

The War on Drugs: Antibiotic Recognition by the ykkCD RNA Toxin Sensor

An Honors Thesis (HONR 499)

by

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A handwritten signature in black ink, appearing to read "Timea Gerczei". The signature is fluid and cursive, with a long horizontal flourish at the end.

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Abstract

Antibiotic resistance is a rapidly growing concern in the global world of healthcare. As our ability to treat infectious becomes more advanced, so do the bacteria's ability to combat it. Currently, more research is needed to uncover how these microorganisms manage to defend themselves against the barrage of treatments available against them. The Gerczei lab is exploring one of the mechanisms of bacterial antibiotic resistance; specifically, how a noncoding RNA regulates the expression of the ykkCD multi-drug resistance efflux pump. This pump removes toxins from bacteria cells thus making the treatment with a wide array of antibiotics useless. Using *Bacillus subtilis* as a model organism, I studied how the ykkCD riboswitch recognizes the target antibiotic, tetracycline, using its derivatives oxycycline and anhydrotetracycline. The expression levels of the ykkCD subunits were analyzed using qRT-PCR technology.

Acknowledgements

First of all I would like to thank my parents for their constant support and motivation towards my academic and personal success. Your love and encouragement helped me to be the woman I am today.

Secondly I would like to thank Dr. Timea Gerczei for her continued guidance throughout this project. You pushed me to become a better researcher and the best scientist I could be.

I would also like to thank Dr. John McKillip for his patience and the use of both his machines and expertise throughout portions of this experiment. Also thanks to Dr. Paul Coan for providing me with the opportunity to research all summer with the CRISP Program.

Beau Champ, Caroline Conley and Evan Jones and the others in the lab who helped me to complete this project and kept the lab filled with laughter.

Endless gratitude belongs to Alysha Ellison and Lauren Williamson for their expertise with the PCR technology and Minitab.

Finally, I would like to thank Mike for all his love, patience and support as I pursued my passion over the course of the past four and a half years.

Author's statement

I performed an independent research project to determine if tetracycline and its commercially available derivatives are recognized using *Bacillus subtilis* as my model organism. Each derivative adds or removes a specific functional group normally found in tetracycline that may be important for recognition. *Bacillus subtilis* was grown in both the presence and absence of tetracycline and its derivatives, oxycycline and anhydrotetracycline. In order to test if any of the derivatives are recognized, quantitative reverse transcriptase PCR (qRT-PCR) was utilized in order to evaluate mRNA levels of the ykkCD efflux pump. If a derivative induced ykkCD multi-drug resistance (MDR) mRNA production, the functional groups altered compared to tetracycline are not essential for antibiotic recognition. If the mRNA is not produced or levels do not increase, the altered functional groups were important for recognition. The outcomes of this project will include a research paper and a poster presentation.

My Honors thesis provided me with an opportunity to conduct research in an independent manner outside of a curriculum laboratory. It gave me insight on how to be self-sufficient in a lab setting, and introduce me to protocols utilized in professional, hypothesis-driven chemistry and biology research labs. Not only that, I used techniques I learned at Ball State University and apply them outside of the classroom while simultaneously learning new techniques. Exposure to all of these techniques prior to my entrance into today's competitive job market makes me a better suited candidate for graduate and professional programs.

This project will be valuable to other biochemistry researchers at Ball State University as well as other members of the scientific community examining antibiotic resistance in bacteria. My research will provide clues on how bacteria recognize antibiotics and better understanding of toxin sensor expression.

Introduction

History of antibiotic resistance

In 1928, Sir Alexander Fleming accidentally discovered a very curious phenomenon. He noticed that the presence of the fungus *Penicillium chrysogenum* growing on plates of bacteria left behind zones of inhibited bacterial growth. This observation led to one of the greatest medical breakthroughs of modern history. Many human diseases are caused by bacteria, and it was soon realized that these naturally produced compounds could be used to treat diseases like tuberculosis, cholera and syphilis. Antibiotics are naturally derived compounds that kill or inhibit the growth of microorganisms. Antibiotics exploit the difference in metabolism or protein synthesis between prokaryotic and eukaryotic cells. These compounds are produced by fungi and even some bacteria. The isolation, analysis, synthesis and even modification of these complexes led to the development of medicines that made the treatment of infectious diseases seemingly flawless. Antibiotics work through different mechanisms. Some inhibit the synthesis of bacterial proteins or interrupt their cell wall production. Today, medications are utilized worldwide and treat a myriad of diseases caused by pathogenic bacteria.

However, as man was creating an effective means to fight against the bacteria, unbeknownst to them, the bacteria had an intrinsic method of fighting back. Antibiotics are naturally derived compounds, and as such, bacteria naturally developed the ability to resist them. Each time a doctor prescribes an antibiotic there is a risk that an antibiotic resistant strain of bacteria will emerge as a result. An antibiotic may effectively kill some of the organisms in a bacterial infection, but those that are resistant will continue to divide and multiply. In fact, over-utilization of antibiotics as a treatment for many illnesses is only exacerbating the development of mutant resistant strains. Doctors and pharmacists have since begun to educate the public on

the importance of completing a regiment of antibiotics when prescribed in order to ensure the treatment is as effective as possible.

Current cost and implications of bacterial resistance

According to the Centers for Disease Control and Prevention (CDC) in 2013, an estimated 2,049,442 illnesses and 23,000 deaths were caused by antibiotic resistance (1). The World Health Organization (WHO) reported 450,000 cases of multi-drug resistant tuberculosis (4). Without potent antibiotic treatment, TB kills 50% of infected individuals. Hospital-linked bacterial infections are caused by highly resistant strains of bacteria, such as methicillin-resistant STAPH (MRSA) and vancomycin-resistant enterococci (4). These two diseases are often contracted while individuals are patients in the hospital, often when immunity is compromised. Methicillin and vancomycin are among a couple of the most potent antibiotics commercially available. People infected with a resistant strain of MRSA are 64% more likely to die as a result of infection than those with a non-resistant strain. Every year in the United States, more people die from bacterial infections than HIV/AIDS and breast cancer combined (1). The CDC estimates that bacterial resistance is responsible for \$45 billions of dollars in direct hospital costs annually (5). Antibiotic resistance is already a large issue in medicine and without proper attention, there is a chance that some of the world's most dangerous diseases will shortly become untreatable.

Mechanisms of antibiotic resistance

The explosion of antibiotic resistance onto the medical scene has caused pharmaceutical companies reluctant to begin the search for new drugs. Research into drug development costs

billions of dollars and can take up-to a decade to reach trial stages. Furthermore, antibiotics are a short-term treatment, meaning that the company may not be able to earn back its investment or achieve a profit. In order for antibiotic research to be more effective, the resistance mechanisms must be better understood. Antibiotic resistance results from one of four mechanisms: 1) inactivation of antibiotic using enzymes that degrade antibiotics; 2) alteration of the antibiotic target, so it is unable to recognize the antibiotic; 3) block entry of the antibiotic into the cell and finally; 4) activation of efflux pumps to pump antibiotics out of the cell. The mechanisms of antibiotic resistance are diagrammed in Figure A.

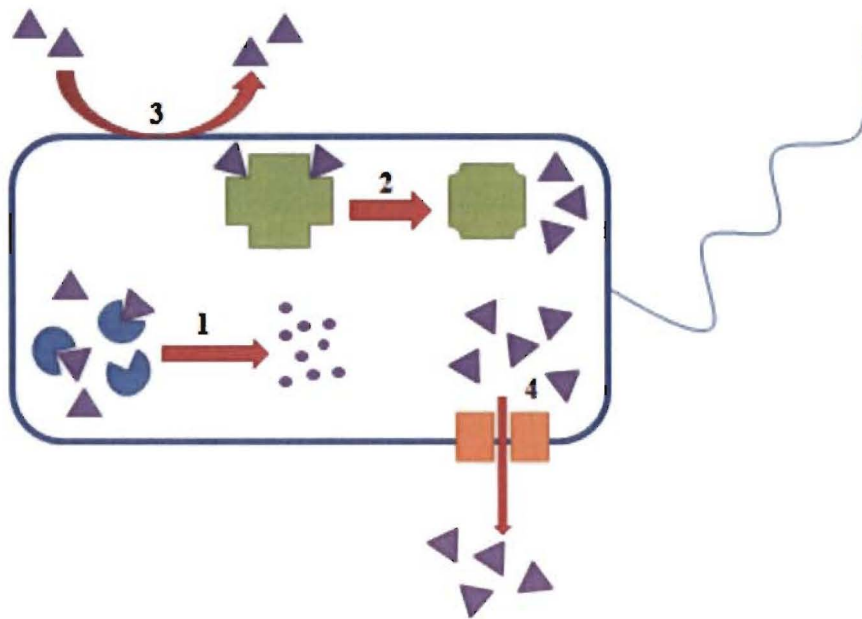


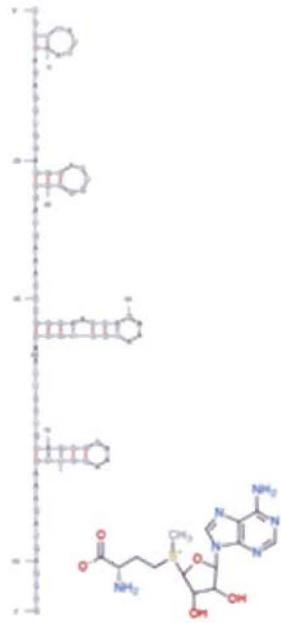
Figure A. Schematic representation of the methods of antibiotic resistance in bacteria. The numbers correspond to descriptions found within the text.

