REGULATION OF THE PUTATIVE YKKCD RIBOSWITCH
BY TETRACYCLINE AND RELATED ANTIBIOTICS
IN BACILLUS SUBTILIS

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ABSTRACT

THESIS: Regulation of the Putative ykkCD Riboswitch by Tetracycline and Related Antibiotics in Bacillus Subtilis

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Multi-drug resistance among bacterial pathogens can be mediated by a number of mechanisms, including multidrug efflux pumps. One such pump in *Bacillus* spp. is ykkCD, a heterodimer of the SMR family consisting of C and D subunits. Previous studies suggest that the expression of ykkCD is controlled by a putative riboswitch and that the antibiotic tetracycline binds to the riboswitch *in vitro*. Additional studies have shown that two derivatives of tetracycline also bind to the putative riboswitch. These findings now need to be validated by an *in vivo* study. In this study, the effects that tetracycline and its commercially available derivatives—doxycycline, minocycline, anhydrotetracycline, and oxytetracycline—have on the expression levels of the *ykkCD* gene in *Bacillus subtilis* were explored. The level of *ykkCD* expression was quantified using two different methods: (1) *ykkCD* protein levels was determined using a *ykkCD* RNA-β-galactosidase reporter gene construct and (2) *ykkCD* mRNA levels was quantified by quantitative RT-PCR. Although the findings from method (1) were inconclusive, upregulation was observed for tetracycline and minocycline, in agreement with the results of the previous binding studies.
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Chapter 1. Background/Significance

1.1. Multidrug resistance in bacteria

Modern medicine has been made possible by the discovery and widespread use of antibiotics to treat bacterial infections. Beginning in the 1930s with penicillin and continuing with the aminoglycosides, the fluoroquinolones, and many other classes of antibiotics, many types of infections have been effectively treated. Many of these antibiotics exploit differences in the processes of protein translation between prokaryotic and eukaryotic organisms at the molecular level.

Resistance to antibiotics emerged early on in antibiotic use, within eight years from the clinical introduction of the antibiotic, on average\(^{(1)}\) and it arose for three reasons. First, patients were prescribed antibiotics unnecessarily. Second, some patients did not finish the whole prescription to completely eliminate the infection. Third, bacteria have an intrinsic ability to evolve in order to survive in toxic environments. When these events converged, new bacterial strains emerged containing one or more genes conferring resistance to these antibiotics. These strains continued to replicate and spread their resistance genes to their progeny and to other species by conjugation, by transformation, or through phages\(^{(1)}\). Antibiotic-resistance genes have been found in nonpathogenic organisms as well as pathogens through metagenomic analysis of various natural
environments, including the human gut, agricultural soil, and seawater. These nonpathogenic bacteria may serve as reservoirs of resistance genes that can be acquired by pathogens. Schmeider and Edwards have proposed a model network of how resistance genes can be spread among various ecosystems.\(^{(1)}\)

Recent research has suggested that in nature antibiotics serve roles in cell-cell communication. This type of communication, known as quorum sensing, is commonly used to coordinate growth and other activities within a bacterial community. For example, individual \textit{Pseudomonas aeruginosa} cells growing in a human tissue may secrete quorum sensing molecules to be received by their neighbors so as to be able to regulate growth and expression of virulence factors as a unit.\(^{(2)}\) Keller and Surette have identified three categories of these quorum sensing molecules: oligopeptides, N-acyl homoserine lactones (AHLs), and LuxS/autoinducer-2. Linares and colleagues have shown that antibiotics such as tetracycline can upregulate growth and motility genes in \textit{P. aeruginosa} at subinhibitory concentration. Since these concentrations resemble those found naturally in the soil, they have suggested a new paradigm where antibiotics are signaling agents within and possibly between species and only become toxic to bacteria at relatively high concentrations.\(^{(3)}\)

In recent decades, multidrug resistant infections have become a significant public health issue. Infections that used to be cured by a simple antibiotic prescription have once again become life-threatening conditions. Hospitals have become breeding grounds for these multidrug resistant organisms, costing more than $25 billion in America and leading to about 100,000 deaths per year.\(^{(1)}\) Although hospitals are taking positive steps to reduce the spread of infections among their patients, a better understanding of
resistance mechanisms on the molecular level is necessary so that new effective antibiotics can be designed to treat those who are infected.

