Optimization of a Beta-Galactosidase Reporter Assay For the Study of the ykkCD Riboswitch As a Mechanism For Antibiotic Resistance in B. subtilis

An Honors Thesis (HONRS 499)

by

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Abstract

Antibiotic resistant strains of bacteria are quickly becoming a major concern in health care. As they evolve and adapt, our most powerful weapons become less and less useful, and very little is known about how to adjust our treatments. Pharmaceutical companies are rushing to create new and more powerful antibiotics, but they are not solving the root problem. This report is an analysis of a proposed mechanism of antibiotic resistance: a special type of mRNA called a riboswitch. The lab group is attempting to study the structure and properties of the ykkCD riboswitch, using *Bacillus subtilis* as a model organism, in hopes of developing a treatment against antibiotic resistant bacteria. The sub-inhibitory concentrations (SIC) were found for selected antibiotics, and a gene reporter assay was used to study the activation of the riboswitch.

Background

Ever since Alexander Fleming noticed the curious bactericidal properties of *Penicillium chrysogenum* and discovered penicillin in 1928, antibiotics have been humanity's most powerful weapon against infectious bacteria. Many of the planet's most deadly diseases, like tuberculosis, cholera, leprosy, and syphilis, could be easily treated with these magical drugs, and everywhere we looked we found more of them. We discovered that they were natural compounds produced by fungi and even certain bacteria, and by synthesizing or modifying them we could produce some amazing medicines. Some antibiotics attacked bacterial protein production, others prevented them from building their cell walls, and simply by prescribing the correct one, a doctor could nearly guarantee his patient's survival. Antibiotics were sold worldwide and would be used to treat anything from a cold to a severe case of bronchitis.

Unfortunately, we were not aware of just how good bacteria were at neutralizing these toxins, and we were caught completely off-guard when our magical medicines started losing their effectiveness. Since these antibiotics are natural compounds, bacteria had been evolving to combat them, and so they easily developed resistance to the medicine. Our overuse and improper use of antibiotics were helping the microbes to build defenses and eventually immunities, and contemporary medical practices were only furthering their cause. Whenever a person takes a course of antibiotics, there is a chance that a mutant bacterium has developed a resistance, and when all of the other bacteria are dead, the resistant strain is free to grow and multiply. This risk multiplies when the full course of antibiotic is not taken, which is why your doctor and pharmacist with both

plead with you to finish your prescription. The medical community rushed to change the way antibiotics were being used in order to stem the tide of drug-resistant bacteria.

Today, antibiotic-resistant infections represent a major concern worldwide. In the United States, the health care system spent $20 billion on these infections in the year 2000, and American households spent an additional $35 billion (1). 440,000 new cases of drug-resistant tuberculosis emerge each year, resulting in a staggering 150,000 deaths (2). Strains like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci make up the majority of nosocomial, or hospital-acquired, infections (2). If you are not familiar with either of these drugs, methicillin and vancomycin are two of the most powerful antibiotics available to us. If left unchecked, these types of infectious bacteria could become completely untreatable in a short amount of time.

While some new antibiotics have been created to fight these resistant bacteria, pharmaceutical companies are very reluctant to research new antibiotics. Drug research costs billions of dollars and takes 10-15 years, and an antibiotic is a short-term prescription, meaning that the drug company might not even make its money back. Even if the pharmaceutical industry was rushing to find the next great antibiotic, they still wouldn't be treating the underlying cause. Very little is understood about how antibiotic resistance works on a cellular level, and by learning more about it we may forge an entirely new weapon to fight the bacteria with. In recent years, three major mechanisms of antibiotic resistance have been proposed: antibiotic-degrading enzymes, antibiotic-altering enzymes, and production of resistance genes triggered by the presence of antibiotic (Fig. 1).In this study we focus on how RNA aptamers called riboswitches trigger production of resistance genes. By characterizing and testing this particular

cellular mechanism, it may become possible to attack infectious bacteria at the very source of their resistance.

In order to understand more about what a riboswitch is, one must have a basic knowledge of the movement of information within a cell. The unifying theory of cell biology, the central dogma, states that cellular DNA is transcribed into messenger RNA, or mRNA, which is then translated into protein. A riboswitch is a specialized piece of mRNA that is made up of two regions: an aptamer, or metabolite-binding, sequence and a variable sequence, or expression platform, region (3). The aptamer can specifically bind to a metabolite, a particular molecule, and this binding causes a conformational change in the expression platform region. This shape change allows the riboswitch to exist as an inactive ("off") state or an active ("on") state depending on presence of the metabolite within the cell (3). In the "on" state, the protein is synthesized and the corresponding gene is said to be expressed, and in the "off" state the gene is not expressed.

There are several possible routes the riboswitch could go through to accomplish this, so it would be most instructive to focus on the one that is most relevant. One study found that a particular riboswitch, typically in the inactive state, could be activated by binding of adenine. Not only was the binding extremely tight, but it was selective for adenine even against close analogs like guanine. The proposed mechanism involved the disruption of a transcription terminator stem loop, which would normally prohibit transcription of the gene (Fig. 2). The binding of adenine turned the riboswitch "on" and thus gene expression was turned on (4).

The proposed mechanism for the ykkCD riboswitch – the topic of this thesis study – is (Fig. 3) is very similar: metabolite binding interrupts formation of a terminator loop

being expressed is a resistance gene and codes for a multi-drug resistant efflux pump (MDR pump), which is then produced and transported to the cell membrane. MDR efflux pumps are a method of antibiotic resistance where a bacterium uses energy to forcibly pump the drug out of the cell (5). These pumps are costly to synthesize and maintain, however, and some kind of regulation is necessary to keep gene expression under control. The riboswitch provides this control by allowing the cell to turn on expression of the pump only when an antibiotic is present.

