OPTIMIZATION OF A BETA-GALACTOSIDASE FLUORESCENCE REPORTER ASSAY FOR
DETERMINATION OF YKKCD RIBOSWITCH REGULATION IN B. SUBTILIS

A RESEARCH PAPER

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BY

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Optimization of a β-galactosidase Fluorescence Reporter Assay for Determination of ykkCD Riboswitch Regulation in *B. subtilis*

Today, 70% of all bacterial strains are resistant to at least one antibiotic. Bacteria have become resistant to antibiotics through several methods, including mutations in their genome. Antibiotic resistance continuities to grow based on several factors. These include over-prescription of antibiotics by physicians, non-completion of prescribed antibiotic treatments by patients and use of antibiotics in animals as growth enhancers by the food industry. Other causes are increased international travel and poor hospital hygiene. Three methods have been discovered that bacteria can employ to resist antibiotics. Bacteria can degrade, modify, or through the use of an efflux pumps, pump the antibiotic out of cells. The focus of this study is on the third mechanism, the triggering of production of these efflux pumps. Efflux pumps are most commonly regulated by transcription factors. However, RNA sensors may also regulate them. A riboswitch is one example of an RNA sensor capable of changing gene expression patterns in response to internal and external signals. It is a highly structured region of the mRNA that specifically binds to a small target, often the product of the gene to be regulated. Once the concentration of this target molecule reaches a threshold, it binds to the riboswitch which can either turn on or turn off expression of this metabolite-producing gene. The *ykkCD* RNA is a small riboswitch that is suspected to act as an antibiotic sensor in *B. subtilis,* and is encoded immediately adjacent to a multidrug-resistant (MDR) ykkCD efflux pump. It has already been demonstrated that the ykkCD riboswitch sensor specifically recognizes tetracycline. This binding event is expected to trigger production of the ykkCD efflux pump causing the removal of the toxic tetracycline from the cell. If tetracycline or its derivatives interact with the putative ykkCD riboswitch, an increase in the ykkCD pump expression levels *in vivo* would be expected when exposed to tetracycline or its derivatives. Two different procedures are necessary to determine if pump expression levels increase when exposed to tetracycline. First, in order to

evaluate the amount of *ykkCD* pump mRNA, *B. subtilis* cells were grown with and without tetracycline or its derivatives, and ykkCD pump mRNA levels were quantified using quantitative real-time PCR. Second, the amount of ykkCD pump protein produced in the presence of tetracycline and its derivatives was quantified with a fluorescence assay using a *ykkCD* RNA-β-galactosidase reporter gene construct. In this construct the ykkCD pump genes were replaced with the β-galactosidase gene. In the case of antibiotics that retain binding, an increase in pump mRNA levels and high β-galactosidase activity would be expected. Together these two results shed light on the *in vivo* antibiotic specificity of the *ykkCD* RNA. The focus of this paper is the optimization of a β-galactosidase fluorescence reporter assay to study the regulation of the ykkCD efflux pump by tetracycline and its derivatives in *B. subtilis*. Using this procedure and the quantitative real-time PCR, preliminary results indicate that when *B. subtilis* cells containing the *ykkCD-lacZ* construct were grown with the antibiotic tetracycline β-galactosidase activity increased, indicating upregulation of gene expression. This would seem to indicate that the presence of tetracycline turns on the gene for the production of a pump for the removal of itself.

Introduction

Today, 70% of all bacterial strains are resistant to at least one antibiotic[1]. Bacteria have become resistant to antibiotics through several methods, including mutations in their genome. Antibiotic resistance continuities to grow based on several factors. These include over-prescription of antibiotics by physicians, non-completion of prescribed antibiotic treatments by patients and use of antibiotics in animals as growth enhancers by the food industry. Other causes include increased international travel and poor hospital hygiene[2]. Three methods have been discovered that bacteria can employ to resist antibiotics. Bacteria can degrade, they can modify, or they can pump out the antibiotic through the use of an efflux pump[3]. The focus of this study is on the third mechanism, the triggering of production of these efflux pumps. Efflux pump production and usage requires a significant amount of energy, leading to this process almost always being regulated.

Efflux pumps are most commonly regulated by transcription factors. However, RNA sensors may also regulate them. A riboswitch is one example of an RNA sensor capable of changing gene expression patterns in response to internal and external signals. Located 100 to 200 nucleotide elements upstream of the target gene in the genome, a riboswitch is a highly structured region of the mRNA that specifically binds to a small target molecule (Figure 1)[4]. The small molecule is often the product of the gene to be regulated. Once the concentration of this molecule reaches a threshold, it binds to the riboswitch that can either turn on or turn off expression of this metabolite-producing gene. There is no need for a protein cofactor because there is direct binding between the riboswitch and the metabolite that triggers or shuts down expression[5].
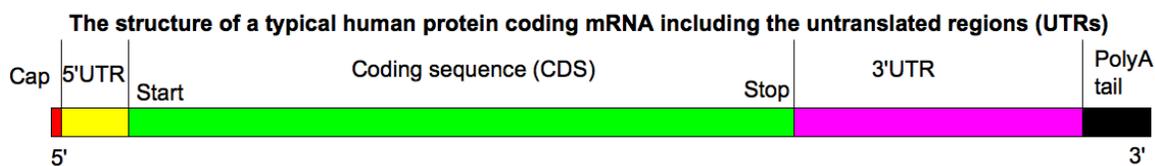

The structure of a typical human protein coding mRNA including the untranslated regions (UTRs)

Figure 1. Riboswitches are found in the 5' untranslated region (UTR) of mRNA[6].

