) in the presence of the inhibitor then there is evidence that either the compound does not completely block floR or a non-efflux mechanism may be aiding resistance. The compound Phenyl-Arginine β-Naphthylamide Dihydrochloride (PAβN, Sigma-Aldrich) (Pagès et al., 2005) was used as a general efflux pump inhibitor (EPI). Cell toxicity of PAβN was assayed using WT and GH grown in different concentrations of the EPI. Culture growth was determined using the Bioscreen C spectrophotometer over a period of 48 to 72 hrs at 37°C, with shaking for 5 sec immediately prior to collecting absorbance values. Cell toxicity was minimal and the three highest concentrations of PAβN tested were used in further experimentation (80, 120, and 160 µg/mL). Growth curves were conducted on WT only using the Bioscreen C. Bioscreen plates were loaded with 200 µL mixtures of LB and varying concentrations of EPI and FF. Fresh cultures of preconditioned and non-preconditioned WT were used as inoculants (at 1:1000).
Results

Growth Curves

Over a period from January 2009 to spring of 2010, growth curve experiments were conducted to examine the ability of WT and GH to withstand concentrations of FF. We removed GH from analysis as its response to FF largely mirrored that of WT. All WT data has been summarized according to preconditioning and growth conditions.

Maximum OD reached during growth was taken from each well and averaged to give a representational view of growth for each condition, and can be seen in Table 1.

**TABLE 1: Wild-type Growth Curve Summary**
Strain AM04528 grown for 48 to 72 hours (depending on the experiment) with or without preconditioning and under various concentrations of FF. Data has been summarized from several experiments. Growth is listed as the number of wells that grew over the total number of wells for that condition. Optical density was measured on a Bioscreen C spectrophotometer at an absorbance of 480-520 nm and is listed as the maximum average value across all wells for each condition.

<table>
<thead>
<tr>
<th>Florfenicol (µg/mL)</th>
<th>No preconditioning</th>
<th>Preconditioned in FF 50 µg/ml</th>
<th>Preconditioned in FF 200 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth / Total</td>
<td>Average Max OD</td>
<td>Growth / Total</td>
</tr>
<tr>
<td>0</td>
<td>100% .95 (estimate)</td>
<td>100% .95 (estimate)</td>
<td>100% .95 (estimate)</td>
</tr>
<tr>
<td>50</td>
<td>19/19 .47</td>
<td>29/29 .59</td>
<td>Not Tested</td>
</tr>
<tr>
<td>100</td>
<td>19/19 .38</td>
<td>39/39 .54</td>
<td>Not Tested</td>
</tr>
<tr>
<td>200</td>
<td>19/19 .42</td>
<td>20/20 .38</td>
<td>25/25 .52</td>
</tr>
<tr>
<td>400</td>
<td>0/10 NA</td>
<td>0/10 NA</td>
<td>25/25 .44</td>
</tr>
<tr>
<td>800</td>
<td>0/10 NA</td>
<td>Not Tested</td>
<td>29/25 .38</td>
</tr>
<tr>
<td>1200</td>
<td>0/10 NA</td>
<td>Not Tested</td>
<td>15/15 .41</td>
</tr>
</tbody>
</table>

We can see that preconditioning WT in FF, prior to exposure to higher concentrations of the antibiotic, is extremely effective in increasing resistance and in stepping up the minimum inhibitory concentration. In cultures with no preconditioning we see 100% growth in concentrations of FF up to 200 µg/mL. The same patterns were observed in cultures preconditioned in FF 50 µg/mL; again, these bacteria will grow in concentrations reaching 200 µg/mL FF. We see that the preconditioned FF 50 µg/mL cultures reach higher max ODs than non-preconditioned bacteria. Bacteria preconditioned in FF 200
μg/mL, on the other hand, are capable of growth in much higher FF concentrations, tested up to 1200 μg/mL. This level of preconditioning also demonstrates the ability of the microbe to grow to a greater density compared with lower levels of preconditioning, as well as the stepwise decrease in OD as FF concentration increases.