A common procedure used to study gene expression is a reporter assay. Originally, reporter assays were developed to study operons, specialized sequences that control gene expression at the DNA level. An operon consists of a promoter region, which allows RNA polymerase to bind to the DNA, an operator region where a metabolite can bind, and the structural genes that are being regulated. Much like a riboswitch, the binding of a metabolite to the operator region can turn the operon on or off depending on the needs of the cell. In a reporter assay, bacterial DNA is modified via plasmid insertion and a reporter gene replaces one of the structural genes. When the operon is turned on, the reporter gene will be transcribed along with the structural genes, and a reporter molecule will be synthesized within the cell. Changes in the concentration of the reporter molecule correspond to the activation or deactivation of the operon, and in this way gene expression can be studied (6).

A similar process can be used to study expression control via a riboswitch. A DNA plasmid is used to insert the reporter gene downstream of the riboswitch, and should be transcribed along with it, thus when the riboswitch is activated the reporter molecule is produced. An increase in the activity of the reporter molecule would

therefore indicate upregulation by the riboswitch. β-galactosidase is a common reporter gene due to its efficient cleavage of lactose analogs and the low cost and availability of those analogs. A glucose or galactose ring can be attached to a spectroscopically active molecule, and cleavage by β-galactosidase will increase the concentration of the detectable compound. Increase in spectroscopic values corresponds to the amount of detectable compound as a function of reaction time per cell volume and is divided by optical density to generate Miller units (7).

Another method of evaluating gene expression would be quantitative RT-PCR. By using specifically designed DNA primers, the mRNA levels of a particular gene can be measured, and the activity of the gene can be evaluated. However, this process is expensive and requires a great deal of training before it can be reliably used. It also assumes that an increase in the amount of mRNA directly relates to an increase in protein concentration. Reporter assays, on the other hand, are relatively inexpensive and simple to perform. They also have the advantages of being able to study mutant strains of bacteria and connect their results to *in vitro* assays.

Methods

SIC Determination

Although production of an efflux pump can protect a cell from antibiotics, the pump itself is costly to produce and maintain, and thus it must be regulated. In this case, binding of the riboswitch by an antibiotic causes an up-regulation in the pump gene and triggers synthesis of the pump itself. In theory, pump production will not be activated in cells grown in an antibiotic-free environment and a reporter molecule would not be

produced. In order to effect production of both the pump and the reporter molecule, a culture must be grown in a medium that contains enough antibiotic to trigger the riboswitch, but not so much as to completely inhibit growth. The sub-inhibitory concentration (SIC) is the concentration of antibiotic at which a culture will grow to about half the density of a control culture. SIC values are unique to each antibiotic and each strain of bacteria, so the first step in the process was to determine these values for selected antibiotics.

Based on quantitative PCR and *in vitro* binding assays in Dr. Gerczei's lab, tetracycline was chosen as the primary candidate for riboswitch activation (Gerczei unpublished), and its SIC had already been obtained. Three tetracycline derivatives were also chosen for study, and their SIC values were unknown: oxytetracycline, anhydrotetracycline, doxycycline. Tetraphenylphosphonium chloride (TPPC) and phosphomycin disodium salt (PDS) were also tested due to their activation of the efflux pump. Each test began with an inoculation of *B. subtilis* in enrichment medium (LB). A single colony was taken from an agar plate using a sterile pipet tip and submerged in the medium. The culture was then covered and placed in an incubator/shaker (37 °C, 220 RPM) for 18-24 hours. The next day a series of test tubes would be prepared with LB medium containing various amounts of antibiotic and the overnight culture would be transferred such that ~1% of the antibiotic culture was cells, i.e. 100 μL in 9900 μL of solution. The antibiotic cultures were then incubated at 37°C/220RPM for 19 hours.

After 19 hours, 200 μL of the antibiotic cultures were placed in the wells of the 96-well microtiter plate along with an aliquot of sterile LB. The optical density (OD) values of these solutions were read at 595 nm, blanked out with the sterile LB, and

plotted by their antibiotic concentration. The result was a bar graph showing the relative amount of growth at each concentration of antibiotic. An SIC value was then extrapolated for the concentration that would produce half the growth of the control culture, and these values were then verified.

It should be noted that during these tests the antibiotics were prepared in large volumes (50 mL) using Millipore water and stored at ~4°C. Each solution was wrapped in aluminum foil, as tetracycline degrades when exposed to light, and it was assumed that its derivatives did as well.

ONPG Assay

In vivo data on binding affinity of the riboswitch was needed in order to support the *in vitro* data already gathered by the Gerczei group. A reporter assay using the enzyme beta-galactosidase was selected, as the enzyme is well-mapped and many procedures exist to detect it. Beta-galactosidase breaks down the disaccharide lactose into its two component rings: galactose and glucose, but it will also separate galactose from other ring structures. By using a compound that is made up of a galactose ring attached to a UV-VIS or fluorescence-active ring, total activity of beta-galactosidase can be determined from a change in spectroscopic properties upon hydrolysis of the compound.