There are primarily four mechanisms by which multidrug resistance occurs (Figure 1). First, the antibiotic can be inactivated by chemical modification or degradation. A classic example is β-lactamase, which inactivates antibiotics of the penicillin family. Second, the antibiotic target can be modified so that the toxic effects of the antibiotic are evaded. Third, the antibiotic can be prevented from entering the cell. Gram-negative bacteria, due to their outer cell membrane, and mycobacteria, due to their
waxy cell envelope, are naturally more resistant to antibiotics in this way.\(^4\) Fourth, the antibiotics can be exported from the cell by active transport, utilizing an efflux pump. These pumps can be specific for one antibiotic or generic for a broad spectrum of antibiotics.

1.2. Efflux pump ykkCD

Many of the multidrug resistance efflux pumps function as secondary transporters, that is, they harness the pH gradient across the cell membrane to pump out the toxic chemicals. Since the extracellular space is slightly more acidic, positively charged toxins or toxins complexed with positive ions are pumped out of the cell using the energy of proton influx (secondary active transport). Efflux pumps are classified into four families based on size and structure. The Major Facilitator Superfamily (MFS) contains transporters of sugars and other metabolites in addition to antibiotics. These are rather large, spanning the cell membrane 12 or 14 times. The Small Multidrug Resistance (SMR) family contains smaller proteins that, in general, transport cationic dyes like ethidium bromide. Due to their small size, it has been postulated that they cluster as homodimers. The Resistance-Nodulation-Cell Division (RND) family, more commonly found in Gram-negative species, interacts with an outer membrane protein to shuttle antibiotics from the cytosol through the intermembrane space. A fourth recently discovered classification is the Multidrug and Toxic Compound Extrusion (MATE) family. Members of this class have been shown to pump out fluoroquinolones and aminoglycosides.\(^4\)
One member of the SMR family is the YkkCD permease, found in the bacterium *Bacillus subtilis*. It is composed of two subunits, YkkC, which contains 112 amino acid residues, and YkkD, which contains 105 amino acid residues. When both subunits are expressed, resistance to a variety of antibiotic compounds—including streptomycin, chloramphenicol, tetracycline, tetraphenylphosphonium chloride (TPPC), and phosphomycin—is increased (Figure 2). However, resistance is eliminated when only one of the subunits is expressed.\(^5\) This contrasts with other members of the SMR family, which are expected to function as homooligomers.

We were interested in which of the compounds extruded by YkkCD is responsible for its regulation. A previous researcher, Ambar Rana, performed NASBA (nucleic acid sequence-based amplification), a method of RNA quantification, on RNA extracts of *B. subtilis* cultures grown with some of the antibiotics relative to untreated cultures. The results are summarized in Figure 3. Only tetracycline shows an increase in mRNA levels for both subunits. This led us to search for a tetracycline sensor that would trigger production of the ykkCD pump.
Several lines of evidence led us to hypothesize that the ykkCD mRNA regulates expression of the ykkCD efflux pump in a riboswitch-like manner at the transcription stage of gene expression: (1) the secondary structure prediction of the ykkCD mRNA resembles a terminator stem at the 3’ end; (2) nucleolytic self-cleavage of this region is reduced in the absence of ligand; (3) even though efflux pump expression in Gram-positive bacteria is strictly regulated, the ykkCD mRNA 5’ untranslated region (UTR) does not resemble the binding site of any known transcription factor. This suggests that the expression of ykkCD may be regulated at the transcription level by the mRNA itself and not by a protein cofactor. The sensor could be a riboswitch that directly detects the presence of toxins.