Riboswitches

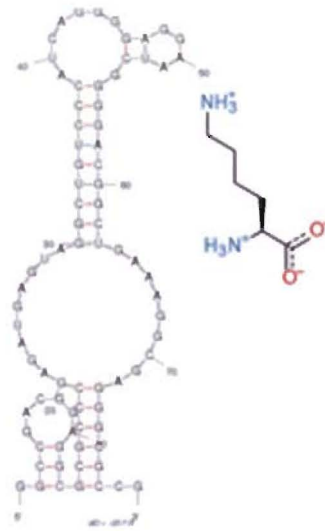
My research focused on how the ykkCD RNA toxin sensor, which is a riboswitch, recognizes its target antibiotic. In a cell, information travels from DNA → RNA → protein, following the central dogma of biology. Usually, a series of extra- and intra-cellular cascades is

necessary for the expression of genes within a cell. A riboswitch is an RNA-dependent method of gene regulation. The name “riboswitch,” is derived from the fact that these mRNA molecules are able to alternate between two conformational states in response to binding of a small ligand. Riboswitches are conserved across prokaryotes. Most riboswitches are located in the 5'-UTR of the gene they regulate and folds into complex 3D structures. The ligand of most riboswitches is produced by the gene that is regulated. Most known riboswitch molecules are involved in the regulation of metabolic genes in bacteria. If the metabolite concentration reaches a given threshold, it is able to bind to the riboswitch. The resulting binding leads to a conformational change which causes a down-regulation of the metabolite producing gene. Hence, the constant, appropriate concentration of the metabolite is maintained.

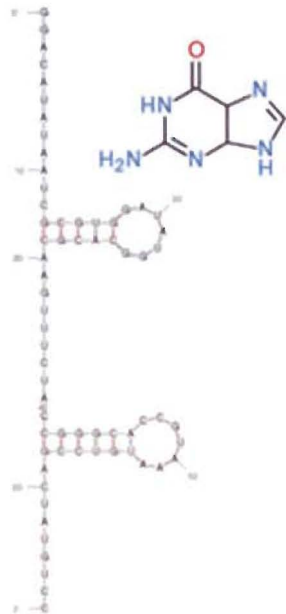
There are eleven known riboswitch classes with a large variety of ligands. Ligands include coenzyme B12, Flavin mononucleotide, purines (adenine/guanine) S-adenylmethionine (SAM), amino acids, glucosamine-6-phosphate (GlcN6P) pre-Q1, cyclic-di-GMP and thiamine pyrophosphate that activates the TPP riboswitch in eukaryotes. Figure B provides an example of the diversity of riboswitches and the specificity of their respective ligands (9).



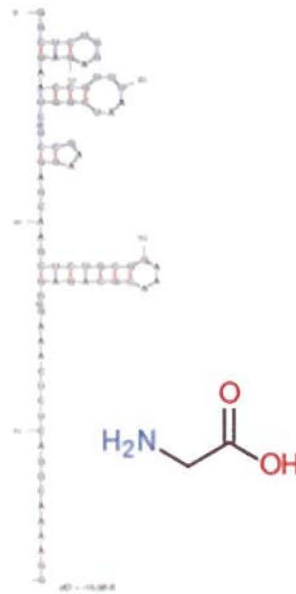
SAM riboswitch



Lysine riboswitch



Guanine riboswitch



Glycine riboswitch

Figure B. *Riboswitch examples and their respective ligands. There is a large diversity in the structure of these molecules and the groups that interact with them.*

The ykkCD riboswitch-type sensor is an evolutionarily conserved portion of the messenger RNA (mRNA) that regulates the expression of the ykkCD multi-drug resistant (MDR) pump. The ykkCD pump is coded in the bacteria's DNA, in the 5' untranslated region of the MDR gene. Its proposed secondary structure is detailed in figure C. Found in 80% of Gram+ bacteria, MDR pumps are able to actively pump antibiotics out of the bacterial cell, rendering the antibiotic ineffective. The ykkCD toxin sensor recognizes and binds tetracycline in *Bacillus subtilis*. This binding causes a structural change that permits transcription of the ykkCD pump mRNA. Translation of the ykkCD MDR efflux pump mRNA forms the MDR efflux pump protein, and the bacterial cell is able to pump out the antibiotic and survive. It is expected that a similar mechanism exists in other Gram+ pathogens (9).

Tetracycline derivatives used in this study

The overall purpose of this experiment is to understand how tetracycline is recognized by the ykkCD riboswitch. By changing the functional groups of tetracycline using commercially available derivatives, the functional groups important for tetracycline recognition can be uncovered. In this experiment, two derivatives of tetracycline were utilized. Traditionally, tetracycline was used for the treatment of bacterial infections like urinary tract infections and pneumonia. Tetracycline antibiotics work by preventing the growth and thus, spread, of infection and are not effective against any viral infections. The chemical structure of tetracycline is pictured in Figure D. Following D is Figures E and F that depict the chemical structures of the two derivatives used in this experiment, oxycycline and anhydrotetracycline. Upon inspection, all three structures contain identical functional groups along the bottom of each image. The major differences are seen along the tops of the pictures. Oxycycline contains an extra hydroxyl group on the third ring and anhydrotetracycline is missing a hydroxyl group on the second ring.

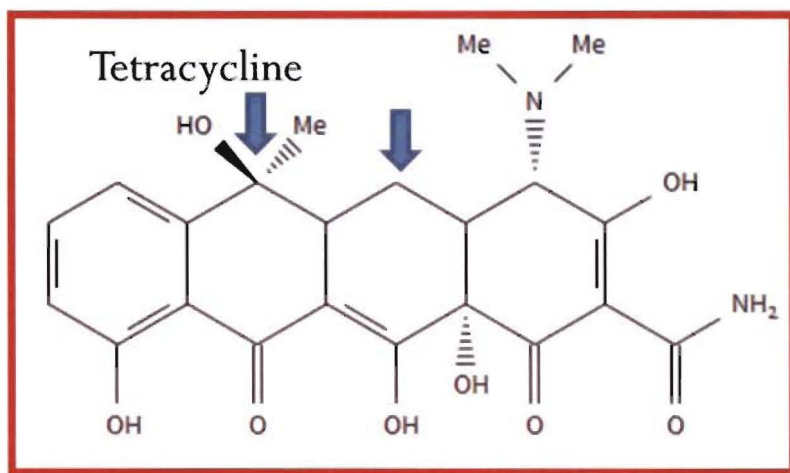


Figure D. *Chemical structure of the antibiotic tetracycline. Differences in functional groups in comparison to oxycycline and anhydrotetracycline are indicated by the blue arrows.*

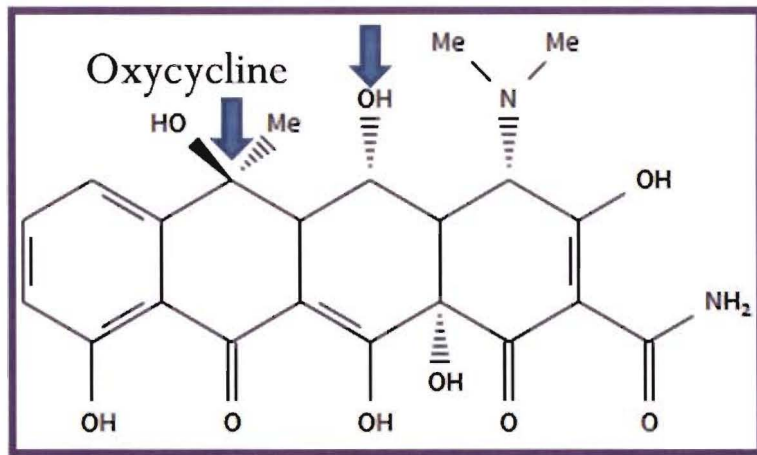


Figure E. *Chemical structure of the antibiotic oxycycline. Differences in functional groups in comparison to tetracycline and anhydrotetracycline are indicated by the blue arrows.*