The first such compound chosen as a reporter molecule was *ortho*-nitrophenyl-β-galactoside (ONPG), which separates as galactose and *ortho*-nitrophenol (ONP)(Fig. 4). ONP emits light in the yellow range, so the relative activity of beta-galactosidase in a solution can be measured by its absorbance at 420 nm (Agilent). The work-up to the assay was the same as an SIC determination: a starter culture was incubated overnight, then a range of antibiotic cultures near the SIC value were prepared. In order to conserve

time and resources, only tetracycline and oxytetracycline were studied using this assay. After the overnight incubation of the antibiotic cultures, their absorbances at 595 nm were read in order to normalize the cell count.

After absorbance was read, the cell cultures were centrifuged based on their absorbance value. 1.5 mL of the control culture would always be used, and the volume of the SIC culture would be adjusted to ensure the same number of cells in each tube. The cultures were centrifuged for 5 minutes at 13,500 rpm and 4°C. A pellet of cells formed at the bottom of the centrifuge tubes, and the supernatant liquid was drawn off using a pipet. The cells were then resuspended in 200 μL of lysis buffer (Table 2) and allowed to sit at room temperature for 5 minutes. The mixture was centrifuged again for 5 minutes at 13,500 rpm and 4°C, and 50 μL of the supernatant liquid was placed in a microtiter well. 152 μL of buffer A (Table 2) was mixed with 8 μL of β-mercaptoethanol, and 110 μL of that mixture was added to the microtiter well with the lysed cells. This solution was incubated for 5 minutes at 37°C, and 50 μL of ONPG was added. After an additional incubation at 37°C, 90 μL of stop solution was added and the absorbance of each well at 420 nm was read.

This protocol comes from the Agilent β-Galactosidase Assay Kit.

MUG Assay

In addition to the ONPG assay, a more sensitive fluorescence assay was used that took advantage of 4-methylumbelliferyl β-D-glucopyranoside (MUG)(Fig. 5). MUG also contains a galactose ring, and its other half, 4-methylumbelliferone (4-MU)(Fig. 6), fluoresces at ~450nm (λ_{ex}=365nm), and thus reports beta-galactosidase activity as a function of intensity at 450nm (8). As with the ONPG assay, the same two-day process

was used to prepare the cells for analysis. After the antibiotic cultures were incubated for 19 hours, their cell densities were read in order to normalize the cell count. In this case, the cells were normalized to 1.5 mL at an absorbance of 0.35 rather than 0.75 in order to reduce the amount of culture used.

Based on whichever normalization constant was being used, corresponding volumes of cells were centrifuged for 1 minute at 13,500 rpm and 4°C. The supernatant liquid was removed and the pellets were resuspended in 600 μL of the Z-buffer reaction mix (Table 3). 200 μL of the MUG solution (Table 3) was added and the solutions were incubated for 40 minutes at 30°C. After the incubation 400 μL of 1 *M* $NaCO_3$ was added to stop the reaction and the solutions were centrifuged for 5 minutes at 13,500 rpm and 4°C. The fluorescence of the supernatant fluid was then read, using the Z-buffer as a blank.

Results

SIC Determination

The results of the SIC determinations are reported in Table 1, and these values are all based on the data reported in Figures 7 through 11. The ideal SIC value lies at a concentration where the cell density of an antibiotic culture is 50% of the control culture. In some cases, a tested concentration produced this result definitively and the SIC value of that antibiotic was chosen at that specific concentration. In other cases, the 50% mark lay between two tested concentrations, so the SIC value was estimated based on the relative trend of the graph. Based on previous work, the value for tetracycline was estimated to be 4 μg/mL, and this value held up when performing reporter assays.

ONPG Assay

There is no reportable data from the ONPG assay series.

MUG Assay

The results of the MUG assay are reported in Figure 14. The data directly obtained from these experiments were in units of counts per second (cps), and were converted to Miller Units using the standard curve shown in Figure 13. The standard curve was constructed using the fluorescent product of MUG: 4-methylumbelliferone (4-MU). Counts per second represent the intensity of the fluorescence of 4-MU and therefore correspond to the amount of 4-MU present. CPS data were converted to Miller units to reflect the activity of β-galactosidase in the solution. In theory, higher β-galactosidase would indicate an up-regulation of the resistance gene, which could be attributed to the activity of the riboswitch.

Discussion

SIC Determination

It should be noted once more that these SIC values are not intended to be ubiquitous. They heavily depend on the antibiotic used, the quality and age of the antibiotic solution, the specific strain of bacteria, and the application being performed. Tetracycline and its derivatives break down with prolonged exposure to heat and light, so fresh solutions needed to be regularly made. In particular, oxytetracycline was found to have an extremely short shelf life and would give erratic data after only a few days. The standard procedure for making up these stock solutions was to dissolve them in water up to 50 mL, wrap them in aluminum foil to prevent light exposure, and store them at 4°C. This method worked well for most antibiotics, but the ultra-sensitive oxytetracycline was quick to break down under these conditions. New preparations were made with dimethyl sulfoxide (DMSO) as the solvent and the solutions were aliquotted into small centrifuge tubes (~1 mL) and stored in a -20°C freezer. These solutions tended to last much longer and gave more consistent data.

The application of the antibiotic was also found to be important when evaluating SIC values. These values were obtained using bacteria cultured in a liquid enrichment media and held at ideal conditions. Any change in the growth parameters shifted the SIC value up or down. For example, cultures grown on solid media tended to require a higher concentration of antibiotic to reach their SIC value, while some liquid applications required a lower concentration. The reported SIC values are intended to be estimations and will mostly likely need to be adjusted based on the needs of the specific research being performed.