1.3. Riboswitches

**Definition.** A “riboswitch” is a part of the untranslated region (UTR) of a
messenger RNA (mRNA) that can detect small molecules in the cytoplasmic environment and regulate gene expression by allostery structural changes.\(^{(7,8)}\) RNA, even though it is single-stranded, has the ability to fold upon itself into complex three-dimensional structures by Watson-Crick base-pairing and non-Watson-Crick interactions. A two-dimensional illustration of this is given in Figure 4. The stem-loop, or “hairpin,” structure is one of the most common secondary structures. These stem-loops can arrange themselves in complex tertiary structures. For example, the loops of two hairpins can come together to form a “kissing complex,” or two nearby stems can interact to form a pseudoknot. (Figure 4.) These higher-order structures allow RNA to form complex protein-like folding patterns with the ability to specifically recognize small molecules. Regions of RNA that specifically recognize small molecules in this way are known as aptamers.\(^{(7)}\) As will be discussed below, these aptamers demonstrate high specificity for their targets and can discriminate between closely related analogues.
Figure 5. A) Transcriptional regulation: In the absence of ligand, the aptamer domain (red) folds into a structure that prevents the formation of the terminator stem. When the ligand is present, the aptamer domain binds with it, allowing for the formation of the terminator stem (blue), and transcription ceases. B) Translational regulation: In the absence of ligand, the ribosome binding site is accessible, and so translation proceeds. When the ligand binds, the RBS is sequestered, so the ribosome does not bind and translation does not start.

In the case of a riboswitch, the aptamer domain, usually located in the 5’-UTR of an mRNA, forms alternate three-dimensional structures in the presence and in the absence of a particular metabolite. This induces an allosteric conformational change in an adjacent expression platform; these expression platforms are much less conserved across species due to the variety of modes of gene regulation and possible overlap with the aptamer domain. Some riboswitches regulate at the transcription stage, and others regulate translation, as illustrated in Figure 5. The image on the left depicts transcription regulation. A terminator stem is sequestered when the ligand is not present, but when the ligand is present in high enough concentration the alternative folding pattern permits the formation of the transcription terminator stem in the expression platform, causing the RNA polymerase to fall off before it reaches the coding region. Thus, the coding region for the gene is not synthesized. Alternatively, translational regulation can occur, as depicted on the right. The aptamer region of the full-length mRNA folds into a form that
Figure 6. Classes of riboswitches, showing secondary structure and molecular structure of ligands. (9-12)

exposes the ribosome-binding site (RBS), allowing translation to proceed. When the ligand binds, the RBS become sequestered, preventing translation. In general, transcriptional regulation is more common in Gram-positive bacteria, and translational regulation is more common in Gram-negative bacteria. (13)
**Examples.** Eleven biomolecules have been specifically identified as riboswitch ligands in prokaryotes—coenzyme B$_{12}$, thiamine pyrophosphate, flavin mononucleotide, guanine/adenine (purines), S-adenosylmethionine (SAM), lysine, glycine, glucosamine-6-phosphate (GlcN6P), Mg$^{2+}$, pre-Q$_1$, and cyclic-di-GMP.$^{(13, 14)}$ Each is highly conserved in structure and even in sequence across species. Some of the ligands and the secondary structures of the consensus sequences are shown in Figure 6. Structures a-k illustrate the diversity of the types of biomolecules that are recognized by RNA aptamers. As these riboswitches involve the biosynthesis or homeostasis of fundamental metabolites, a great deal of effort has been channeled to characterize them, and more are being discovered on a regular basis. Let us look at two examples to illustrate the details of riboswitch function.$^{(13)}$

**SAM riboswitch—an OFF riboswitch.** S-adenosylmethionine (SAM) is involved in a variety of metabolic pathways, including methyl transfers and sulfur metabolism. In certain bacterial genera, especially of the *Bacillus* species, the SAM riboswitch appears multiple times in the genome where it regulates a number of genes

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![Figure 7. SAM-I riboswitch. a) In the ON-state, the riboswitch takes on an open structure. SAM binding causes the P1 stem to form and fold around SAM. The terminator stem then forms, halting transcription. b) 3-D model of SAM-I riboswitch bound to SAM. Images from Sashital and Butcher, 2006, ref. 16.](image-url)
involved in SAM synthesis and import as well as synthesis of cysteine and methionine.\textsuperscript{(10)}\textsuperscript{15)} Such a diverse function indicates that this riboswitch plays an extensive role in cellular homeostasis. When the SAM concentration is relatively low in the cell, these genes are constitutively expressed to maintain homeostasis. In this state, the transcription terminator stem does not form, and transcription can proceed. When SAM is present, the aptamer domain changes its conformation, causing the terminator stem to form, preventing the RNA polymerase from continuing, and shutting off gene expression. This is the typical pattern by which metabolites shut down gene expression when their concentration reaches a threshold, i.e, feedback regulation. For this reason, such riboswitches are known as OFF-riboswitches.\textsuperscript{(16)}