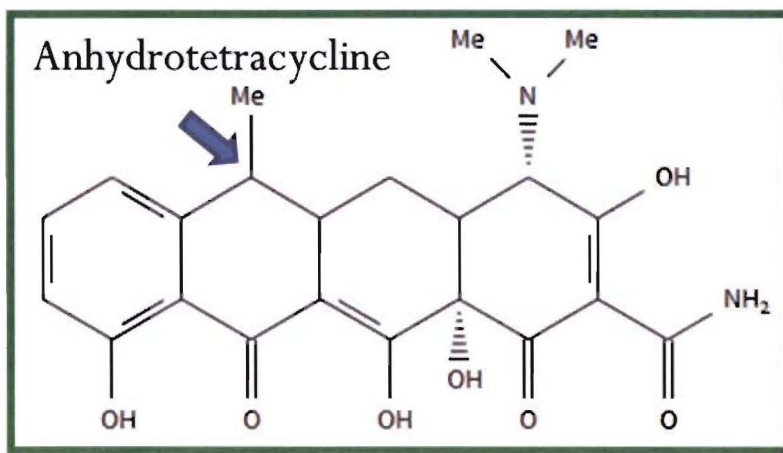


Figure F. *Chemical structure of the antibiotic anhydrotetracycline. Differences in functional groups in comparison to tetracycline and oxycycline are indicated by the blue arrows.*

qRT-PCR technology

Protein expression levels can be estimated by quantifying the levels of mRNA within a cell. If the expression of the mRNA is increased in a cell, it is likely that the levels of protein expression are increased in correlation. In this experiment, our lab utilized qRT-PCR technique, which quantifies mRNA levels of a specific gene as opposed to amplifying DNA, which is what

traditional PCR technology was established for (3). In qRT-PCR, the RT stands for reverse transcriptase. Reverse transcriptase, an enzyme that copies RNA into its complementary DNA strand. Once the cDNA has been created, traditional PCR methods amplify the gene-of-interest with the use of specifically designed primers. PCR follows the steps below.

1. High temperatures separate the strands
2. Lower temperatures allow the primers to anneal to the cDNA
3. Optimal temperature allows for DNA polymerization

Due to the high temperatures required for this process, DNA and RT from thermophilic organisms are used. Typically, DNA doubles each cycle under 100 percent efficiency and with 40 cycles per experiment, the level of amplification is remarkable. In real-time PCR, fluorescent dyes are used that intercalate with DNA. Many cyclers have built-in fluorescent detectors that record the fluorescence of cDNA in real-time. That way, samples with more mRNA fluoresce and are detected earlier in amplification than those samples with less RNA, allowing for the quantification of mRNA levels.

My experiment

I performed an independent research project to determine if tetracycline and some of its commercially available derivatives alter the expression of the ykkCD MDR pump using *Bacillus subtilis* as my model organism. The purpose of this study was to understand how the antibiotic tetracycline is recognized by the riboswitch. Each derivative adds or removes a specific functional group normally found in tetracycline that may be important for recognition. *Bacillus subtilis* was grown in both the presence and absence of oxycycline and anhydrotetracycline. In order to test if any of the derivatives are recognized, quantitative real-time PCR (qRT-PCR) was

performed in order to evaluate mRNA levels of the ykkCD efflux pump. If a derivative induced ykkCD MDR mRNA production, the functional groups altered compared to tetracycline are not essential for antibiotic recognition. If the mRNA is not produced or levels do not increase, the altered functional groups were important for recognition.

Materials & Methods

Cell growth in the presence and absence of antibiotics

Total mRNA was isolated from *Bacillus subtilis* spp. 168. Cells were streaked for isolation on LB agar and grown overnight in the incubator at 37°C. Time of growth did not exceed 20 hours. Cells were obtained from *Bacillus subtilis* Genetic Stock Center and streaked for isolation. In a sterile 250mL Erlenmyer flask, a single colony from the streak plate was dropped into 50mL of LB media. Cells were shaken at 260rpm for 18-24 hours at 37°C.

Samples of cells were then grown both in the presence and absence of the antibiotics oxycycline and anhydrotetracycline. 250µL of cells from the previous growth broth growth were added to 25mL of autoclaved LB in a sterile flask. Stock concentration of antibiotic was diluted and added according to the values listed in Table 1. Table 1 lists the stock concentrations and sub-inhibitory concentrations (SIC) used for this experiment. Samples were grown overnight in the shaker at 37°C for no more than 19 hours.

Table 1. Antibiotic stock concentrations, SIC levels and volumes used in growth for mRNA isolation.

Antibiotic	Stock concentration	Dilution	Volume
Oxycycline	5000 µg/mL	1:15	500 µL
Anhydrotetracycline	1000 µg/mL	1:60	300 µL

The SIC level is the antibiotic concentration that causes cells to grow at an optical density (OD) that is approximately half of a control culture. For the antibiotics in question, the SIC values were determined by growing cells overnight in varying concentrations of oxycycline and anhydrotetracycline. The following day, the OD values were measured at 595nm on the TECAN infinite F200 fluorescent plate reader OD values of 0.45-0.65 were accepted. This value provided the information necessary to determine how much liquid cell culture was needed for mRNA extraction.

mRNA extraction

To quantify the levels of ykkCD expression in response to the antibiotic treatment, it was necessary to extract the mRNA from the cultures grown. mRNA extraction aims to remove all other cellular components from the sample, including genomic DNA, which could interfere with analysis. Total mRNA extraction was performed using the Qiagen® RNeasy Mini Kit (catalogue #74104). The manufacturer's protocol was followed, complete with the on-column DNase digestion (catalogue #79254). After the final elution step, the concentration of mRNA extracted was determined using the ThermoScientific Nanodrop1000 spectrophotometer. Typical yields were between 120-500 ng/ μ L.

Formaldehyde denaturing gel electrophoresis

In order to ensure that the mRNA was of good quality, denaturing agarose gel electrophoresis was done. Good quality mRNA should yield two distinct bands, representing the 16S and 18S subunits of the cellular ribosomal RNA (rRNA). For the following protocol, 10X Formaldehyde (FA) gel buffer was needed.

Table 2. 10X FA Buffer and FA running Buffer components.

10X FA Buffer, pH 7	FA running buffer
200 mM 3-(N-morpholino)propanesulfonic acid (MOPS)	50 mL 10X FA Buffer
50 mM sodium acetate	10 mL 37% formaldehyde
10 mM EDTA	440 mL MilliQ water

To prepare the gel, 100mL of FA running buffer was added to 1.0g Sigma agarose powder and that mixture was heated until boiling, which took approximately 1-2 minutes. The flask was allowed to cool and then 5 μ L of ethidium bromide (EtBr) was added. The gel was poured into the apparatus and allowed to polymerize.

In order to prepare the mRNA samples, 7 μ L of RNase-free water and 3 μ L FA loading buffer was added to 5 μ L mRNA samples for a total volume 15 μ L. The components of FA running buffer are as follows:

- 80 μ L 500 mM EDTA (pH~8)
- 720 μ L 37% formaldehyde
- 2 mL 100% glycerol
- 3.084 mL 100% N,N-dimethyl formamide
- 16 μ L bromophenol blue (BPB)
- 4 mL 10X FA Buffer
- MilliQ water to 10 mL

Samples were heated in 65°C water bath for 5 minutes and cooled on ice for another 1-2 minutes. The gel was covered in remaining FA running buffer and samples were loaded into the wells. Gel was run at 45V for 1 hour and then UV imaged with Bio-Rad Gel-Doc™ XR+ on the ethidium bromide setting.

Quantitative RT-PCR (qRT-PCR)

After the cultures had been grown, the mRNA extracted and checked for quality using agarose gel electrophoresis, qRT-PCR was performed in order to quantify the mRNA levels of the *ykkCD* pump. The genes *ykkC*, *ykkD* and *gyrase* were obtained from GeneBank and placed into the Primer3 software at <http://primer3.wi.mit.edu/>. The primers designed are seen in Table 3. The designed primers were obtained from Integrated DNA Technologies and the lyophilized samples were resuspended in RNase-free H₂O.

Table 3. Primers used for RT-PCR.

Primer	# bases	T _m (°C)	Sequence 5' → 3'
ykkC forward	20	58.0	ATG GGG ATT GGT CGT GCT TG
ykkC reverse	20	55.4	AAA GAC GGC ATA CAC GGT TC
ykkD forward	20	59.7	GGC GTG CTC TTA GTC GGT GT
ykkD reverse	20	60.5	TAA CAT GGA GCG GCA CTG CC
gyrase forward	20	55.2	GAG AAA GCC CGT TAC CAC AA
gyrase reverse	20	55.4	CAC GTT TCC CCT GTT GAA CT

RT-PCR was performed using the Applied Biosystems® Power SYBR® Green RNA-to-Ct™ 1 step kit (catalogue #4389986). For each primer set, a pre-mix was prepared by multiplying the volumes seen in Table 4 by the number of reactions for each primer. A no-template control and an RT-control were also used to check against contamination, which could have included residual DNA. Each experimental tube and the No-RT mix received a volume of extracted RNA equal to 50ng. Table 5 details the contents of each experimental Corbett Research reaction tube. For each trial, tubes were prepared in duplicate, though the controls were only prepared once per trial.