ONPG Assay

Unfortunately, the ONPG experiments did not turn up any useful data. During the incubation after addition of ONPG, a yellow color should develop in any solution containing β-galactosidase, but this color never developed. Even when the absorbances of the solutions were measured at 420 nm, the data were not significant enough to report. It was concluded that the ONPG assay was not sensitive enough for the low concentrations of enzyme being measured, which is why the MUG assay was later employed. Although activation of the riboswitch causes an up-regulation in the production of the efflux pump, the effect is not huge, and subtle changes in concentration can be difficult to measure. The colorimetric assay was simply not able to detect the low concentrations of β-galactosidase effectively, but it was instructive. Many of the processes and techniques from the ONPG protocol were used in the MUG assay, so the transition was not difficult.

MUG Assay

Of the many fluorescent assays available for β-galactosidase detection, this method was chosen due to its high sensitivity, low cost, and relative ease of use. The data shown in Figure 8 is only for oxytetracycline; tetracycline was used mainly as "guinea pig" molecule for optimizing the assay. The graph shows a marginal increase in the activity of β-galactosidase, which would correspond to activation of the riboswitch. However, the usefulness of this data must be called into question due to the large error bars on both columns. The standard error of each data set is at least half the magnitude of the mean, and this casts doubt on any conclusion one would try to draw.

Taking the standard error into account, there are three options for what this data could mean. Option 1: the error is too high and no reliable conclusion can be drawn at all. Option 2: the marginal increase in measured enzyme activity is reasonable evidence for an up-regulation in the resistance gene. This would indicate that oxytetracycline is able to bind to the riboswitch and change it to an "on" conformation. Option 3: enzyme activity of the control culture and the SIC culture are extremely similar and there is no correlation between the presence of oxytetracycline and production of β-galactosidase. A lack of correlation would mean that oxytetracycline is unable to bind to the riboswitch and that it will not trigger this resistance mechanism.

Each option is a reasonable conclusion, and further testing must be done to give this data context. Firstly, more antibiotics must be studied in order to tease out some kind of meaning from the MUG assay data. There is no reference point for the oxytetracycline data, and additional antibiotic studies may make it possible for a more definitive conclusion. These additional tests may also reveal a flaw in the assay and lead to changes in the protocol or selection of a new procedure altogether.

Further normalization may also be necessary in the construction of the data, particularly normalization by total protein content. If the MUG assay were performed alongside a Bradford assay (9), the data might fall into a more predicable pattern. A Bradford assay is typically used to normalize enzymatic activity by measure total protein in a solution. Even though each assay normalizes cell count so that the same amount is present in both cultures, there may be differences in cell breakage, resulting in inaccurate measurements of β-galactosidase activity. By measuring total protein content after cell lysis, this contingency could be accounted for.

Tables/Figures

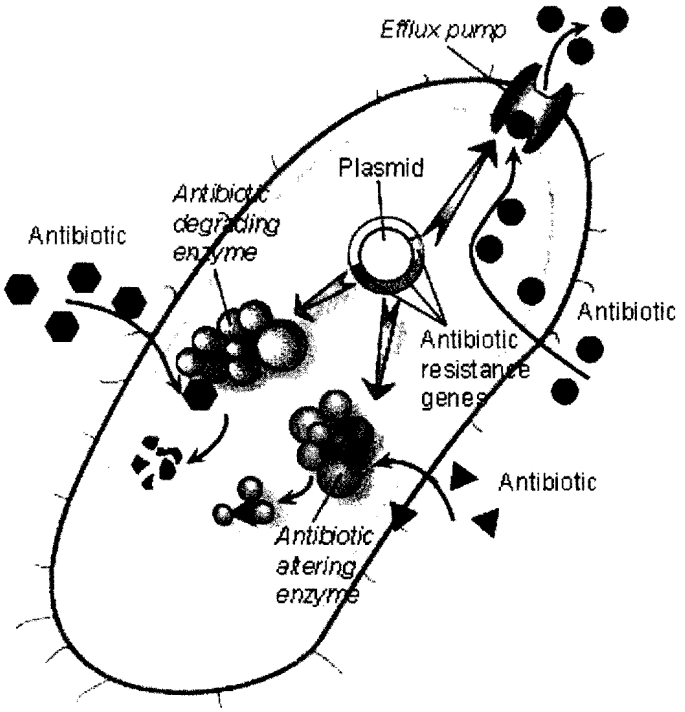

Figure 1: Mechanisms of Antibiotic Resistance

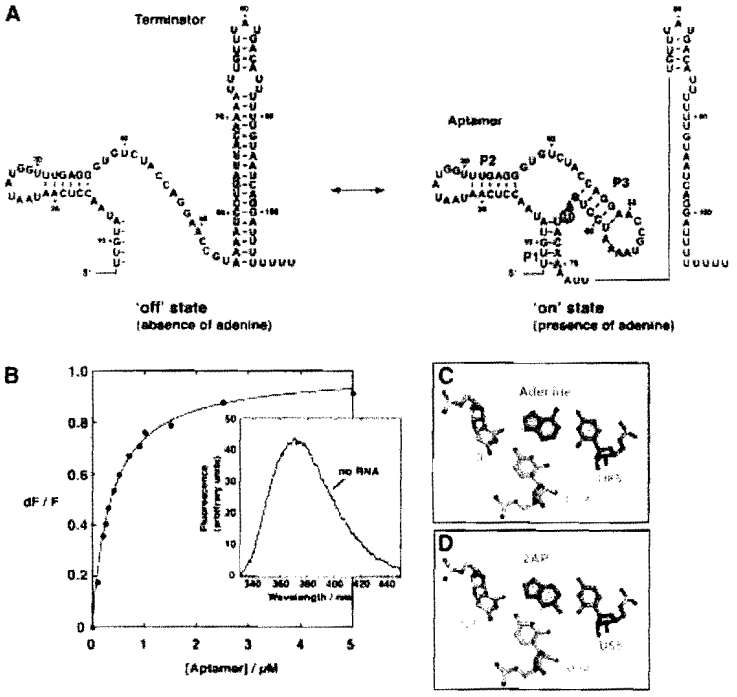

Figure 2: Depiction of the Adenine Riboswitch (4)