Investigation of the three-dimensional structure of the SAM riboswitch provides insights into how these allosteric changes exert their effect. These changes are illustrated 2-dimensionally in Figure 7a. In the absence of SAM, the aptamer domain folds into a particular pattern of stem-loops including a key pseudoknot, preventing the terminator stem from forming. When SAM is present, the P1 stem forms and completely surrounds the ligand. SAM is held in place through extensive electrostatic interactions and hydrogen-bonds.\textsuperscript{(16)} Since this structure is very compact and stable, the terminator stem is able to form (Figure 7b). Thus, structural changes in the aptamer region affect the expression platform. The specificity of the SAM riboswitch is illustrated by the evidence that a related molecule, S-adenosylhomocysteine, which lacks a sulfonium ion, is not able to bind to the aptamer domain.\textsuperscript{(16)}
Figure 8. A) Secondary structure of adenine riboswitch. P1, P2, and P3 are the three stem structures. L2 and L3 and the loops associated with P2 and P3, respectively. J1-2, J2-3, and J3-1 are the bases connecting the respective stems. Free adenine forms Watson-Crick bond with U74. B) The 3-D structure of the adenine riboswitch. P2 and P3 are held together due to Watson-Crick interactions between bases of L2 and L3. This forms a tight binding pocket for free adenine (shown in red) to bind. This represents the ON state for the pbuE gene. C) Adenine is held in place through Watson-Crick pairing with U74, as well as other interactions with neighboring bases. In the guanine riboswitch, guanine forms a Watson-Crick pair with the mutated C74. Figure from Serganov, et al., 2004, ref. 18.

Adenine riboswitch. In *B. subtilis*, the purine riboswitch class has two well-characterized members. The *xpt-pbuX* RNA encodes the genes for purine biosynthesis and contains a riboswitch that recognizes guanine, xanthine, and hypoxanthine, but has a much lower affinity for adenine.\(^{(17,18)}\) A related riboswitch with a critical C to U mutation has been found in the *pbuE* mRNA. This riboswitch selectively binds to adenine and is less sensitive to guanine.\(^{(17)}\) *PbuE*, the protein product of *pbuE* gene, is a purine efflux pump, expressed when purine levels become high enough.\(^{(19)}\) The adenine riboswitch was the first ON-riboswitch to be identified, that is, one that turns on gene expression when
the ligand is bound. When adenine binds, a transcription terminator is interrupted, and the efflux pump is produced.

X-ray crystallography of the *pbuE* riboswitch illustrates how adenine is specifically recognized by the RNA aptamer. The aptamer domain folds into a three-stem junction that tightly surrounds the adenine binding pocket. In Figure 8, free adenine, shown in red, is surrounded by the two-stem arch and held in place by Watson-Crick base-pairing to U-74 on the J3-1 region. U-74 is the point mutation that determines the selectivity between the adenine and guanine riboswitches. Guanine bound to the *xpt-PbuX* riboswitch displays a similar structure, with guanine interacting with the cytosine on J3-1, illustrated in part C of the image.

We find the adenine riboswitch particularly interesting because a similar ON-switch mechanism is expected for the ykkCD riboswitch, although its structure and target recognition is expected to be different.

**Features of ykkCD the Putative Riboswitch.** The predicted secondary structure of the 5’ end of the *ykkCD* mRNA is a stem-loop containing a large central bulge adjacent to a second stem-loop (Figure 9). This adjoins a potential terminator stem. A computer-assisted alignment of many bacterial genomes has revealed a region of significant sequence similarity among Gram-positive species, including *B. subtilis*, *B. cereus*, *B. halodurans*, and *B. furiosus*. In *B. subtilis* this sequence appears 19 times. These observations suggest that this noncoding RNA might be a regulatory element for the associated genes. In *B. subtilis*, its presence at the 5’ end of the *ykkCD* operon suggests that it could function as a toxin sensor and trigger the production of the ykkCD operon.
efflux pump. If so, this would be the first known riboswitch regulated by an antibiotic.