Typical growth for AM04528 in LB with no selection is rapid, starting just 2 hours after inoculation, and exhibiting normal early and late log-phase growth. Stationary phase is reached after about 18 hours at an optical density of around one. Stationary phase also remains relatively steady until the final OD measurement taken at 36 hours. Comparing growth without selectivity to growth in the presence of FF highlights drastic changes in the growth kinetics. For example, growth starts much later (as much as 20 hours after inoculation) and displays depressed and abnormal log phase activity. Stationary phase is reached after about 24 hours and is often unsteady, as the population appears to spike and die. Maximum OD reached during growth is also affected by FF concentration and preconditioning. The greater the concentration of FF in the media, the lower the maximum OD; but the greater the concentration of preconditioning, the greater the maximum OD achieved. In all, growth is bolstered by previous exposure to FF and permits the bacteria to survive in much higher concentrations of FF.

floR Expression

The main method used to analyze floR expression was RT-qPCR. To accomplish this, RNA was extracted from WT grown in FF 50, 200, and 800 μg/mL, as well as Ampicillin (AMP) 100 μg/mL (a derivative of penicillin, to which AM04528 is resistant). Ampicillin acted as a control for comparison against expression of the floR gene in samples grown in the presence of FF.
FIGURE 1: floR Expression
Data compiled and analyzed using REST2009 software and is represented as a ratio of CT value for floR to rpoD. Each sample is a composite of three biological replicates. Error is represented as the standard error between each biological replicate.

It is clear that floR is up-regulated in FF when compared to AMP (Fig. 1). We encountered enough variation in the 800 μg/mL group to prevent statistically significant differences, but we anticipate that addition of one or two more replicates will show that 100 μg/mL AMP shows significantly lower expression compared to FF exposure. Between FF treatments, there is no obvious difference in floR expression, which is counter to the expected direct correlation with doses. Instead, floR expression behaves more like an on-off signal, at least at the time point tested.

We also analyzed floR expression using a microarray (data not shown). This process relied on the same RNA extracted for RT-qPCR and also indicates up-regulation of floR in samples exposed to FF. Microarray analysis of plasmidic gene expression also indicated consistent up-regulation of another gene, referred to as pAM04528_0024,
located upstream of floR. This hypothetical protein, when examined using NCBI Basic Local Alignment Search Tool (BLASTx), was found to be homologous to genes belonging to a family of sequence-specific DNA binding proteins. The implications of this finding are quite important as they suggest the possibility that pAM04528_0024 is the regulator of floR, although more work is needed to determine if this is the case.

We also examined floR expression using a floR promoter-GFP gene fusion construct. In this experiment, induction of floR should be correlated with the amount of GFP that is produce. When the WT+GFP strain was grown under 50, 100, and 200 µg/mL concentrations of FF, this resulted in consistent expression of GFP. At the 50 and 100 µg/mL concentrations the GFP fluorescence declines indicating a decrease in GFP expression and degradation of remaining fluorescent proteins. This loss of expression occurs when AM04528 reaches exponential growth phase. The 200 µg/mL concentration never reaches a point of expression decrease, as exponential growth is not achieved within the timeline assayed. WT+GFP grown without FF show a steady increase in fluorescence that represents background expression.
**FIGURE 2: Expression of Green Fluorescent Protein**
Relative Fluorescent Units refers to the double normalization of the data as follows: (WT+GFP Fluorescence / OD95) - (WT Fluorescence / OD95). Best-fit lines have been added to illustrate the pattern of fluorescence for each condition. No standard error is available as this experiment was only conducted once.

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**23S Ribosomal RNA Expression**

Up-regulation of FF's target, the 23S component of the 50S ribosomal subunit, was analyzed using semi-quantitative northern blots. Samples from all three biological replicates were run through formaldehyde denaturing gels and then transferred onto nylon substrates. The resulting blots were hybridized with DIG 23S and *rpoD* probes, immuno-detected, and developed using a colorimetric substrate. The largest band that appears on the blot has been sized to be about 2.9 kbp. The second largest band has been sized at 1.8 kbp. These band sizes correspond with expected sizes for 23S and *rpoD*, respectively. Figure three shows a representative northern blot. The 23S and *rpoD* bands are fairly well defined; however there is a great deal of background, indicating possible RNA degradation (Fig. 3). Based on these experiments we were unable to detect significant differences in the copy number of 23S rRNA for any condition or biological replicate...
(Fig. 4). These results indicate that up-regulation of 23S rRNA plays no role in increased resistance to FF.