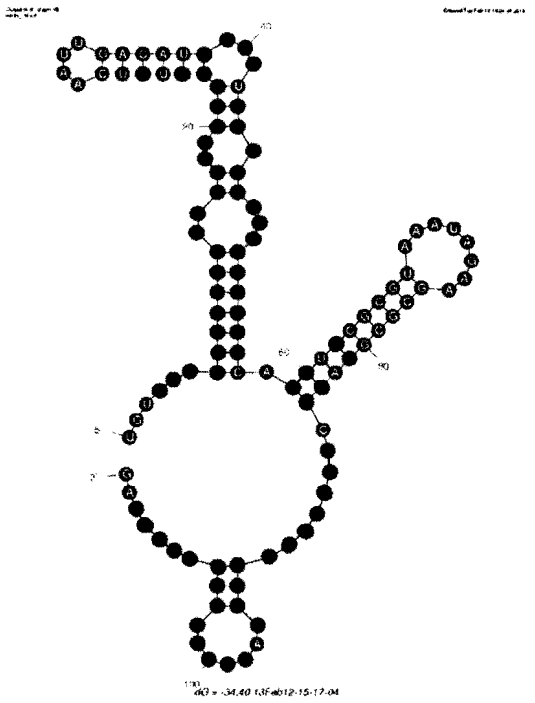

Figure 3: Schematic of the ykkCD Riboswitch Aptamer Domain

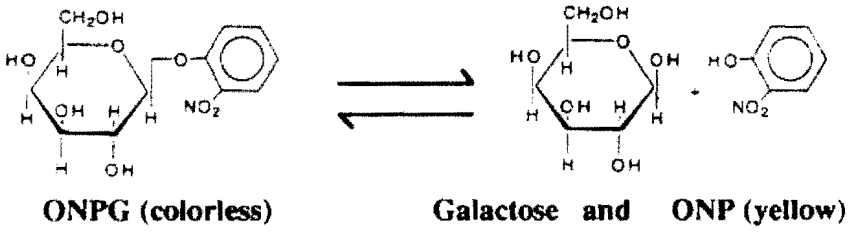

Figure 4: Hydrolysis of ONPG

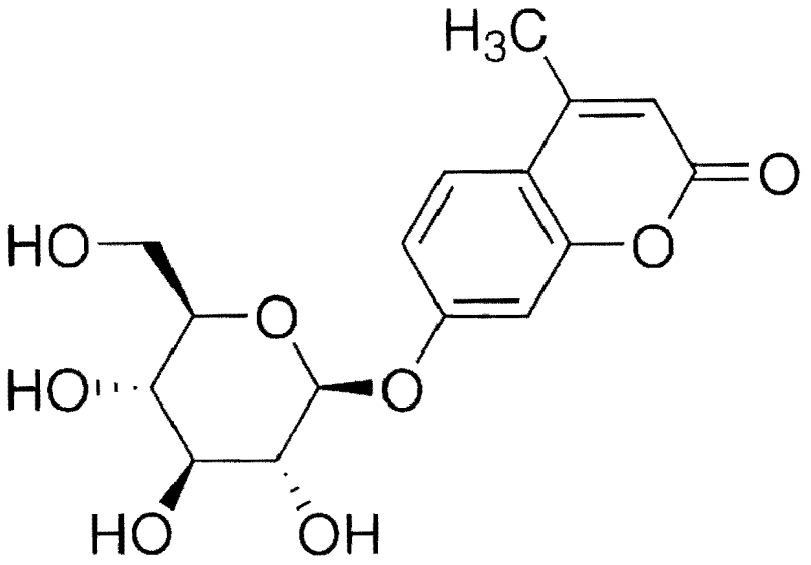

Figure 5: Structure of MUG

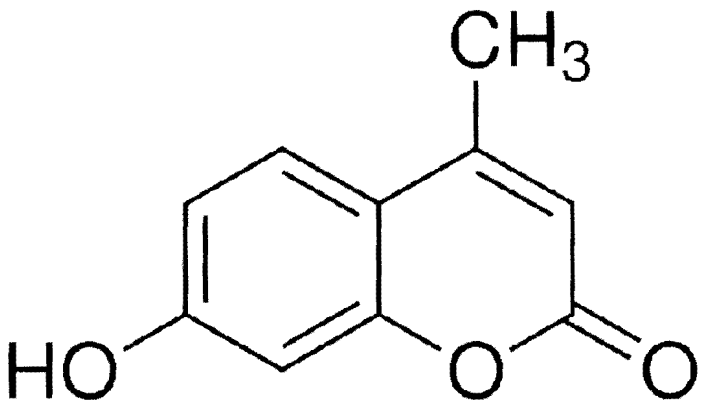

Figure 6: Structure of 4-MU

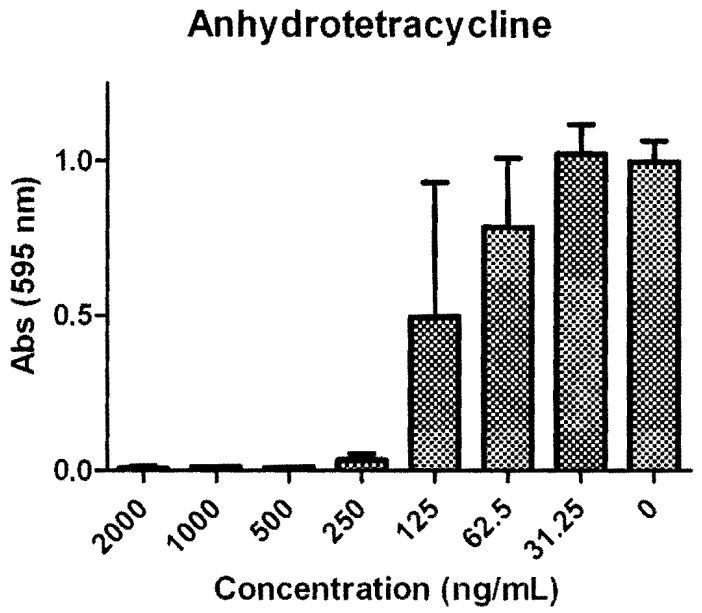

Figure 7: Results of SIC testing using anhydrotetracycline

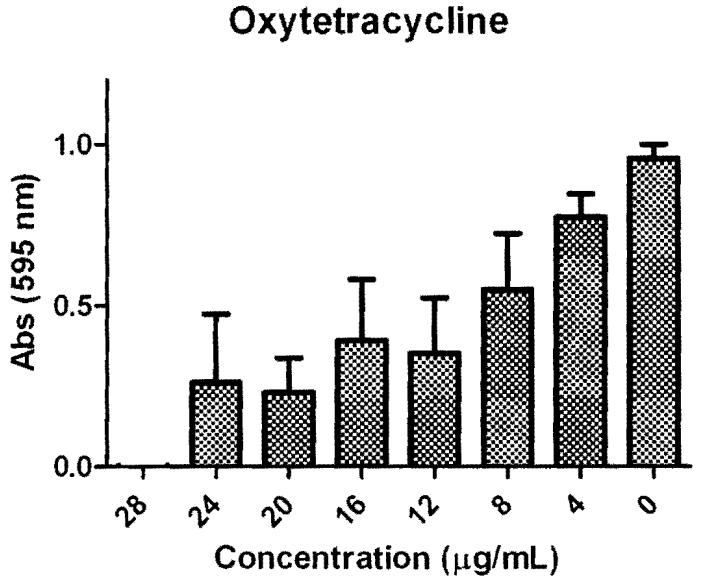

Figure 8: Results of SIC testing using oxytetracycline

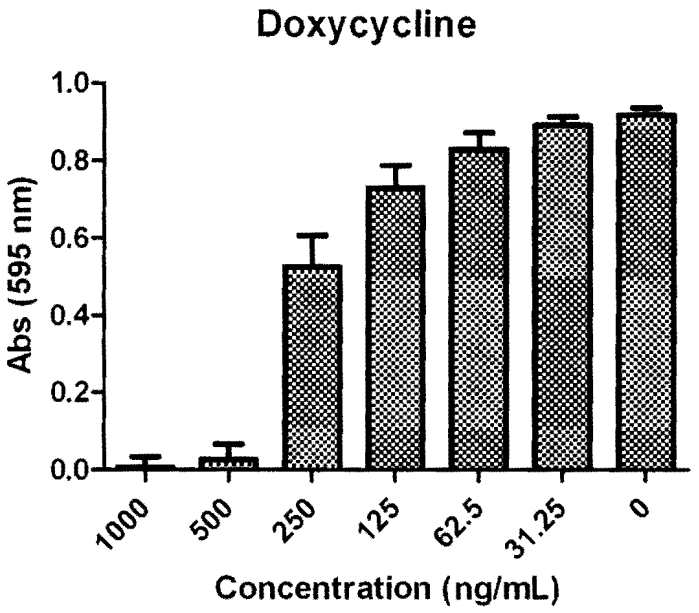

Figure 9: Results of SIC testing using doxycycline

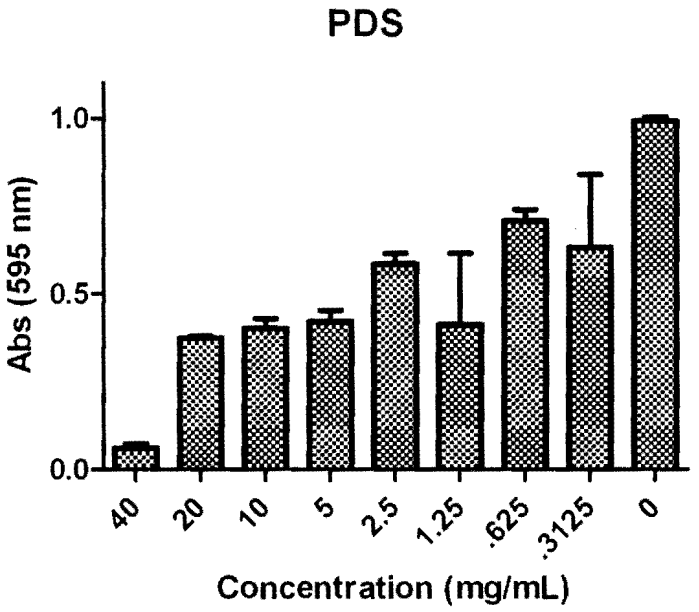

Figure 10: Results of SIC testing using phosphomycin disodium salt

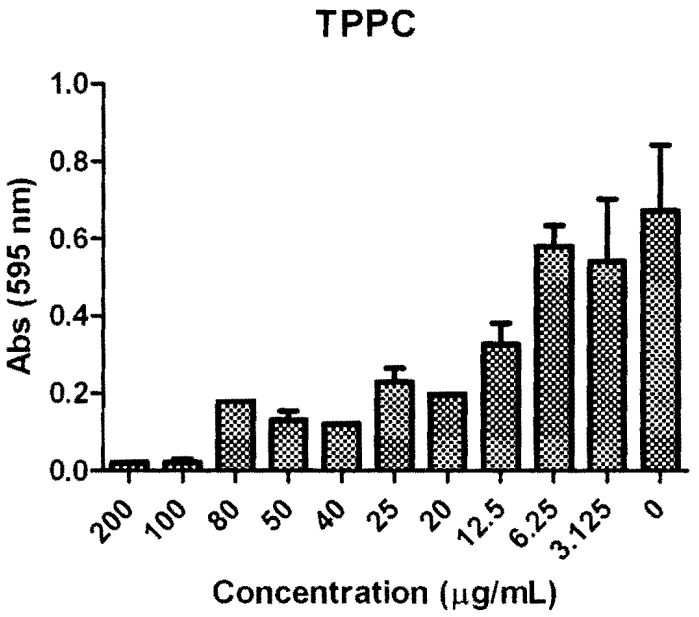

Figure 11: Results of SIC testing using tetraphenylphosphonium chloride

SIC values determined using Figures X-Y	