Riboswitches have been shown to be highly specific sensors. Numerous classes of riboswitches are present in bacteria. Riboswitches comprise a common metabolite-sensing system. There have been nearly 200 unique riboswitches categorized into ten distinct classes, with each class having up to nineteen known examples of riboswitches per class[7]. When the riboswitch detects these small molecules in the cellular environment, it regulates gene expression by allosteric structural changes. Most riboswitches are made up of two regions: an aptamer domain and an expression platform. The aptamer domain in each class of riboswitches binds to the same metabolite and shares a highly conserved sequence and secondary structure. The expression platform is responsible for gene control by these allosteric structural changes (Figure 2)[8].
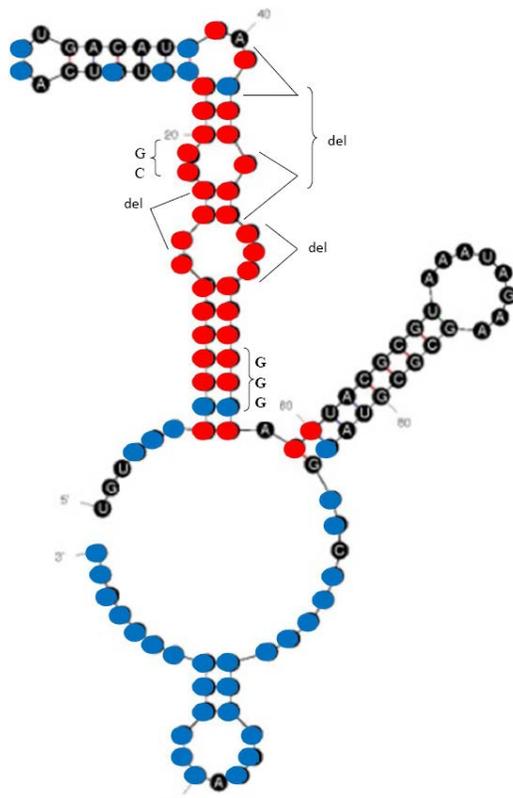
Figure 2. The secondary structure of the *ykkCD* riboswitch aptamer domain of non-binding mutants. Red indicates bases that are 100% conserved, blue indicates bases that are frequently conserved, and black indicates little conservation. The highest sequence conservation is clustered on the central bulge of the riboswitch indicating the importance of this region to tetracycline binding.

In order to demonstrate that pump expression is regulated by an antibiotic, it must meet three criteria: a) the pump expression must respond to the antibiotic; b) something must specifically recognize the antibiotic; and c) this recognition must trigger a conformational change which allows for the synthesis of the open reading frame of the pump. Since some riboswitches regulate at the transcription stage, and others regulate the translation stage, this structural change would either unfold a transcription terminator stem or uncover a ribosomal binding site, allowing for production of the pump gene[9]. Figure 3 illustrates these processes.
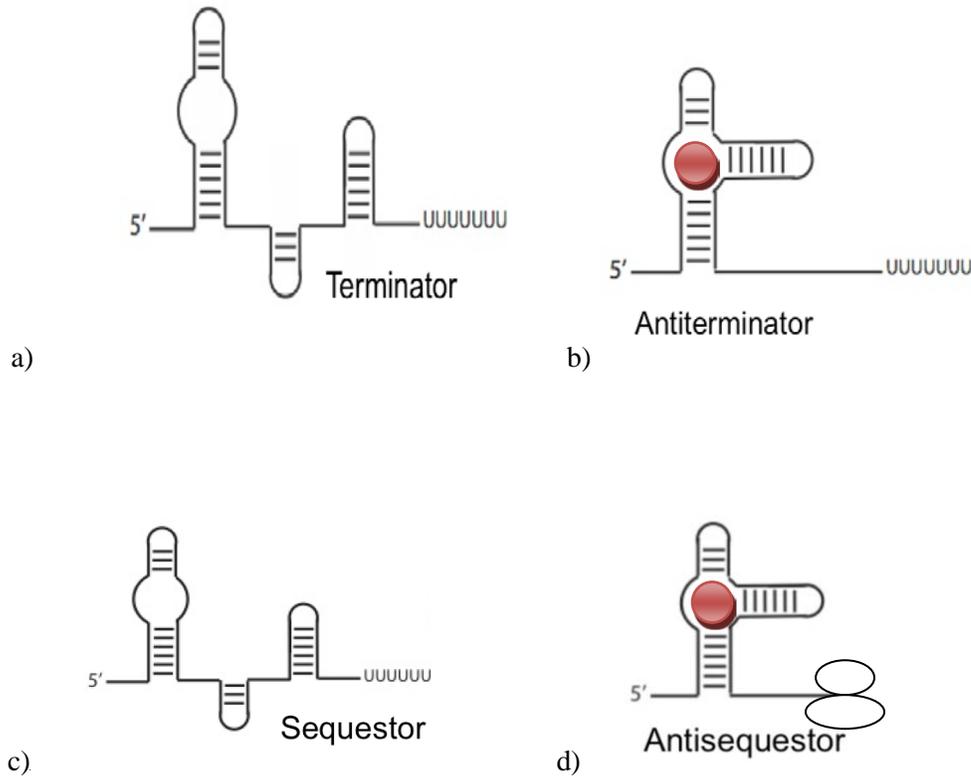
Figure 3. Transcription regulation. a) In the absence of antibiotic, because the terminator stem is present, transcription is halted preventing mRNA for the efflux pump from being made. b) When enough antibiotic is present, it binds to the aptamer domain causing formation of the antiterminator structure that does not include the terminator stem, allowing transcription of the pump mRNA. Translation regulation. c) In the absence of antibiotic, because the ribosomal binding site is not accessible, translation is halted preventing protein for the efflux pump from being made. d) When enough antibiotic is present, it binds to the aptamer domain causing formation of the antisequestor structure that exposes the ribosomal binding site, allowing translation of the pump protein.