Previous work in our lab has shown that the *ykkCD* mRNA forms specific interactions with tetracycline. This was achieved through binding assays, which take advantage of the natural fluorescence of tetracycline. When tetracycline binds to RNA, its fluorescence is quenched. Fluorescence quenching due to the *ykkCD*-tetracycline interactions can be due to (1) non-specific stacking interactions of the aromatic ring in tetracycline with those of the nucleotide bases, similar to the non-specific interactions of intercalating dyes like ethidium bromide; or (2) a specific recognition of the three-dimensional structure of the RNA. Krystal Roark and Delores James in our lab have

Figure 9. Nonbinding mutants mapped to the secondary structure of the *ykkCD* putative riboswitch aptamer domain showing sequence conservation. Red indicates bases that are 100% conserved, blue indicates bases that are frequently conserved, and black indicates little conservation. Nonbinding mutants cluster in the regions of high sequence conservation, indicating a site for tetracycline binding.
developed a series of small mutations in the theoretical aptamer domain of the ykkCD RNA. If the binding interactions were nonspecific, such small mutations would have no effect on the quenching. However, they identified a series of mutations that reduced fluorescence quenching, located mainly in the central bulge of the extended stem-loop structure.\(^{(20)}\) (Figure 9) We can conclude that those bases are involved in specific recognition of tetracycline. Our lab is continuing to study additional mutants in order to identify other points of interaction.

From a pharmaceutical point of view, it would be desirable to identify which functional groups of tetracycline are significant for binding so as to design a new generation of derivatives that would not bind to the riboswitch and thus not induce efflux pump production. Delores investigated this question by performing binding assays on the unmutated ykkCD mRNA with tetracycline and four commercially available derivatives—minocycline, anhydrotetracyline, doxycycline, and oxytetracycline. These compounds display similar fluorescence characteristics as tetracycline. Her results

![Figure 10. Tetracycline derivatives used in binding assay. Top) molecules that retained binding; Bottom) molecules that had reduced binding.](image)
showed that tetracycline, minocycline, and anhydrotetracycline retained binding, but doxycycline and oxytetracycline display reduced binding.\(^{(20)}\) This result is very surprising, since doxycycline and oxytetracycline share the most structural similarity to tetracycline. (See Figure 10) Both of these have an additional hydroxyl group on the top face of ring C, adjacent to a tertiary amine on ring D, likely making the structure too crowded for the riboswitch to form around. By contrast, adding aromaticity to ring B (anhydrotetracycline) and adding an amino group to ring A (minocycline) did not affect the ability to bind. This can serve as a guide for future derivative screening.

As valuable as these \textit{in vitro} studies are for identifying the specifics of the tetracycline-RNA structure, they are meaningful only if an effect is observed in living cells with all the complexity of factors they contain. If tetracycline, minocycline, and anhydrotetracycline really do interact with the putative \textit{ykkCD} riboswitch, there should be an increase in the \textit{ykkCD} pump expression levels \textit{in vivo} when exposed to these antibiotics. This requires a method of \textit{in vivo} protein quantification. Several methods will be considered.

\textbf{1.4. Protein Quantification}

\textbf{Western blotting.} The gold standard of protein quantification is Western blotting. In this method, all the cellular proteins are extracted and separated by electrophoresis on a denaturing polyacrylamide (SDS-PAGE) gel. Then they are transferred by mechanical means onto a nitrocellulose membrane. The membrane is then soaked in a solution of antibodies designed against the protein of interest. It can then be visualized in one of two ways. If the antibody has a UV active tag or is radioactively labeled, the membrane can
be imaged directly. Alternatively, a secondary antibody specific for the Fc region of the primary antibody can be applied. This antibody can either be radioactively labeled or colorimetrically detected using horseradish peroxidase assay. Both of these methods create a dark spot on the membrane where the antibody is present. In either case, the presence or absence of the protein can be detected, and the quantity can be estimated by the intensity of the band.

The chief advantage of Western blotting is its specificity, which comes from the antibody. The antibody is also its chief disadvantage. A number of primary human, mouse, and rat antibodies are commercially available. For most other research purposes, a unique antibody has to be designed by exposing a small animal to the protein (antigen).