Table 4. Pre-mix volumes for one reaction tube.

Volume (μL)
12.5 2X SYBR®Green Taq ReadyMix
0.125 μL 200 U/ μL moloney murine leukemia virus reverse transcriptase(M-MLV RT)
1 μL 100 μM Forward primer
1 μL 100 μM Reverse primer
0.375 μL RNase-free water

Table 5. Volumes and contents for experimental and control tubes for RT-PCR.

Experimental tube	No-template control	No-RT tube
15 μL Pre-mix	15 μL Pre-mix	12.5 μL 2X SYBR®Green Taq ReadyMix
50ng total RNA	10 μL RNase-free water	1 μL forward primer
RNase-free water to 25 μL	-	1 μL reverse primer
-	-	50ng total RNA
-	-	RNase-free water to 25 μL

Samples were then placed into the Corbett Research RotorGene RG-3000 and run under the following cycling conditions:

- Reverse transcription 43°C for 30 minutes
- Initial denaturation 95°C for 2 minutes
- Denaturation 95°C for 15 seconds
- Annealing 52°C for 30 seconds
- Extension and fluorescence reading 60°C for 60 seconds
- Melt curve ramp- 0.2°C/s from 45°C-95°C

Samples were repeated for 40 cycles (denaturation → extension) and the results were viewed on Quantitation mode.

Data Analysis

After the completion of a round of qRT-PCR, a variety of analyses were performed on the trials. (a) In qRT-PCR, mRNA is converted to DNA. The C_T values were gathered from the

RotorGene. A CT value is the “cycle threshold,” or more simply, the amount of cycles it takes for the machine to detect fluorescence. The higher the C_T value, the lower the presence of the gene-of-interest in the sample. Melt temperature analysis was performed in order to identify each of the replicons used in the experiment. (b) I performed a one-way analysis of variance (ANOVA) for the treated versus untreated samples to determine difference in expression. (c) REST complete statistical analysis is a tool that takes into accounts the individual amplification efficiencies and changes in expression of a housekeeping gene in response to antibiotic treatment.

The Relative Expression Software Tool (REST) was utilized in order to compare the mRNA expression levels of *ykkC* and *ykkD* in comparison to DNA gyrase, the reference gene in this experiment. This technique uses randomization to calculate 95% confidence intervals and p-values and is specifically designed to be used with the RotorGene cyler. The values used in REST were gathered using a comparative quantitation of the amplification curve for each trial, providing “take-off” (TO) values and the amplification efficiency. One hundred percent polymerase efficiency would cause a doubling of material for each cycle. For each cycle, and average efficiency is calculated and reported as a value between 1 and 2. The TO and amplification values were to be entered into REST for each gene of interest (*ykkC*, *ykkD* and *DNA gyrase*) for both treated and untreated samples.

Once the values have been entered, the efficiency (E) is calculated on a scale from 0-1, with 0 being no amplification and 1 being 100% efficiency. Then, pairs of TO values of treated and untreated samples were randomly paired and a concentration was calculated using the equation $C = E^{TO_{untreated} - TO_{treated}}$. Then, a relative concentration was calculated using the equation $RE = C_{GOI}/C_{ref}$, where GOI stands for the gene-of-interest and ref indicates the reference

(housekeeping) gene. The calculation is repeated thousands of times with random pairs creating the range of expression values. Because of this, some values will be greater or lesser than the actual expression levels. The software has a built-in hypothesis test to estimate the likelihood that the difference between the samples is not just due to random chance. REST uses the Pair Wise Fixed Randomized Reallocation Test. If there is no difference in expression in the treated and untreated samples, the data could be exchanged and the expression ratio should not increase. GOI and references points are randomly exchanged between the treated and untreated groups thousands of times and the percent of times the expression value increases is recorded. The lower the frequency, the more likely that the results seen are not due to random chance and a p-value is calculated for this.

Results

SIC values

The sub-inhibitory concentration (SIC) values were reported in Table 1 of the Methods section. The SIC level is the antibiotic concentration that causes cells to grow at an optical density (OD) that is approximately half of a control culture. For the antibiotics in question, the SIC values were determined by growing cells overnight in varying concentrations of oxycycline and anhydrotetracycline. The following day, the OD values were measured at 595nm on the TECAN infinite F200 fluorescent plate reader OD values of 0.5-0.65 were accepted. For oxycycline it was 0.45 and for anhydrotetracycline 0.65. Table I presents the SIC values from the concentrations in the medium and K_D values. The K_D values were determined *in vitro* by a previous researcher in the lab, Delores James.

Table I. SIC and KD concentrations for the antibiotic treated samples. KD determinations come from previous research in the lab.

Antibiotic	[Antibiotic] in medium	[K _D]
<i>Oxycycline</i>	14.4μM	No binding observed
<i>Anhydrotetracycline</i>	444nM	24.5±9.2nM

Melting temperatures

Melt curve analysis was performed in order to ensure the identity of all replicons produced in this experiment. The values were determined using the Rotorgene analysis to produce a melt curve. An example of the melt curve can be seen in Appendix C. Thresholds were recorded at 0.5-1.6. The primary *ykkC* replicon melted at 82.16°C. The primary *ykkD* replicon melted at an average temperature of 80.33°C. Finally, the *gyrase* replicon melted at an average temperature of 80.5°C. In Table IV, a comparison of expected and actual melt temperatures is presented for the *ykkC* and *ykkD* replicons. Based on this data, the correct replicons for each GOI were created from the sample mRNA.

Table IV. Melting temperature comparison of predicted and actual T_m for the replicons of the GOI.

Replicon	Predicted melting temp (°C)	Actual melting temp (°C)
<i>ykkC</i>	82.5	82.16
<i>ykkD</i>	80.9	80.33

Formaldehyde denaturing gel electrophoresis

Figure I depicts the results from cell from the formaldehyde denaturing gel electrophoresis. For each sample, two distinct bands are present, representing the 16S and 18S subunits of ribosomal RNA, indicating that the total mRNA extracted is of good quality, which is required for qRT-PCR.

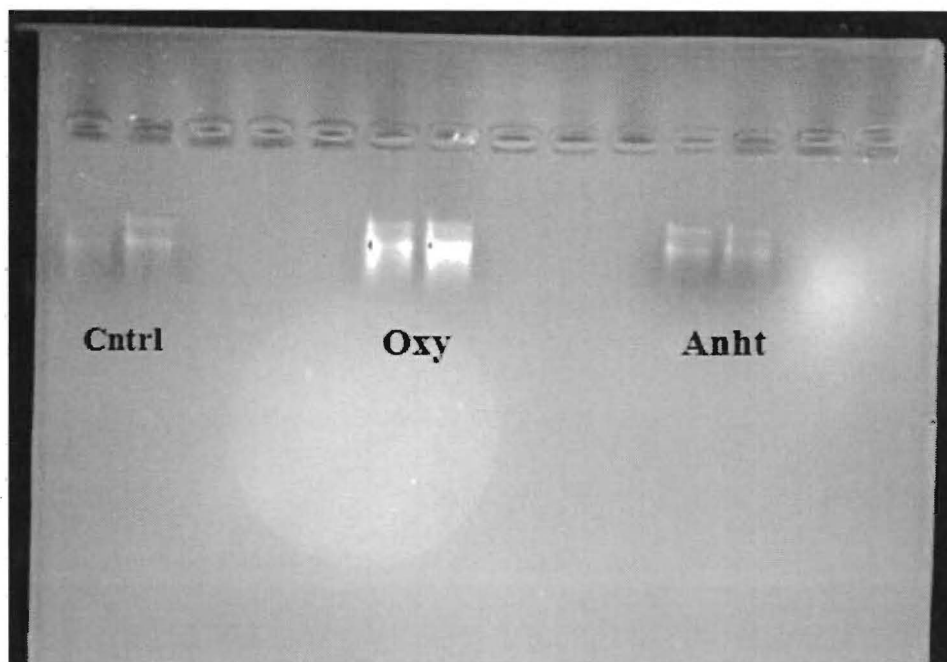


Figure I. Gel image from formaldehyde denaturing gel electrophoresis. From left to right: Cntrl-control total mRNA, Oxy-oxycycline treated total mRNA and Anht-anhydrotetracycline-treated total mRNA.

qRT-PCR

The results of the qRT-PCR provided information that then required further analysis. The C_T values indicate that a particular mRNA has detectable concentrations. As a result, if an mRNA is present in cells at a low concentration, it takes more amplification cycles to result in detectable fluorescence levels. Hence, the mRNAs with low concentration have higher C_T values than mRNAs with high concentrations. The C_T values for each experimental condition are listed in Table II. A total of four trials were run with each sample performed in duplicates for each trial. An example of the fluorescence curve from the RotorGene once cycling is seen in Appendix A. In Appendix B, a curve of the C_T determination is depicted.