Antibiotic	**SIC Value**
Anhydrotetracycline	**125 ng/mL**
Oxytetracycline	**8 μg/mL**
Doxycycline	**250 ng/mL**
PDS	**3 mg/mL**
TPPC	**8 μg/mL**

Table 1: SIC values for selected antibiotics

ONPG Assay Components	
Solution Name	**Contents**
Buffer A	**100 mM NaH_2PO_4** **10 mM KCl** **1 mM $MgSO_4$** **pH 7.5**
Lysis Buffer	**Proprietary?**
β-mercaptoethanol	**1 *M* β-mercaptoethanol**
ONPG	**4 mg/mL *ortho*-nitrophenyl-β-galactoside in Buffer A (pH 7.5)**
PBS	**137 mM NaCl** **2.7 mM KCl** **10 mM Na_2HPO_4** **KH_2PO_4** **pH 7.4**

Table 2: Components of ONPG assay

MUG Assay Components	
Solution Name	**Contents**
Z-Buffer	**60 mM Na_2HPO_4** **40 mM NaH_2PO_4** **10 mM KCl** **1 mM $MgSO_4$** **pH 7.0**
Lysozyme	**200 µg/mL**
β-mercaptoethanol	**100 µg/mL**
DNAse 1	**40 µg/mL**
MUG	**50 mM 4-methylumbelliferyl β-D-glucopyranoside in DMSO**