The *ykkCD* RNA is a small putative riboswitch that is suspected to act as an antibiotic sensor in *B. subtilis* and is encoded immediately adjacent to a multidrug-resistant (MDR) ykkCD efflux pump. The putative ykkCD riboswitch is likely to be an antibiotic sensor and regulates expression of the ykkCD pump for the following reasons: a) it is located upstream of an MDR efflux pump gene, b) it is found in pathogenic bacteria, c) more than 80% of its sequence is conserved, d) occurs 19 times in *B. subtilis*, and e) it does not resemble the binding site for transcription factors[4].

In order to demonstrate that up-regulation of gene expression is due to the *ykkCD* RNA acting as a riboswitch, it must be shown that the *ykkCD* RNA specifically binds to an antibiotic that is a ligand of

the pump, and that this binding alters the conformation of the *ykkCD* RNA leading to up-regulation of gene expression. Two methods, footprinting and mutagenesis, were used to demonstrate that tetracycline binds specifically to the *ykkCD* RNA. Footprinting helped to identify the sites of antibiotic binding because, in the binding sites, the nucleotides were protected from fragmentation in the presence of tetracycline. Further studies will allow the structure of the RNA to be visualized by using nucleases with difference specificity. Thus if a major structural change is needed for tetracycline binding, it will be apparent from analyzing footprinting patterns. Mutagenesis studies are necessary as a complementary method, because sites of protection could occur as a result of a conformational change instead of the binding of tetracycline. To distinguish between these two possibilities, the site-directed mutagenesis studies test which part of the regulatory element is important for antibiotic recognition. This is because conserved nucleotides that cause impaired tetracycline binding when altered are likely to be important for binding. These studies together revealed that the 5' highly conserved stem of the ykkCD is responsible for specifically recognizing tetracycline.

We have demonstrated that the ykkCD riboswitch sensor specifically recognizes tetracycline. This binding event is expected to trigger production of the ykkCD efflux pump causing the removal of the toxic tetracycline from the cell. If tetracycline and its derivatives interact with the putative ykkCD riboswitch, an increase in the ykkCD pump expression levels *in vivo* would be expected when exposed to tetracycline or its derivatives. Two different procedures were set up to determine if pump expression levels increase when exposed to tetracycline. First, in order to evaluate the amount of *ykkCD* pump mRNA, *B. subtilis* cells were grown with and without tetracycline or its derivatives, and ykkCD pump mRNA levels were quantified using quantitative real-time PCR (qRT-PCR). In those samples with greater starting amounts of the specific mRNA, the amplification would be expected to rise to detectable levels before those with less target mRNA. However, this method uses the assumption that mRNA levels correlate well with protein levels, which is not always the case. Therefore, the amount of ykkCD pump protein produced in the presence of tetracycline or its derivatives was also quantified using a *ykkCD* RNA-β-galactosidase reporter gene construct. In this construct the ykkCD pump genes were replaced