FIGURE 3: Northern Blot
This is the northern blot of biological replicate #3. The dark top band was sized to be around 2.9 kbp, corresponding to the appropriate size of 23S rRNA. The second dark band was sized to be around 1.8 kbp, corresponding to the appropriate size of the rpoD housekeeping gene.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT – AMP 100 µg/mL</td>
</tr>
<tr>
<td>2</td>
<td>GH – AMP 100 µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>WT – FF 50 µg/mL</td>
</tr>
<tr>
<td>4</td>
<td>GH – FF 50 µg/mL</td>
</tr>
<tr>
<td>5</td>
<td>WT – FF 200 µg/mL</td>
</tr>
<tr>
<td>6</td>
<td>GH – FF 200 µg/mL</td>
</tr>
<tr>
<td>7</td>
<td>WT – FF 800 µg/mL</td>
</tr>
<tr>
<td>8</td>
<td>GH – FF 800 µg/mL</td>
</tr>
</tbody>
</table>

FIGURE 4: 23S Expression
The bar graph below displays the ratio of 23S to rpoD. Each bar is an averaged value from three biological replicates. There were no statistical differences between treatments (P > 0.05).
**Efflux Pump Inhibitor**

Contribution of other non-efflux pump resistance mechanisms was determined by using the efflux pump inhibitor PAβN (Phenyl-Argenine β-Naphthylamide Dichlorohydride). Preconditioned and non-preconditioned cultures of WT were grown in various concentrations of PAβN and FF to evaluate resistance in the presence of the efflux pump inhibitor (EPI). Cultures grown in the presence of EPI only show PAβN to have minimal cell toxicity (Fig. 5). Cultures grown in the presence of FF and PAβN exhibited little to no growth. Most interestingly, WT without preconditioning was unable to grow in media containing EPI and FF. WT preconditioned in 50 μg/mL FF was able to survive at that same concentration of FF in all three concentrations of PAβN, but not when antibiotic concentration was increased to 100 μg/mL FF. Moreover, in preconditioned WT grown at 50 μg/mL we see a stepwise decrease in maximum OD achieved during growth as concentration of PAβN increases. This type of growth is indicative of a dose-response relationship to the concentration of the EPI. Collectively, these results indicate that efflux from floR is most likely responsible for the majority of FF resistance.
FIGURE 5: Efflux Pump Inhibitor Growth Charts
Preconditioned and non-preconditioned cultures of WT pAM04528 were grown in concentrations of EPI and FF. FF and EPI only controls were run as well. Maximum OD (at 72 hrs) is an averaged value of each condition. Error is represented by the standard deviation between technical replicates.

Non-preconditioned Wild-type

Preconditioned FF 50 ug/mL Wild-type
**Discussion**

We have shown that AM04528, once exposed to clinical concentrations of FF, is able to grow in much higher concentrations of the antibiotic. From these preliminary findings, we made five hypotheses as to what could attribute to the super-resistant phenotype, four of which we have tested and rejected.

Our initial hypothesis was that super resistance resulted from a genetic mutation in the promoter or coding region of floR. When super-resistant strains are passaged on LB agar plates they lose the phenotype (data not shown). This loss of preconditioning makes the bacteria vulnerable to lower concentrations of FF again, indicating that gene regulation or expression is involved but it is unlikely that mutations are occurring. To confirm this conclusion we sequenced the floR coding and promoter regions and found no mutations present in the super-resistant phenotype (data not shown).

These findings led to our second hypothesis that preconditioning WT in low concentrations of FF acted to “prime” the system and resulted in consistent floR up-regulation. Results from RT-qPCR showed evidence for up-regulation of floR in WT grown in FF when compared to WT grown in AMP. However, expression of floR appeared to be constant across each concentration of FF, leading us to reject our hypothesis that super resistance corresponded to consistent up-regulation of floR.