Table 3: Components of MUG assay

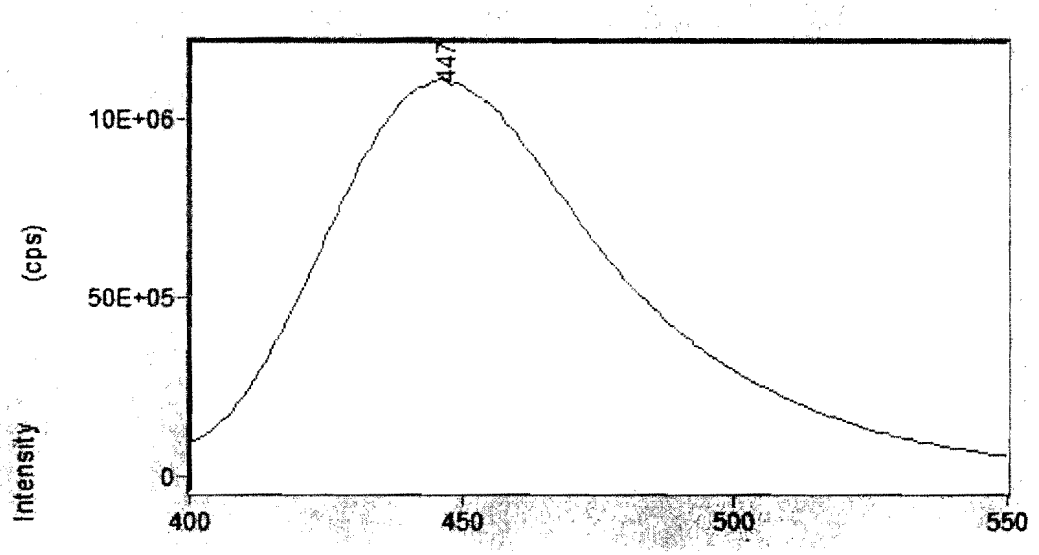

Figure 12: Sample Fluorescence Reading

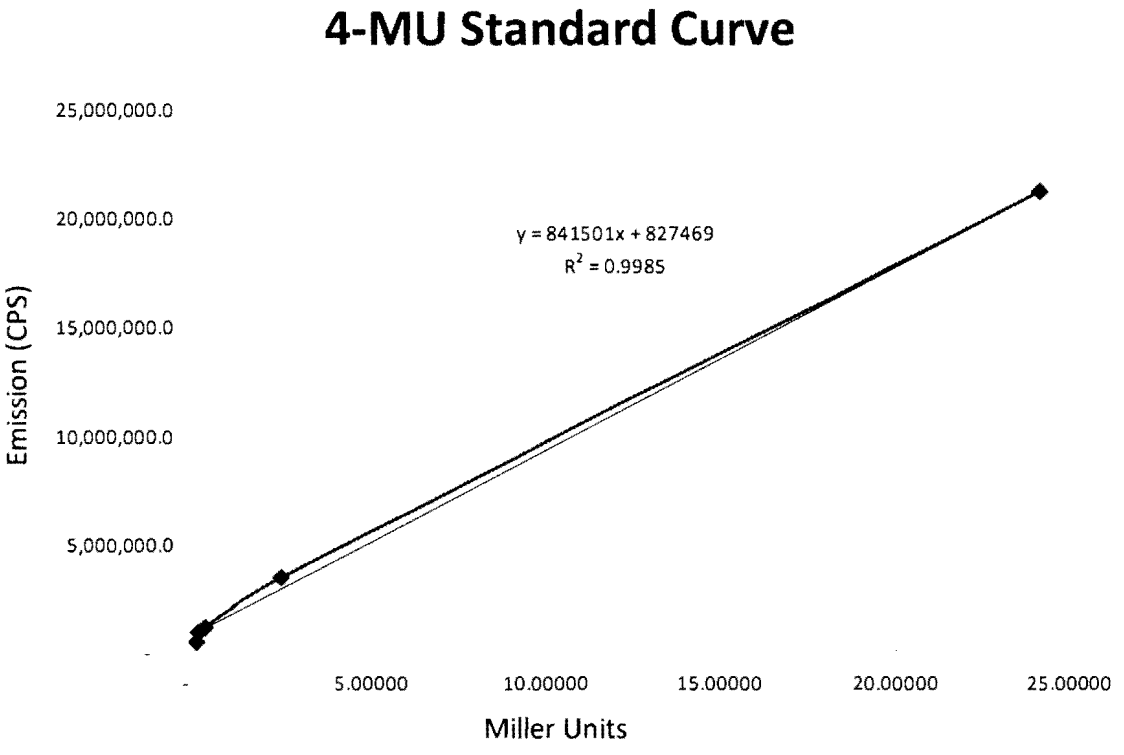

Figure 13: Standard Curve for MUG assay

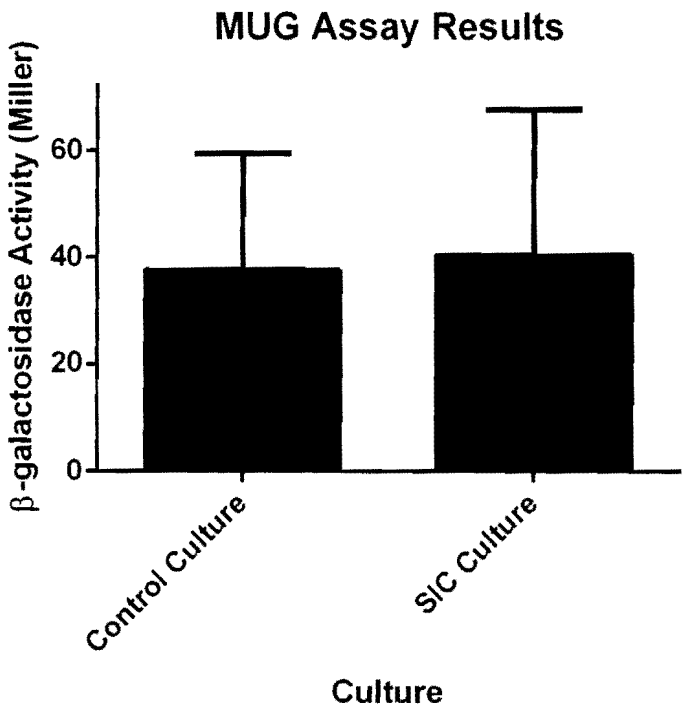

Figure 14: Results of MUG Assays Using Oxytetracycline

References

1. Alliance for the Prudent Use of Antibiotics. The Cost of Antibiotic Resistance to U.S. Families and the Health Care System. http://www.tufts.edu/med/apua/consumers /personal_home_5_1451036133.pdf (accessed Apr 15, 2013).

2. World Health Organization. Antimicrobial Resistance Fact Sheet. http:// www.who.int/mediacentre/factsheets/fs194/en/ (accessed Apr 15, 2013).

3. Barrick, Jeffrey E. et. al. New RNA Motifs Suggest an Expanded Scope for Riboswitches in Bacterial Genetic Control. *PNAS.* **2004**, *101*, 6421-6426.

4. Mandal, Maumita & Breaker, Ronald R. Adenine Riboswitches and Gene Acitivation By Disruption of a Transcription Terminator. *Nature Structural & Molecular Biology.* **2004**, *11*, 29-35.

5. Paulsen, Ian T. Multidrug Efflux Pumps and Resistance: Regulation and Evolution. *Current Opinion in Microbiology.* **2003**, *6*, 446-451.

6. Dale, Jeremy and Malcolm von Schantz. *From Genes to Genomes: Concepts and Applications of DNA Technology*; John Wiley & Sons: Chichester, West Sussex, England, 2007.

7. Li, W. et. al. Scanning Assay of Beta-Galactosidase Activity. *Applied Biochemistry and Microbiology.* **2012**, *48*, 603-607.

8. Harwood, Colin R. and Simon M. Cutting. *Molecular Biological Methods for Bacillus*; Wiley: Chichester, West Sussex, England, 1990.

9. Bradford, Marion M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry.* **1976**, *72*, 248-254.

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