with the β-galactosidase gene using a double crossover event, followed by the quantification of β-galactosidase enzymatic activity with a fluorescence assay. In the case of derivatives that fail to bind tetracycline, low pump mRNA levels and low β-galactosidase production would be expected. In the case of derivatives that retain binding, an increase in pump mRNA levels and high β-galactosidase activity would be expected. Together these two results shed light on the *in vivo* antibiotic specificity of the *ykkCD* RNA. Figure 4 shows the structure of tetracycline and its derivatives. The differences in structure are highlighted in red.
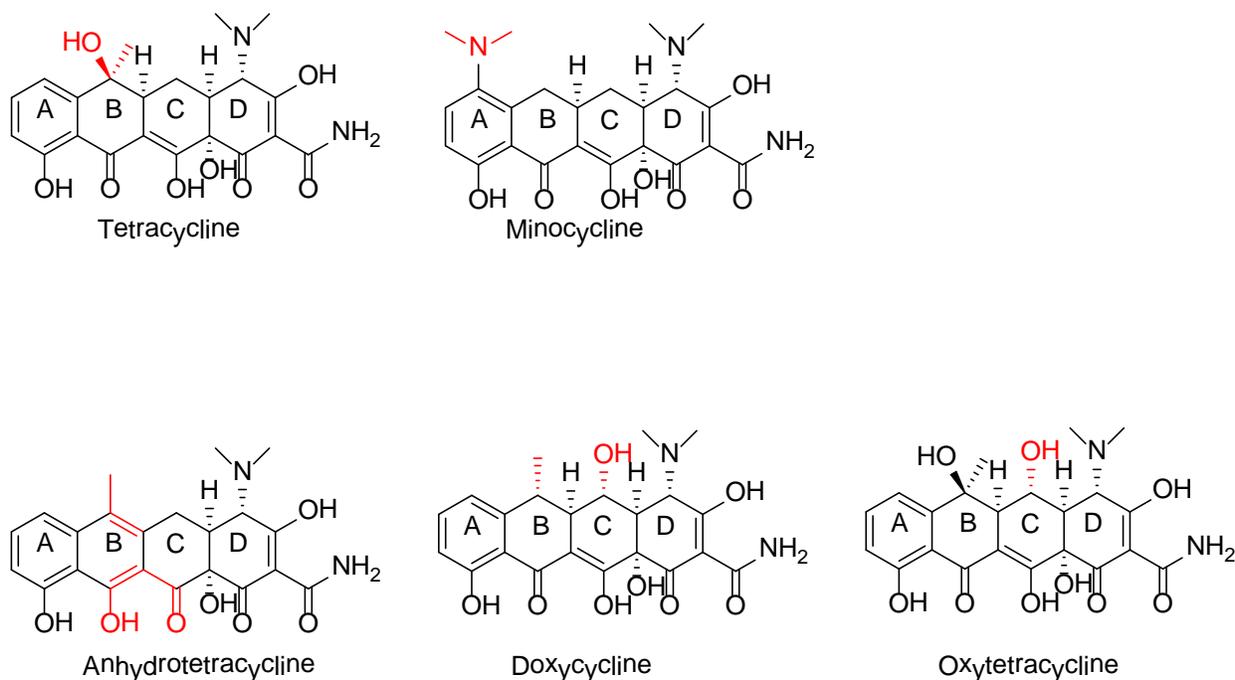

Figure 4. Structure of tetracycline and its derivatives. Changes from tetracycline are shown in red.

Results

The β-galactosidase assay using the conventional ortho-Nitrophenyl-β-galactoside (ONPG) substrate was not sensitive enough based upon assays testing a *B. subtilis* strain with known β-galactosidase activity. This assay measures absorbance of the product of the enzymatic reaction at

420nm. Therefore, a fluorescence assay was chosen using 2-nitrophenyl-β-D-galactopyranoside (4-MU) as substrate to test the β-galactosidase activity of the *B. subtilis* strain containing the *ykkCD-lacZ* construct. To increase the accuracy of the assay and to reduce experimental error the following a Bradford assay normalizing β-galactosidase activity by protein content of the sample (per 1 mg protein) was also deemed necessary. This was to allow for experimental errors and to account for fluctuation of total protein content in each sample. As tetracycline and its derivatives are known protein synthesis inhibitors it is expected that in cells grown with these antibiotics protein synthesis is inhibited and thus total protein content is reduced. An optical density reading at 600 nm (OD_{600}) was taken for each culture to ensure that equal number of cells was processed in each assay. Experimental errors may arise from the settling of cells in cultures leading to incorrect OD_{600} measurements or form pipetting errors. The normalization data of the Bradford assay was performed the same time as the β-galactosidase assay to further safeguard against data fluctuation. Figure 5 illustrates the advantages of normalizing β-galactosidase activity by protein content using Bradford assay. For example, in trial 84 involving cells grown without an antibiotic (AB) and cells grown with 15 µg/mL of minocycline, the outcome of a β-galactosidase assay (activity increase or decrease) was influenced by this protein normalization procedure. Without the protein normalization, the number of Miller units for cells grown without AB was 53.7 MU vs. 38.7 MU for those grown with minocycline, leading to a conclusion that β-galactosidase activity decreased in response to minocycline. After protein normalization, which factors in the total protein content of the cell cultures, the numbers changed to 154.4 MU vs. 159.9 MU respectively, leading to a conclusion that β-galactosidase activity indeed slightly increased in response to minocycline.
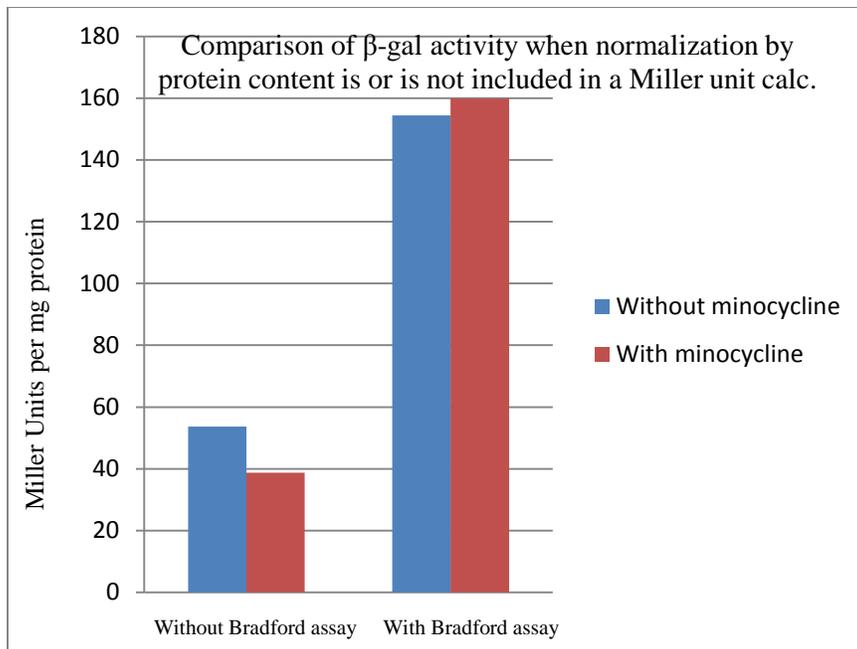
Figure 5. Comparison of β-galactosidase activity of *B. Subtilis* cells grown with and without minocycline: data processed without normalization to total protein (left); data processed with normalization to total protein (right).