To address our third hypothesis, we examined the 23S ribosomal RNA to determine if this target of FF increased in copy number to mitigate for higher drug concentrations. AM04528 would, in addition to actively pumping FF out of the cell, mitigate for FF by producing a molar excess of 23S rRNA molecules. This would permit protein synthesis to continue even though some ribosomes were bound by FF. Our
analysis of 23S rRNA, via semi-quantitative northern blots, indicated no evidence for a change in 23S rRNA copy number. Assuming there were no issues with the analytic sensitivity of our assay, we can reject this hypothesis.

Our fourth hypothesis conjectured that unknown non-efflux pump mechanisms could play a role in super resistance. We assessed this alternative by "subtracting" the contribution of floR and determining if there was any residual resistance. We used Phenyl-Arginine β-Naphthylamide Dihydrochloride, which is a general efflux pump inhibitor, to block FloR function. From this we determined that the majority of resistance to FF is conferred by efflux pump mechanisms. The primary drawback to this experimental design lies in the fact that PAβN inhibits all efflux pumps in a cell. Thus we do not know if there are secondary efflux pumps that aid floR in shuttling FF from the cell. To partially address this, we used microarray analysis in an effort to find additional differentially regulated genes on the AM04528 plasmid. Experimental results from the microarray corroborated our RT-qPCR findings regarding floR expression, and identified a handful of plasmid encoded genes to be differentially regulated (several of which are conserved hypothetical proteins). While no known efflux pump genes were shown to be up-regulated in tandem with floR, one hypothetical protein, identified as pAM04528_0024, was found to be a member of a family of sequence-specific DNA binding proteins. This indicates the possibility that pAM04528_0024 is a transcriptional regulator of the floR gene. We were not in a position to assess the contribution of other chromosomally encoded efflux pumps, but at this point we lack any data to the contrary and we have tentatively rejected the hypothesis.
In eliminating our four original hypotheses, we narrowed the scope of resistance mechanisms that AM04528 could use to achieve the super-resistant phenotype. From our data we can surmise that resistance to FF does not rely on genetic mutational changes, up-regulation of the target, or on constant up-regulation of the floR gene. floR seems to be a constitutively expressed gene whose expression is increased in the presence of FF. The GFP fusion experiment fits with the model of floR expression demonstrated by the RT-qPCR analysis. We see accumulation of GFP in the bacteria until exponential growth is reached. Once this occurs, GFP fluorescence declines to its basal level, indicating a decrease in expression. Taking this into account, our fifth hypothesis is that super-resistance is a product of steady floR transcription leading to accumulation of efflux pumps in the cellular membrane.

Understanding this resistance mechanism could have a major impact on understanding other efflux mechanisms and on the development and implication of new antibiotic treatments. While we do not know the clinical relevance of super resistance, efflux pumps are considered major contributors to antibiotic resistance (Lomovskaya & Watkins, 2001). Some multi-drug resistant bacteria, such as Pseudomonas aeruginosa, are considered to be resistant to a broad spectrum of antibiotics. In the case of P. aeruginosa, it was discovered that the disruption of the MexB efflux pump gene increased susceptibility to several antibiotics (Van Bambeke et al., 2003). This is clear evidence that efflux pump inhibitors can, and should, be taken into account for use in future methods of treatment of antibiotic resistant infections. This could range from dual use of antibiotics with EPIs in treatment to examine the susceptibility of new antibiotics to efflux pumps (Lomovskaya & Watkins, 2001). In addition to this, other studies have
shown antibiotic MIC to increase after serial passages. In some of these cases, after passaging without the antibiotic, increased resistance was not be retained and in these studies efflux pumps were cited as possible contributors (Clark et al., 2011). This is good evidence for efflux pumps conferring increased resistance to other antibiotics in other bacterial strains. Thus, the ramifications of our investigation extend beyond AM04528 and floR, and could impact the way we treat infectious strains of bacteria harboring multidrug efflux pump resistance mechanisms.