Figure 11. Western blot procedure. Proteins are separated on a gel, then transferred to a nitrocellulose film. An antibody for the protein of interest in applied to the film, and when imaged, only the protein of interest is visualized.
Figure 12. Plasmid vector for reporter gene assay. The gene of interest is inserted at the multiple cloning site in front of the *spoVG-lacZ* locus.

...of interest, followed by harvesting and purifying the antibody. Then the UV active tag or radioactive label has to be added. This can be a laborious and expensive process that often is reserved for projects where a commercially available antibody is available or where protein quantification is expected to play a major role in the project; thus developing an antibody and optimizing Western blot conditions is justified. Less direct methods of protein quantification have been developed as an alternative for protein quantification in other situations.

**Reporter gene assays.** One example, the reporter gene assay, takes advantage of the gene activation process. Conditions that turn on gene expression will cause transcription factors to gather 5′ to the gene or operon and recruit RNA polymerase to
The normal function of β-galactosidase is to break down lactose into galactose and glucose. (top) In the β-galactosidase assay, ONPG is added, which is hydrolyzed to produce galactose and the colored product ONP. (bottom)

start transcription. Downstream genes will be transcribed and eventually translated into protein. In a reporter gene assay, the promoter region for the gene of interest is cloned into a plasmid vector (e.g., Figure 12) in front of a gene whose protein product has a readily available detection protocol. This might be a fluorescent product, like green fluorescent protein (GFP), or an enzyme that produces a colored or fluorescent product. A very common example of the latter is the *E. coli* lacZ gene, which produces β-galactosidase. The normal function of β-galactosidase is to hydrolyze lactose into glucose and galactose (Figure 13, top). In the assay, after lysing the cells, o-nitrophenyl-β-D-galactopyranoside (ONPG) is added, which β-galactosidase breaks down into the yellow product o-nitrophenol when present (Figure 13, bottom). Both up- and down-regulation of various transcription factors can be investigated in this way.

There are several disadvantages to this technique. First, it involves precise cloning and extensive strain validation. Second, these studies are often carried out in *E. coli*
rather than the native organism, which can affect the degree of expression. Third, the sensitivity is rather low, with a dynamic range of about two orders of magnitude. On the other hand, after strain validation, it is a simple and straightforward technique, and thus can be useful as a screening test by entry-level researchers.

**Quantitative PCR Methods.** Protein expression levels can be estimated by measuring the intracellular mRNA concentration for the gene of interest. If the protein is overexpressed, it is highly likely that production of its mRNA will increase as well. Quantification of a specific mRNA can be achieved by quantitative PCR methods.

Traditional PCR (polymerase chain reactions) was developed for quantifying DNA. In this technique, the total DNA isolated from cells is amplified by *in vitro* DNA synthesis, by combining the DNA template with DNA polymerase, dNTPs, and primers specific for the region of interest. One primer starts amplification on the coding (top) strand, and the other starts amplification on the template (bottom) strand. Amplification is achieved by cycling through the following steps: (1) a high-temperature step (~95 °C) for strand separation; (2) a lower temperature step (45-60 °C) for primer annealing; and (3) a step for DNA synthesis that matches the optimal temperature (~70 °C) for polymerization. Since the process cycles through very high temperatures, DNA polymerases from thermophilic organisms, such as *Thermus aquaticus (Taq)* or *Pyrococcus furiosus (Pfu)*, are used. If the polymerase functions with 100% efficiency, the DNA of interest doubles during each cycle, which results in tremendous amplification after 40 or 50 cycles.
To develop a quantification method for a given cellular mRNA, the mRNA first must be converted to DNA by means of a reverse transcriptase and then the resulting cDNA is amplified as described before: hence the name reverse transcription PCR or RT-PCR. Reverse transcriptase is the enzyme used by retroviruses to convert their RNA genome into DNA. This step can occur at a more mild temperature (42-44 °C). Commonly used reverse transcriptases are from avian myeloid virus (AMV-RT) and moloney murine leukemia virus (M-MLV RT). Once the mRNA is converted to cDNA, PCR can proceed as usual, using primers specific to the gene of interest. The early RT-PCR methods used end-point detection, in which the amplified cDNA was visualized on a polyacrylamide gel. This method can determine the presence or absence of the mRNA of interest (the source of the cDNA), but does not readily permit quantification of the mRNA.