Table II. CT values from qRT-PCR. From left to right, CC-untreated (control) *ykkC* primer, OC-oxycycline treated *ykkC* primer, AC-anhydrotetracycline treated primer. CD-untreated *ykkD* primer, OD-oxycycline treated *ykkD* primer, AD-anhydrotetracycline treated *ykkD* primer. CG- control gyrase, OG-oxycycline gyrase primer, AG-anhydrotetracycline treated primer.

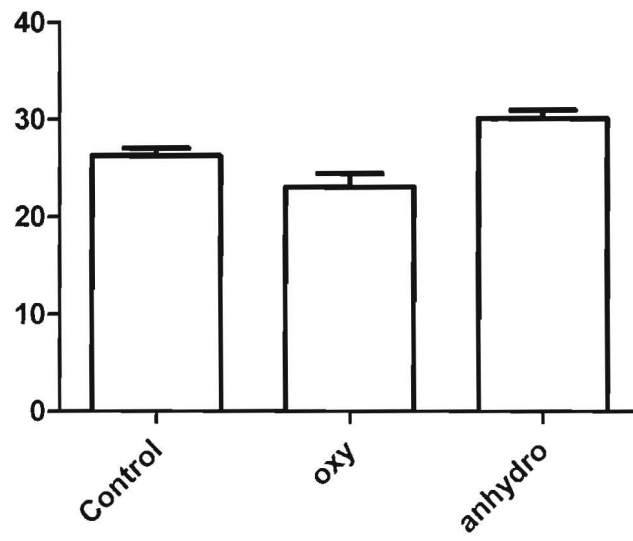
CC	OC	AC	CD	OD	AD	CG	OG	AG
24.31	24.52	25.25	31.88	32.33	32.18	25.57	21.63	29.93
23.67	23.12	24.59	31.68	33.31	31.42	25.84	20.98	30.08
24.49	23.99	21.01	30.68	31.25	30.91	26.36	22.27	36.12
24.2	23.36	22.59	31.49	30.95	33.5	26.67	20.72	29.37
22.95	24.22	22.61	30.3	31.66	30.22	31.36	32.32	28.35
23.04	23.39	22.87	30.92	31.24	30.97	25.04	21.28	29.18
26.61	26.62	24.23	31.5	31.45	31.63	24.33	24.67	29.02
26.39	26.69	25.25	30.78	32.21	31.51	25.14	20.79	29.23

Since the C_T values tell you which cycle fluorescence were first detected at, a quick visual inspection of the data provides a good indication as to the expression levels. For the *ykkC* pump, the untreated and treated samples do not appear to be drastically different, meaning that gene expression was neither significantly down- or up-regulated. In the treated and untreated samples of the *ykkD* pump, again there is little difference in the C_T values recorded.

Interestingly, the *gyrase* samples appear to be down-regulated in the anhydrotetracycline-treated samples. The inspection of the housekeeping CT values for the treated and untreated cells showed that a down-regulation of overall gene expression may have occurred. Remember, the higher the C_T value, the lower the mRNA concentration.

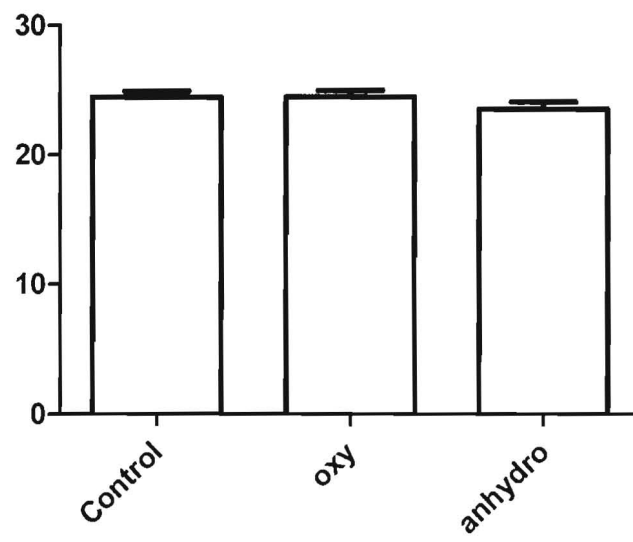
A

DNA gyrase C_T values



B

ykkC C_T values



C

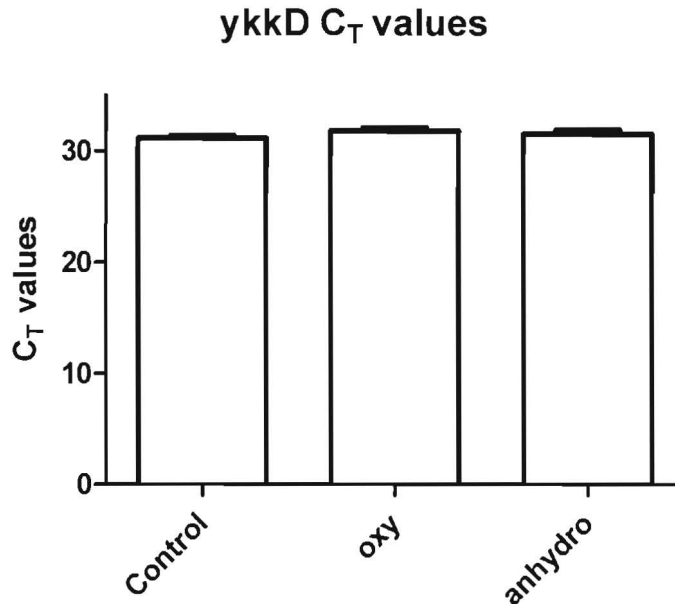


Figure II. Bar charts of all C_T values collected from the RotorGene analysis. (A) The C_T values of the control DNA gyrase samples as compared with the oxycycline and anhydrotetracycline treated samples. (B) The C_T values of the control ykkC samples as compared with the oxycycline and anhydrotetracycline treated samples. (C) The C_T values of the control ykkD samples as compared with the oxycycline and anhydrotetracycline treated samples.

Analysis of C_T values

A one way analysis of variance (ANOVA) with Tukey was performed on the C_T values in order to see if the expression of the treated and untreated samples was statistically significant. No statistically significant difference between any of the treated and untreated samples was seen based on this analysis. Conventionally, a p-value <0.05 indicates evidence to support significant difference between datasets. Any p-value greater than 0.1 indicates no statistically significant to, and a 0.05<p<0.1 indicates that there is a statistically significant difference between datasets. Table V presents the p-values determined by the ANOVA. With the ykkD pump, the oxycycline-treated versus untreated sample had a p-value between 0.05 and 0.1, which indicates some

evidence that the *ykkD* may be down-regulated in the presence of oxycycline. Further analysis should include changes in housekeeping gene expression.

Table V. *p*-values for the treated versus the untreated samples reported by ANOVA. *P*-values further discussed are in bold.

Gene of interest	Oxycycline treated	Anhydrotetracycline treated
<i>ykkC</i>	0.965	0.231
<i>ykkD</i>	0.077	0.347
<i>DNA gyrase</i>	0.102	0.042

The *p*-value of the anhydrotetracycline sample was <0.05 , which shows a statistical significance when compared to the untreated sample. A down-regulation of DNA gyrase expression might indicate that the total mRNA levels in general were lower in the anhydrotetracycline samples. Taking this into consideration, no change in the *ykkCD* pump mRNA levels might mean that *ykkCD* expression is up-regulated in the anhydrotetracycline treated samples. To definitively make this conclusion about the data, it needs to be re-analyzed using a program that takes changes in housekeeping gene expression levels into consideration. At the time of this thesis, the program REST that could complete this analysis was temporarily unavailable.

TO and amplification efficiency

The TO values and amplification efficiencies from the RotorGene are detailed for each primer in Table VI. An example of the TO curve can be seen in Appendix D.

Table VI. Take-off values and amplification efficiencies (AE). Key for other values follows same pattern as in Table I.