The overall results of the β-galactosidase activity assay, including normalization for total protein using Bradford assay, for cell cultures grown with and without different antibiotics is shown in Figure 6.
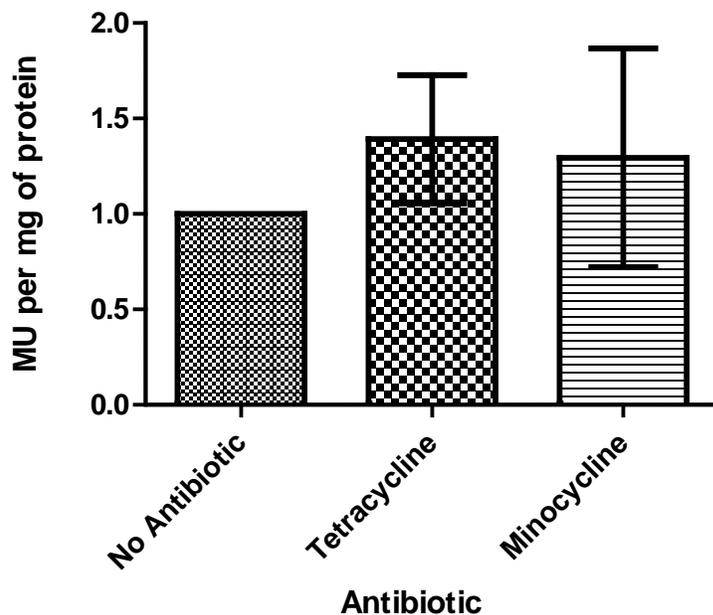
Figure 6. Comparison of Miller Unit values per mg of protein for cultures grown without antibiotic vs. cultures grown with tetracycline or minocycline using a β-galactosidase fluorescence reporter assay per 1mg total protein.

These results indicate that when *B. subtilis* cells containing the *ykkCD-lacZ* construct were grown with the antibiotic tetracycline expression of the β-galactosidase was upregulated as shown by an increase of β-galactosidase activity in cells grown with tetracycline. The presence of tetracycline is associated with an increase in β-galactosidase activity, possibly indicating that the tetracycline turns on expression of the ykkCD pump gene to remove the toxic tetracycline from the cells. This conclusion can't be made for the same type of cells grown with minocycline, because β-galactosidase of cells grown with and without minocycline are the virtually the same within error. More experimental trials need to be performed with minocycline to reduce experimental error before a conclusion can be made. In addition, other antibiotics and ykkCD RNA mutants will be tested using *B. subtilis* cells containing the *ykkCD-lacZ* reporter construct.