Therefore, reverse transcription quantitative PCR (RT-qPCR) was developed to quantify the mRNA in a sample. In those samples with greater starting amounts of the specific mRNA, the amplification will rise to detectable levels before those with less target mRNA. In real-time PCR, detection occurs at the same time as amplification through the use of a fluorescent dye that intercalates with DNA. As the concentration of the amplified cDNA increases, so does fluorescence. Fluorescent probes that bind specifically to the region being amplified have also been developed. Modern thermal cyclers have a built-in fluorescence detector that plots the fluorescence as a function of cycle number and records the data in an associated computer software system, which can aid in the analysis.
A further development of PCR for RNA quantification is nucleic acid sequence-based amplification (NASBA). The process is diagramed in Figure 14. This technique uses three enzymes: AMV-RT, RNase H, and T7 RNA polymerase (T7 RNAP). After initial denaturation at 65°C, the first primer binds to the target RNA (a) and is extended by AMV-RT (b). This primer has a 5’ extension of the T7 promoter, which is important in a subsequent step. The RNA of the resulting RNA-cDNA is degraded by RNase H, allowing the second primer to bind (c). The DNA-dependent activity of AMV-RT creates a double-stranded cDNA with a T7 promoter at one end (d). The T7 RNAP is then able to
transcribe the cDNA into a reverse copy of the original RNA (e). At this point a cycling process begins. The second primer binds to the RNA (f), and AMV-RT converts it into an RNA-cDNA hybrid (g). RNase H degrades the RNA (h), the first primer binds to the cDNA, and AMV-RT synthesizes the complementary DNA (i). T7 RNAP is then recruited to this freshly synthesized DNA and makes more RNA (j). The process continues, resulting in exponential increase of the target RNA concentration.\(^{(21)}\)

NASBA possess a number of advantages over RT-qPCR. The reverse transcription is incorporated in the amplification process, so it is more conducive to RNA targets than RT-PCR. Since it is an isothermal process, NASBA does not require a thermal cycler and only needs one melting step at the beginning. Also, in NASBA the target is continuously amplified, in contrast to the stepwise amplification of RT-PCR.\(^{(21)}\)

Each cDNA copy in NASBA can produce multiple RNA copies, resulting in rapid exponential increase. In NASBA the amplification can exceed the initial primer concentration by at least one order of magnitude,\(^{(21)}\) in contrast to RT-PCR, where the primer concentration limits the amount of increase. Additionally, NASBA selectively amplifies mRNA in the presence of genomic DNA background, whereas RT-PCR cannot distinguish between cDNA and genomic DNA, necessitating a DNase treatment prior to analysis.

There are, however, several disadvantages of NASBA compared to RT-PCR. It requires three enzymes that must be active under the same reaction conditions, whereas RT-PCR only uses one at a time. The NASBA enzymes are not thermostable either, and so they have to be added after the initial denaturation step. The lower temperature of
NASBA compared to RT-PCR permits more non-specific primer interactions. Finally, since the NASBA end product is single-stranded RNA, melting curve analysis cannot be used. With these differences in mind, each researcher has to decide which method is better for the type of study considering the available resources.
Chapter 2. Materials and Methods

2.1. Methods for Quantification of ykkCD Pump Protein Levels

A direct way of quantifying expression levels is by measuring pump protein levels. However, quantifying a membrane-bound antibiotic efflux pump would require developing an antibody for Western blotting. A reporter-gene construct, which replaces the ykkCD pump ORF with the β-galactosidase ORF, is more practical and economical, as the β-galactosidase assay has been validated by many sources. The process involves: a) preparation of competent *B. subtilis*, b) transformation of *B. subtilis* with plasmid construct, and c) the β-galactosidase assay.

Preparation of Competent *Bacillus* cells

The plasmid pDG1661, provided by the Bacillus Genetic Stock Center (BGSC), was utilized. They recommend transforming it into *B. subtilis* strain 1A771. Prior to transformation, these cells must be made competent, using this protocol.