CG	AE	CC	AE	CD	AE	OG	AE	OC	AE	OD	AE	AG	AE	AC	AE	AD	AE
27.1	1.77	25.6	1.78	32.9	1.82	23.1	1.74	26.6	1.75	33.2	1.76	32.1	1.74	27	1.78	33.5	1.87
27.4	1.77	25.1	1.74	33	1.73	22.3	1.73	24.9	1.78	33.7	1.92	32.1	1.75	26.5	1.72	33.2	1.8
28.2	1.76	25.9	1.75	32.5	1.81	23.9	1.76	25.8	1.79	33	1.81	34.2	1.48	22.5	1.73	32.1	1.75
28.5	1.76	25.7	1.73	32.9	1.82	22.5	1.75	25	1.75	32.2	1.75	31.1	1.75	24.1	1.73	33.5	1.87
33.3	1.83	25.2	1.78	32.1	1.8	32.3	1.55	26.4	1.77	33.2	1.87	30.3	1.78	24.3	1.8	31.7	1.78
27.2	1.83	25	1.78	32.8	1.86	23	1.79	25.2	1.79	32.8	1.83	31.1	1.8	24.6	1.81	32.4	1.79
27	1.87	29.5	1.89	33.5	1.96	24.2	1.85	29.4	1.94	33.5	1.97	31.6	1.89	26.9	1.91	33.4	1.88
27.7	1.9	29	1.88	33	1.96	23.4	1.89	29.4	1.93	34	1.95	31.3	1.87	27.9	1.88	33.2	1.87

REST analysis

Due to temporary inaccessibility to the REST analytical software, REST analysis was unable to be performed at the time of this thesis.

Discussion

In this study, the recognition of two tetracycline derivatives, oxycycline and anhydrotetracycline, by the ykkCD riboswitch RNA toxin sensor were examined. This was achieved by growing *Bacillus subtilis* both with and without the presence of the sub-inhibitory concentration of antibiotic in culture medium. The total RNA was then extracted from these samples. In order to determine how the ykkCD pump expression was affected by the sensor, qRT-PCR was utilized.

SIC value determination

The sub-inhibitory concentration (SIC) values are the antibiotic concentrations that cause cells to grow at an optical density (OD) that is approximately half of a control culture. SIC values are not ubiquitous, and many factors have to be taken into account when utilizing them in research. The derivatives used in this experiment were light sensitive and stored in a 4°C freezer

until use. Samples were kept out of direct light as much as possible, but there is still the chance that environmental factors affected the effectiveness of the antibiotics *in vivo*.

Formaldehyde denaturing gel electrophoresis and melting temperature

Once the RNA had been extracted, the denaturing formaldehyde gel electrophoresis depicted that the RNA was of good quality to proceed with the qRT-PCR. Four trials were performed, and each trial had duplicates of each sample for a total of eight individual trials that were to be analyzed. The melting temperature analysis indicated that the *ykkC* and *ykkD* replicons were assumed to be the mRNA generated, as the predicted melting temperatures correlated with the actual observed values, as seen in Table IV of the Results section

qRT-PCR analysis

A quick visual interpretation of the C_T values and statistical analysis both indicated that there was no significant up- or down-regulation for any of the oxycycline or anhydrotetracycline samples when compared to the untreated samples for each individual operon.

The C_T values from each trial were plotted on a bar chart in order to compare the untreated and treated samples for each gene (*DNA gyrase*, *ykkC* and *ykkD*), and a visual comparison of these charts suggested no change in gene expression. One-way ANOVA indicated no statistically significant difference in expression of the *ykkC* or the *ykkD* pumps due to the presence of these antibiotics was seen. The error bars for these samples were very small, as the data was of good quality. These signs all point to the fact that functional groups altered on the derivatives were important for recognition by the riboswitch, as the expression levels of the *ykkCD* efflux pump were unchanged.

Compared to the structure of tetracycline, presented in Figure D of the Introduction, oxytetracycline has an extra hydroxyl (-OH) group from the left, causing crowding in the upper portion of the molecule. This crowding may lead to steric clash at the active site, hence positioning of the oxy into the active site is prevented. Anhydrotetracycline is missing a hydroxyl group on the second ring of the structure. Hydroxyl groups are important hydrogen bond donors and acceptors, and its absence may have led to the altered binding.

Past work from this laboratory has showed that anhydrotetracycline tightly binds to the riboswitch mRNA with a K_D value of 24.5 ± 9.2 nM determined in an *in vitro* binding assay (7). In my experiment, the *in vivo* concentration of the anhydrotetracycline was 444 nM, well above the K_D value. Thus, the ykkCD would be expected to be saturated at this level, and increased levels of ykkCD pump mRNA would be expected. However, this experiment shows that there is no elevation in the expression of the ykkCD MDR pump. This discrepancy is possible due to one of two reasons. The first discrepancy is that the cytosolic concentration of anhydrotetracycline might not be equal to the concentration of the antibiotic in the media. Thus, there may not be enough antibiotic in the cell to initiate binding and subsequent up-regulation of ykkCD pump gene expression. The second reason stems from the fact that the overall mRNA levels in the anhydrotetracycline treated samples appear to have been down-regulated based on the analysis of housekeeping gene expression levels of the untreated and treated samples, as seen in Figure II of the results section. This phenomenon requires a more careful analysis in reference to a housekeeping gene. It may reveal that the ykkCD pump mRNA was in fact up-regulated, but a program like REST is required to make that conclusion.

For oxytetracycline, a preliminary trial indicated down-regulation of the pump (7). However, oxytetracycline was also shown to be non-binding, and a K_D value could not be determined. This

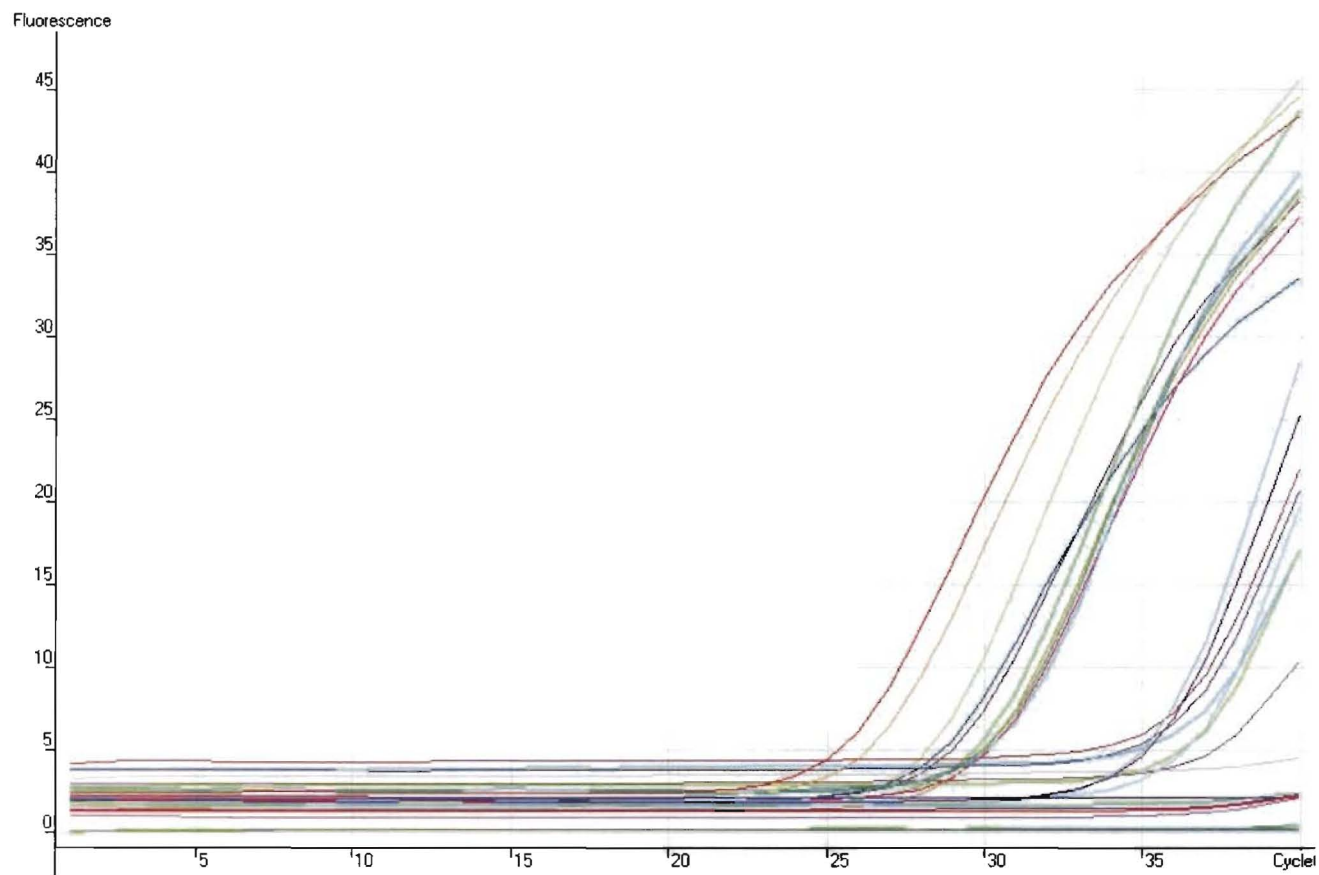
experiment showed that there is neither up- nor –down-regulation of the pump in the presence of either tetracycline derivative. It is important to note that the *in vivo* concentration of oxycycline was quite high (14.4 μ M) and still no up-regulation appeared to take place, indicating that the functional groups altered were probably important for ligand recognition.

ykkCD regulation

The *ykkCD* MDR efflux pump is a dimer composed of two subunits: the *ykkC* and the *ykkD*. Without both of these subunits, the MDR efflux pump cannot be expressed, so it was vital in this experiment to explore the effects of antibiotics on both subunits. What the results of this experiment indicate is that the expression of both the *ykkC* and *ykkD* were unaffected by the presence of these two derivatives, meaning that the functional groups altered were important for recognition. If the functional groups altered were not important changing them would have retained recognition of these derivatives by the *ykkCD* riboswitch and resulted in up-regulation of the pump. It is vital to note that an increase in mRNA expression correlates with increased protein levels in a cell. The results of this experiment will aid in the search for a method to deal with antibiotic resistance in bacteria. By understanding how the bacteria recognize the antibiotics, researchers are better able to design a drug that can evade the bacterial defense mechanisms.