Discussion

To test how tetracycline and its derivatives contribute to riboswitch function *in vivo,* two different procedures were used. This is because, although protein expression levels can usually be estimated by measuring the intracellular mRNA concentration for the gene of interest, the assumption that mRNA levels correlate well with protein levels is not always valid. Therefore both mRNA and protein levels were measured. Both of these methods involve the growth of *B. subtilis* cells in media with and without tetracycline or its derivatives. The mRNA method involves quantifying levels of the ykkCD gene in *B. subtilis* through measuring ykkCD pump mRNA levels by qRT-PCR. Prior to the qRT-PCR, the mRNA was extracted, and its concentration and quality were determined. The other method involves the quantification of protein production through the use of the β-galactosidase assay. Because quantifying the level of a particular protein in the cell is difficult in the absence of a good antibody against the protein, this particular assay was performed as a means of estimating pump gene expression *in*. This assay uses a ykkCD RNA-β-galactosidase reporter gene construct where the riboswitch regulates the expression of the β-galactosidase gene. The reporter assay is most useful to test the *in vivo* effect of ykkCD RNA mutants that were shown not to recognize tetracycline *in vitro*. These mutants were created for the purpose of identifying which region of the ykkCD RNA is responsible for recognizing tetracycline, thus when *in vitro* binding to tetracycline does not occur, a situation that would be expected for some mutants when mutations, increase in β-galactosidase activity is not expected *in vivo*. In this situation, using qRT-PCR would not be possible, because that assay uses wild type *Bacillus* strains, but a reporter assay uses cloned ykkCD RNA where the relevant mutations could be created and tested. Another advantage of this procedure is that reporter assays tend to be simple and can thus be performed by less trained personnel, for example to use it as a high-throughput screening process.

Both experiments described in the previous paragraph involving the qRT-PCR or the β-galactosidase assay used various antibiotics including tetracycline, minocycline and oxytetracycline. In the presence of either tetracycline or minocycline, expression of the ykkCD gene quantified through qRT-

PCR was shown to be upregulated[10]. Oxytetracycline was not tested. When the β-galactosidase assay

was used, tetracycline was shown to upregulate gene expression while the minocycline did not.

Oxytetracycline was not tested. Laura Howell from our lab performed a primer extension experiment,

another method of quantifying mRNA levels *in vivo* in the presence of various antibiotics. This assay

quantified ykkC mRNA using a specific primer and it is a less-sensitive endpoint version of RT-PCR.

Her results indicated an increase of ykkC pump mRNA levels in *B. subtilis* cells grown with tetracycline

and oxytetracycline. Those grown with minocycline showed no change in ykkCD mRNA levels. Table 1

summarizes the findings of Nicholas Frecker, Laura Howell and my experiments.

Table 1: Summary of results using qRT-PCR, primer extension and β-galactosidase assays for three antibiotics.

Antibiotic	qRT-PCR	Primer extension	β-galactosidase assay
Tetracycline	upregulation	upregulation	upregulation
Minocycline	upregulation	no change	no change
Oxytetracycline	NA	upregulation	NA

Based on these data we can conclusively say that tetracycline upregulates expression of the ykkCD pump

in *B. subtilis* (all three methods reach the same conclusion). Oxytetracycline was not tested with all three

methods and the results for minocycline were in disagreement (a slight upregulation was only shown with

qRT-PCR). Since the minocycline concentrations used to grow cells were below the K_D value of the

ykkCD RNA-minocycline complex (Table 2) one way to reconcile this discrepancy is to point out that

qRT-PCR is the most sensitive of these methods. Thus it is plausible that a slight upregulation is

detectable with qRT-PCR and not with the other methods. These results also agree with the data

summarized in Table 2 where both tetracycline and oxytetracycline used are at saturation with respect to

binding such that upregulation of gene expression is expected (even for the weak binder oxytetracycline).

For minocycline, because binding is not saturated and the K_D value is low with respect to the SIC

concentration, significant upregulation would not be expected. We must stress that SIC values used for a

given antibiotic are a result of empirical determination of the antibiotic concentration that resulted in 50% reduced growth of cells and is solely dependent of the strain used and not a value that can be adjusted by the experimenter. Thus when data from qRT-PCR, reporter assay and *in vitro* binding assays are analyzed together we must keep in mind what we can expected at SIC level antibiotic concentrations based on binding assays performed *in vitro*.

Materials and Methods

Cloning & strain validation of ykkCD-βgal construct. In the reporter-gene construct, the ykkCD pump ORF will be replaced with the β-galactosidase ORF. The process involves cloning of the *ykkCD-lacZ* construct, preparation of competent *B. subtilis* and transformation of the *B. subtilis* with the plasmid construct. This *ykkCD-lacZ* construct was generated using the pDG1661 plasmid vector (Bacillus Genetic Stock Center). This plasmid has a polyclonal site, a copy of the *lacZ* gene flanked by forward and reverse fragments of the *B. subtilis amyE* locus. Using a double crossover event, these flanking sequences allow integration of the *ykkCD-lacZ* construct into *B. subtilis*. The *cat* gene is also integrated into the *amyE* locus replacing the *ery^R* gene with a chloramphenicol-resistance gene. The ykkCD riboswitch including its promoter region was PCR amplified using *B. subtilis W23* strain genomic DNA as a template and the following primers: top primer 5' GCG **GAATTC** AAAATCTTACAATTGAA<u>AGATAGAG</u>ctgag 3' (**EcoRI**), bottom primer 5' GCG **GGATCC** TTTGGAGTCATTCCTTTCTATCTAT 3' (**BamHI**). The amplicon and the pDG1661 vector were treated with EcoRI and BamHI restriction enzymes (NEB), ligated and transformed into *E.coli* Dh5α cells. The resulting plasmid DNA was purified using standard procedures and transformed into *B. subtilis* strain 1A771. Prior to transformation, these cells were made competent using the following protocol. Two colonies of *B. subtilis* 1A771 were placed into 5 mL of HS medium (66.5 mL milliQ water, 10 mL 10X S-base, 2.5 mL 20% glucose, 5 mL 0.1% tryptophan, 1 mL 2% casein, 5 mL 10% yeast extract, 10 mL 8% arginine/0.4% histidine) and grown at 37°C overnight. 500 µL of this culture was placed into 50