**Future Directions**

Variations in RT-qPCR for the current study included an unacceptably high standard error and we believe that this variability results from RNA degradation. It would seem that the stress of high concentrations of antibiotic unavoidably results in deterioration of RNA transcripts. Another key issue, discovered in hindsight, is the variability of the rpoD housekeeping gene. While generally considered a suitable transcript for normalizing results, we believe that rpoD expression is altered under stress from FF. Some of the variance for rpoD is also likely attributed to RNA degradation or, quite possibly, bacterial stress. We know very little about bacterial behavior in such extreme concentrations of antibiotics, as bacteria rarely survive at these levels, and once reliable housekeeping genes could become less steadily regulated as the bacterium struggles to survive. In any case, the simplest solution is to increase the number of replicates to limit the impact of variance on the analysis. It is important to note, however, that based on the fusion protein results, transcript analysis at a single time point could lead to inaccurate conclusions.
To test the final hypothesis we need to determine if the number of efflux pumps differs significantly between different FF treatments. If an antibody were available, this could be done using a western blot or enzyme-linked immunosorbent assay. Unfortunately, antibodies are not commercially available, although it would be possible to generate polyclonal antisera if we are able to produce a recombinant FloR protein. A simpler alternative would be to use a procedure that inverts the cell membrane so that we can separate the cell membrane from the cytosol. Using this approach we may be able to detect differences in total protein for the membrane fraction for the different FF treatments. If total protein in the membrane increases for super-resistant strains, this would be indirect evidence in support of our hypothesis. Finally, we may have unique opportunity to identify the transcriptional regulator for floR based on the microarray data for gene pAM04528_0024. Deletion and gel shift assays are the tools that we could employ to determine if the gene is necessary for floR expression and to determine if pAM04528 binds the promoter region of floR.

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support during a project such as this is unparalleled. Last, but certainly not least, I would like to extend my gratitude to Dr. Douglas Call for remaining a wonderful mentor and teacher. His willingness to employ me as an undergraduate researcher has significantly changed my experience at WSU. I must say that research, although often fraught with frustration, has been the single most important learning experience in my education to date. I am truly grateful for having been given this opportunity.

Appendix A

<table>
<thead>
<tr>
<th>qPCR Primers</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>qPCR rpoD Forward</td>
<td>5'-CAGGTTCAATGCTCCGTTGC-3'</td>
</tr>
<tr>
<td>qPCR rpoD Reverse</td>
<td>5'-GCGACCTTTTCGCTTTGATGG-3'</td>
</tr>
<tr>
<td>qPCR floR Forward</td>
<td>5'-GACGCCCCGCTATGATCCAAC-3'</td>
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<tr>
<td>qPCR floR Reverse</td>
<td>5'-CCTGCCATCCCCAAGAACTCG-3'</td>
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</table>

*Primer efficiency – rpoD: 100%; floR: 87%

<table>
<thead>
<tr>
<th>Northern Blot Probes</th>
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<tbody>
<tr>
<td>23S Probe Left</td>
<td>5'-CGAAATTCTTTGTCGGGTAA-3'</td>
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<tr>
<td>23S Probe Right</td>
<td>5'-TCGTGCTCTCCGTTAATCT-3'</td>
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<tr>
<td>qPCR proD Forward</td>
<td>5'-CAGGTCTAATGCTCCGTTGC-3'</td>
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<td>qPCR rpoD Reverse</td>
<td>5'-GCGACCTTTTCGCTTTGATGG-3'</td>
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<tr>
<td>floR Pro Forward</td>
<td>5'-GCGAAGCAAAGATATAATCGG-3'</td>
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<td>floR Pro Reverse</td>
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<tr>
<td>GFP Forward (2)</td>
<td>5'-AGTCGGATCCGAAAGAAGACCTTTTTC-3'</td>
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<tr>
<td>GFP Reverse no tag (2)</td>
<td>5'-AGTCGAGCTCTTAGTTCATCATGCC-3'</td>
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Appendix B

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<th>Ingredient</th>
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<td>Na$_2$HPO$_4$</td>
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<td>KH$_2$PO$_4$</td>
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<td>Thiamine</td>
<td>1 mg/mL</td>
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<tr>
<td>MgSO$_4$</td>
<td>1 mM</td>
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<tr>
<td>CaCl$_2$</td>
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<tr>
<td>Glucose</td>
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References


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Date Spring 2011

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