Appendix

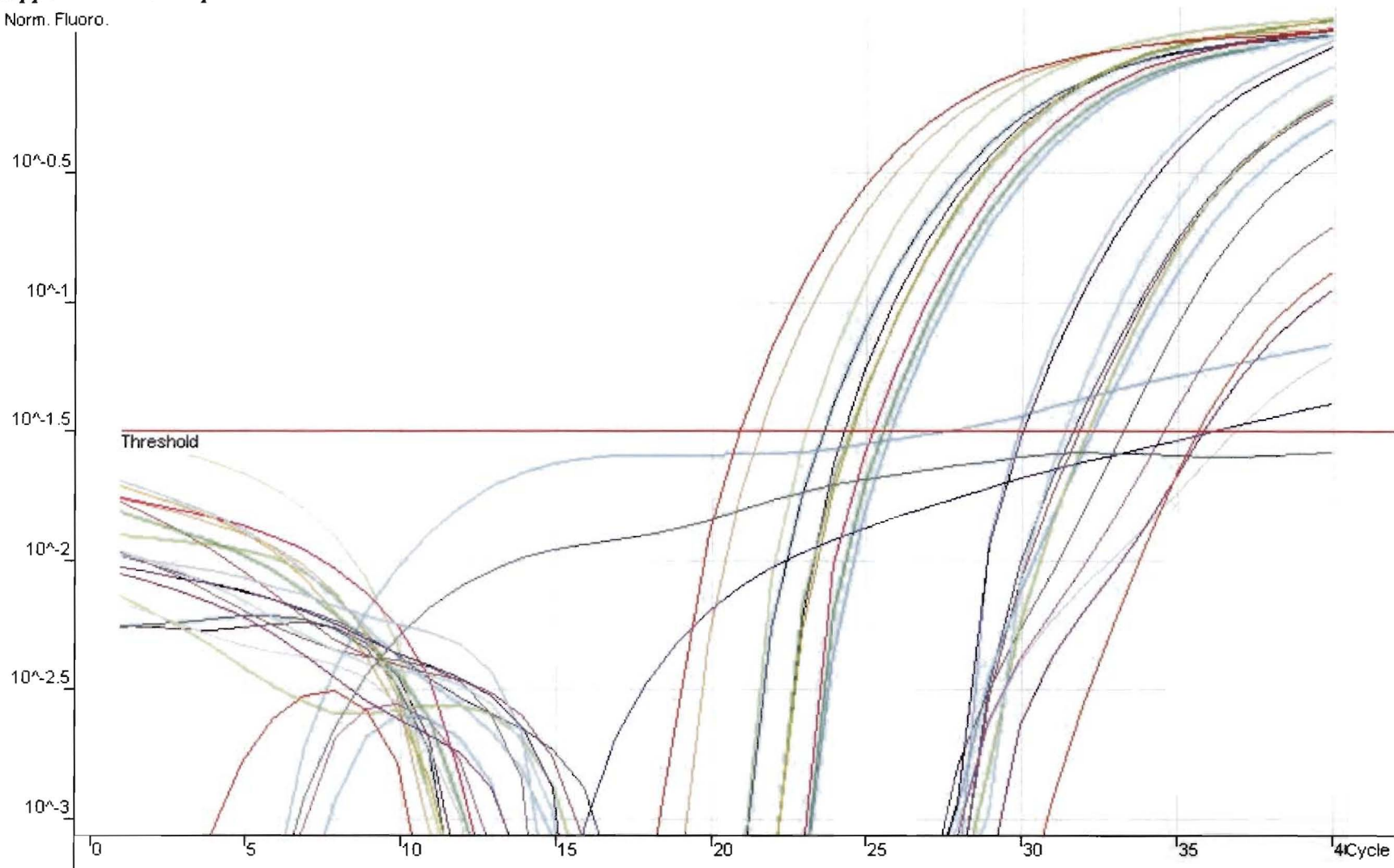
A. An example of the fluorescence curve from the RotorGene once cycling was complete. The key color corresponds to the appropriate line on the chart.



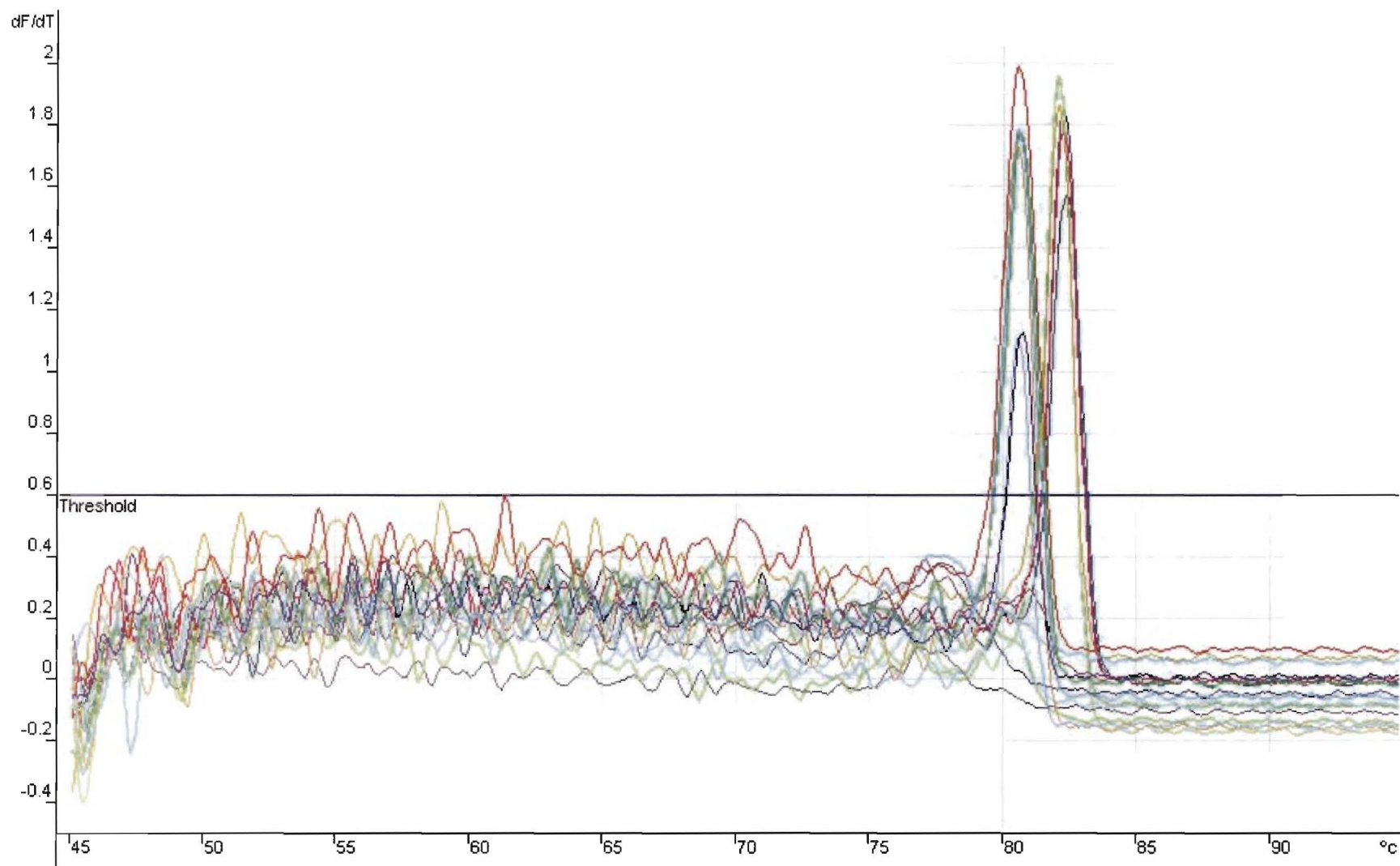
1	CC 1
2	CC 2
3	OC 1
4	OC 2
5	AC 1
6	AC 2
7	No RT C
8	blank
9	CD 1
10	CD 2
11	OD 1
12	OD 2
13	AD 1
14	AD 2
15	No RT D
16	blank
17	CG 1
18	CG 2
19	OG 1
20	OG 2
21	AG 1
22	AG 2
23	No RT G
24	blank
25	no template C
26	no template O
27	no template G