mL of HS medium and incubated with shaking at 37°C, after which an OD_{600} measurement was taken every 20 min until stationary phase was achieved. 10 mL of culture was harvested every 15 min for the next hour. 1 mL of sterile 87% glycerol was added to each portion and cooled on ice for 15 min. Each sample was aliquotted and frozen in liquid nitrogen for approx. 1 min. Samples were stored at -80°C. Following the competency procedure, transformation into *B. subtilis* strain 1A771 was performed using the following protocol. The competent 1A771 *B. subtilis* cells were thawed in a 37°C water bath. The contents were poured into 20 mL of LS medium (80 mL MilliQ water, 10 mL 10X S-base, 2.5 mL 20% glycerol, 500 µL 0.1 % DL-tryptophan, 500 µL 2% casein, 5 mL 2% yeast extract, 250 µL 1 mM $MgCl_2$, 50 µL 1 mM $CaCl_2$) in a 250 mL Erlenmeyer flask and incubated in 30°C water bath with low shaking for 2 hours. The cell culture was separated into 1-mL portions in a sterile test tube for each plasmid. 10 µL of 0.1 M ethylene glycol tetraacetic acid (EGTA) was added. This was incubated at room temperature (RT) for 5 min, 3-4 µg of pDG1661-ykkCD plasmid DNA was added, and incubation was performed in a 37°C water bath at 220 rpm for 2 hours. The culture was transferred to sterile 1.5-mL centrifuge tubes and centrifuged gently at ~3000 rpm for 5 min. Approx. 900 µL of supernatant fluid was removed and the pellet was re-suspended in the remaining 100 µL. The suspension was spread onto LB agar plates containing 5 μg/mL chloramphenicol and grown for approx. 18 hours at 37°C.

Beta-galactosidase assay. The specific activity of β-galactosidase was determined with 2-nitrophenyl-β-D-galactopyranoside (4-MU) as a substrate and expressed as nanomoles of 4-MU hydrolyzed per minute per μg of total protein (mU/μg). The protocol for the β-galactosidase assay consisted of growing 1A771 cells containing the *ykkCD-lacZ* construct in LB medium with and without antibiotic for 18-19 hours.

The cells were grown as a series of cultures using antibiotic concentrations slightly above and below the SIC value for the particular antibiotic used. The cultures processed further with the β-galactosidase assay were 1) the culture without antibiotic and 2) the culture closest to an OD_{600} value of 50% of the culture without antibiotic. The SIC value for a particular antibiotic was determined through

growth for 18-19 hours at various antibiotic concentrations. The SIC value is the average concentration at which the OD_{600} reading is 50% of the culture without antibiotic, which equals approx. half the bacterial growth over that period. Some SIC values for antibiotics are listed in Table 2.

Table 2: SIC values for tetracycline and its derivatives as compared to KD values of tetracycline derivative-ykkCD RNa complexes determined in vitro using fluorescent quenching.

Antibiotic	SIC, mg/mL	SIC (M)	K_D (nM)	Binding at SIC
Tetracycline	0.004	8.32E-6	(1.07±4.5)E-8	at saturation
Doxycycline	0.0004	7.80E-7	1.5 E-6	below K_D
Anhydrotetracycline	0.0005	1.08E-6	(2.45±9.2)E-8	at saturation
Oxytetracycline	0.019	3.82E-5	4E-6	at saturation
Minocycline	0.00004	8.10E-8	(9.74±17.2)E-8	below K_D

Mixing Buffer was prepared prior to the end of the 18 to 19-hour period by using the following ingredients *per pair of samples*: 1530 μL Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), 31.5 μL chicken lysozyme (Sigma), 8.4 μL DNase I 7500 U/mL (NEB), and 2.8 μL β-mercaptoethanol (Sigma)). Buffer 2 was prepared just prior to the end of the 18 to19-hour growth period by using the following ingredients *per pair of samples*: 230μL of 200 μg/mL 4-methylumbelliferyl β-D-glucopyranoside (MUG) (Sigma) and 920 μL Z-buffer. An equal number of cells were harvested based upon the measured optical density (OD) of the culture, according to the equation: (0.375 mL)/OD = number of μL of culture to process. These cultures were pelleted by centrifuging at high speed for 1 min and re-suspended in 600 μL of Mixing Buffer. The protein standard curve was prepared using solutions of bovine serum albumin (BSA) at the following concentrations: 0.1, 0.25, 0.50, 1.00 and 1.40 μg/mL. Into a flat-bottomed, transparent 96-well plate was placed 5 μL of each of these five standards and 5 μL of each sample (cells plus Mixing Buffer – no dilution necessary), as well as a blank consisting of pure Bradford solution. 250 μL of Bradford solution was added to each well. Following incubation at RT for 20 min, OD_{600} readings were taken for the 96-well plate. During this 20-minute incubation period for the 96-well plate, 200 μL of Buffer 2 was added to the samples of cells in Mixing Buffer, and incubation was