B. *An example of the curve used to determine the CT values. This curve was for a single trial of PCR. The key from the Figure in Appendix A corresponds to this curve.*

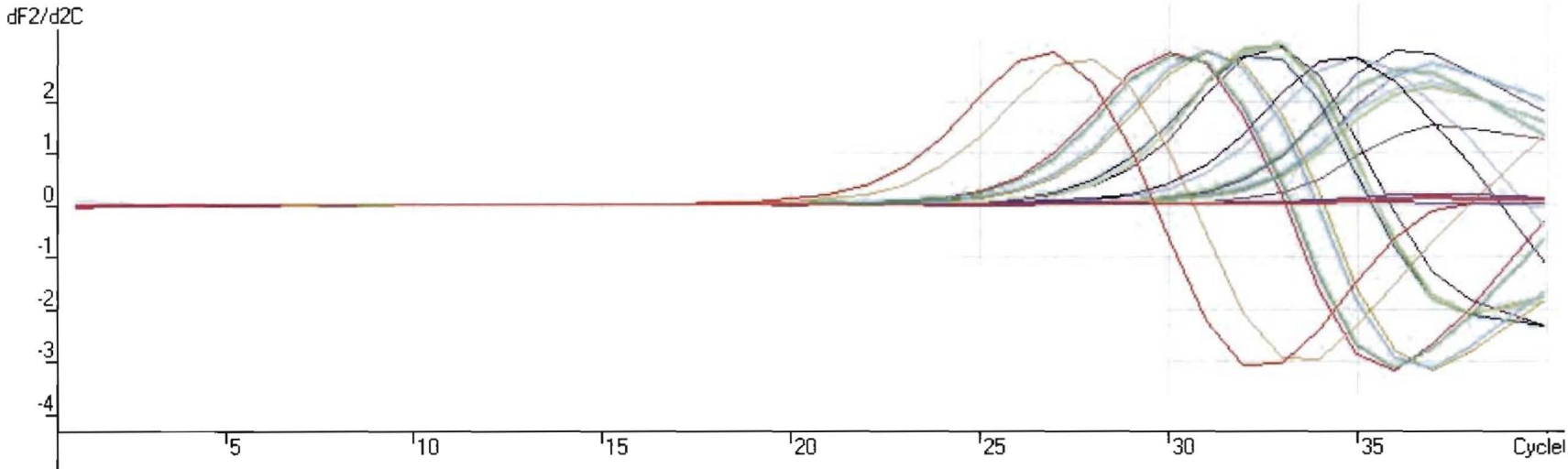
Norm. Fluoro.



C. An example of the curve used to determine the melting temperatures for the different replicons during the qRT-PCR experiments in the RotorGene. The key from Appendix A corresponds to this curve.



D. An example of the curve used to determine the TO values and amplification efficiencies. The key from Appendix A corresponds to this curve.



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Office of Research Integrity
Institutional Biosafety Committee (IBC)
2000 University Avenue
Muncie, IN 47306-0155
Phone: 765-285-5070

DATE: November 11, 2014
TO: Brenna Wermers
FROM: Ball State University IBC
RE: IBC Protocol # 633371-1
Title: The War on Drugs: Antibiotic recognition by the ykkCD RNA toxin sensor
Submission Type: New Project
Action: APPROVED
Decision Date: November 11, 2014
Expiration Date: November 10, 2017

The Institutional Biosafety Committee (IBC) has recently reviewed and approved the above protocol.

Approved Biosafety Level: BSL-1 BSL-2

If human participants are involved in this research, IRB reference #: [enter IRB reference #]

Approved Animal Biosafety Level: ABSL-1 ABSL-2

If animals are used as part of the research, IACUC reference #: [Enter IACUC reference #]

Editorial Notes

Approved

PI's and their research team are required to abide by any requirements listed above, outlined in their protocol and/or by the following (as applicable):

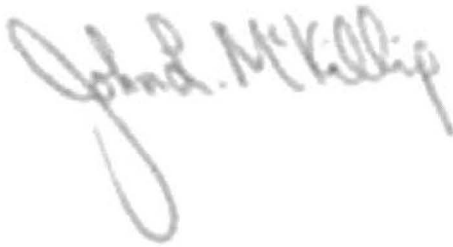
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition
- NIH Guidelines for Research Involving Recombinant DNA Molecules
- 7 CFR Part 331 and 9 CFR Part 121 USDA Possession Use and Transfer of Select Agents and Toxins
- Any other applicable Federal or State regulation or University policy applicable to the above research

As a reminder, it is the responsibility of the P.I. and/or faculty supervisor to inform the IBC in a timely manner:

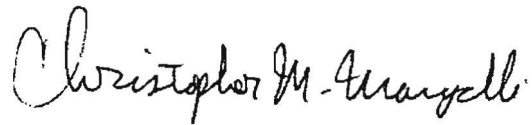
- when the project is completed,
- if the project is to be continued beyond the approved end date,

- if the project is to be modified,
- if the project encounters problems, or
- if the project is discontinued.

Any of the above notifications must be submitted electronically to the IBC through IRBNet (<https://www.irbnet.org/release/index.html>). Please reference the IBC protocol number given above in any communication with the IBC or the BSU Office of Research Integrity (ORI) regarding this project. Be sure to allow sufficient time for review and approval of requests for modification or continuation. If you have questions, please contact Chris Mangelli at (765) 285-5070 or cmmangelli@bsu.edu.



John L. McKillip, PhD/Chair
Institutional Biosafety Committee



Christopher M. Mangelli, JD, MS, MEd, CIP/Director
Office of Research Integrity

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)

BASIC BIOSAFETY TRAINING CURRICULUM COMPLETION REPORT

Printed on 11/07/2014

LEARNER Timea Fernandez (ID: 4492372)
DEPARTMENT Chemistry
EMAIL tgerczei@bsu.edu
INSTITUTION Ball State University
EXPIRATION DATE 11/07/2015

TRAINING FOR INVESTIGATORS, STAFF AND STUDENTS HANDLING BIOHAZARDS. : Initial training targeted for researchers handling or who will handle biohazards in a research or clinical laboratory.

This training addresses an awareness in biohazards, risk assessment and key risk management principles, including work practices, personal protective equipment, engineering controls, and emergency response.

COURSE/STAGE: Biosafety/Biosecurity/1
PASSED ON: 11/07/2014
REFERENCE ID: 14449740

REQUIRED MODULES	DATE COMPLETED	SCORE
Biosafety Course Overview	10/29/14	8/8 (100%)
Laboratory Acquired Infections	10/29/14	4/5 (80%)
Biohazard Risk Assessment	10/29/14	8/8 (100%)
Medical Surveillance	10/30/14	7/8 (88%)
Risk Management - Work Practices	11/07/14	2/2 (100%)
Work Safely with Sharp Instruments	11/07/14	2/2 (100%)
Disinfection and Sterilization	11/07/14	2/2 (100%)
Risk Management - Personal Protective Equipment	11/07/14	8/8 (100%)
ELECTIVE MODULES	DATE COMPLETED	SCORE
OSHA Bloodborne Pathogens Standard	11/07/14	2/2 (100%)
Universal Precautions and Work Practices	11/07/14	2/2 (100%)
Centrifuge Precautions	11/07/14	2/2 (100%)

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)
BIOSAFETY RETRAINING COURSE CURRICULUM COMPLETION REPORT
Printed on 09/30/2014

LEARNER Brenna Wermers (ID: 4251898)
DEPARTMENT Chemistry
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INSTITUTION Ball State University
EXPIRATION DATE 07/14/2015

BIOSAFETY RETRAINING COURSE : This course is designed as a refresher Biosafety training course option for researchers handling biohazards. This periodic refresher training can be scheduled by the institution either annually or other period.

COURSE/STAGE: Biosafety/Biosecurity/1
PASSED ON: 07/14/2014
REFERENCE ID: 13472574

REQUIRED MODULES	DATE COMPLETED	SCORE
Laboratory Acquired Infections	07/14/14	5/5 (100%)
Biohazard Risk Assessment	07/14/14	7/8 (88%)
Risk Management - Work Practices	07/14/14	2/2 (100%)
Risk Management - Personal Protective Equipment	07/14/14	7/8 (88%)
NIH Guidelines for Research Involving Recombinant and Synthetic Nucleic Acid Molecules	07/14/14	13/15 (87%)
ELECTIVE MODULES	DATE COMPLETED	SCORE
Universal Precautions and Work Practices	07/14/14	2/2 (100%)

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