performed at 29-30ºC for 40 min. 400 µL of Stop Solution (1M Na₂CO₃) was added followed by centrifugation at high speed for 5 min. Fluorescence readings were taken using the following parameters: Fluoromax®-3 fluorimeter, excitation value of 365 nm, emission value of 400-550 nm, scan with an integration time equal to 0.250 seconds, and a volume of 100 µL in the cuvette. The blank solution was prepared with 25 µL of Buffer 2, 50 µL of Stop solution and 75 µL of Mixing Buffer. The following concentrations of 4-MU were used for the fluorescence standard curve: 100, 200, 300, 400, 500, 750 and 1,000 nM, with the five standards chosen that best encompass all of the sample readings. For data manipulation, the 4-MU concentrations were multiplied by 0.1 mL (100 µL cuvette) causing the conversion to pmol, then divided by 40 (incubation time) to obtain Miller Units. These values (x-axis) were plotted vs. the measured fluorescence (y-value) in order to determine the Miller Units of the samples based on their fluorescence values. Once the Miller Units were determined, the protein standard curve was used to normalize these Miller Unit values based upon their protein content.

Cloning of mutant ykkCD- βgal constructs. The structure of *B. subtilis* ykkCD putative riboswitch was compared to several other specimens' secondary structures predicted by Mfold. Structural elements within the toxin sensor that did not vary throughout evolution, or invariable blocks, were identified by visual inspection of structures predicted by Mfold. The sequences were then put into Multialin to identify the regions of sequence conservation (invariable blocks). After selecting specific mutations of these invariable blocks, four mutations were generated (see Table 3 for primer sequences). Primers were synthesized by Integrated DNA Technologies (IDT Inc.). The plasmids were prepared for mutation using QuikChange Site-Directed Mutagenesis Kit (Stratagene). The first step in this procedure, PCR, was done with the following procedure. To a PCR tube, 25µL of Pfu master mix buffer, 1 µL of the top primer, 1 µL of the bottom primer, 1 µL of wild type DNA, and 23 µL of RNase free water were added. All liquids were centrifuged before being placed into the PCR. After the DNA was made, 1 µL of Dpn1 (NEB) enzyme was added to digest the wild type DNA. Once the wild type DNA has been digested the transformation can be completed. The remaining mutant DNA was transformed into Dhα5 supercompetent cells for Mid-Scale plasmid prep. Transformation was performed as follows. 10 µL of

the mutated DNA was added to 100 µL of the Dh5α competent cells. The cells were stored on ice for 20 min. The cells were heat shocked for 2 min at 42°C. The cells were cooled on ice for 2 min. The cells were incubated and grown in a shaker at 37°C, 120 rpm for one hour. 900 µL LB media was added to labeled sterile glass tubes. The 110 µL of cells were transferred to the media in the glass tubes. After the cells were grown, the cells were transferred to sterile centrifuge tubes. The cells in solution were spun for 2 min. 900 µL of supernatant was removed and the cells were re-suspended in the remaining 100 µL of LB media. The cells were plated on LB agar with 150 µg/mL ampicillin (Sigma, #A0166-25G). The plate was placed into a 37°C incubator overnight for approx. 19 hours. Once the colonies were grown, they were inoculated in 20-40 mL of LB broth and grown overnight for approx. 19 hours. The plasmid DNA was purified from the cell cultures using the QIAGEN Plasmid *Plus* Midi Kit according to the manufacturer's directions. Mutant *ykkCD-lacZ* constructs were transformed in 1A771 *B. subtilis* cells. Strain validation & βgal assays were performed as listed before.

Mutant No.	Top Primer: 5' - 3'	Bottom Primer: 5' - 3'
4	TGT AAA GTT TTC TAG GGC GCC GCA TGT CAA TTG	TCA ATT GAC ATG CGG CGC CCT AGA AAA CTT TAC
5	AAG CGC GTA TGC ACA CAA CAA AAA AAA AGC CC	CTC TCC CGG GCT TTT TTT TTTG TTG TGT GCA TAC
8	GAC ATG GAC TGG TCC AGA AAA CAC ATA CGC GTA	TAT TTA CGC GTA TGT GTT TTC TGG ACC AGT CCA
11	CTG GTC CGA GAG GGG ACA CAT ACG CGT	ACG CGT ATG TGT CCC CTC TCG GAC CAG

Table 3. Primers used for corresponding mutation in 5' to 3' sequence.

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