**Materials**

- 10X S-base
  - 2 g \((\text{NH}_4)_2\text{SO}_4\)
  - 14 g anhydrous \(\text{K}_2\text{HPO}_4\)
- 6 g anhydrous KH$_2$PO$_4$
- 1 g trisodium citrate
- MilliQ water to 100 mL
- Autoclave
- Add 0.1 mL 1M sterile MgSO$_4$

- Individually autoclave:
  - 20% glucose
  - 2% casein
    - Note: add 6 M NaOH until casein turns milky white prior to autoclave
  - 10% yeast extract
  - 8% arginine w. 0.4% histidine
  - 1 M MgSO$_4$

- Prepare 0.1% tryptophan and filter sterilize

- 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

Protocol

1. Place 2 colonies of *B. subtilis* 1A771 into 5 mL of HS Medium and grow at 37°C overnight.
2. Place 500 µL of this culture into 50 mL of HS Medium.
3. Incubate with shaking at 37°C.
4. Measure OD$_{595}$ every 20 min until stationary phase is achieved.
5. Harvest 10 mL of culture every 15 min for the next hour.
6. Add 1 mL sterile 87% glycerol to each portion and cool on ice for 15 min.
7. Aliquot each sample and freeze in liquid nitrogen for about 1 min.
8. Samples are stored at -80°C.

*Bacillus* Transformation

Materials

- LS Medium:
  - 80 mL MilliQ water
  - 10 mL 10X S-base
Protocol

1. Thaw competent 1A771 *B. subtilis* cells in 37 °C water bath.
2. Pour contents into 20 mL LS medium in 250 mL Erlenmeyer flask.
3. Incubate in 30 °C water bath with low shaking for 2 hours.
4. Remove cells; heat water bath to 37 °C.
5. Separate culture into 1-mL portions in sterile test tube for each plasmid.
6. Add 10 μL of 0.1 M ethylene glycol tetraacetic acid (EGTA).
7. Incubate at RT for 5 min.
8. Add 3-4 μg of pDG1661 plasmid DNA.
9. Incubate in 37 °C water bath at 220 rpm for 2 hours.
10. Transfer culture to sterile 1.5-mL centrifuge tube.
11. Centrifuge at 5000 rpm for 5 min.
12. Remove approx. 900 μL supernatant fluid.
13. Resuspend pellet in remaining 100 μL.
14. Spread suspension onto LB agar plates w. chloramphenicol at 5 μg/mL.
15. Grow overnight at 37 °C.

**Beta-galactosidase assay**

When the *ykkCD-lacZ* construct is inserted into the *B. subtilis* genome, it is cultured with potential ligands to determine whether they increase gene expression. Those that activate the riboswitch would be expected to produce more β-galactosidase, the protein product of *lacZ*. The substrate 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) is added to the cell lysate, which is broken down by β-galactosidase into the fluorescent product 4-methylumbulliferone (4-MU). The amount of 4-MU produced is directly related to the amount of β-galactosidase produced by the cells. The 4-MU can be quantified by setting up a standard curve.
Materials
- Z-buffer
  - 60 mM Na$_2$HPO$_4$
  - 40 mM NaH$_2$PO$_4$
  - 10 mM KCl
  - 1 mM MgSO$_4$

Preparation of 4-MU Solutions for Standard Curve
1. Prepare 0.1 M 4-MU by dissolving 0.176 g Aldrich 4-MU in 10 mL 1 M Na$_2$CO$_3$ (Stop Solution).
2. Prepare 10 mM 4-MU by diluting 100 μL 0.1 M 4-MU in 900 μL Stop solution.
3. Repeat Step 2 to prepare standard 4-MU solutions of 1 mM, 100 μM, 10,000 nM, 1000 nM, 100 nM, 10 nM, and 1 nM.
4. Measure fluorescence with the Fluoromax®-3 fluorimeter of 100 μL 10,000 nM, 1000 nM, 100 nM, 10 nM, and 1 nM 4-MU solutions, using excitation at 365 nm and scanning emission at 400-550 nm. Also measure fluorescence of 100 μL Stop Solution as the blank.
5. Store 4-MU solutions at -20 °C.

Protocol
1. Grow transformed 1A771 cells containing the ykkCD-lacZ construct in LB medium with and without antibiotic for 18 hours.
2. Prepare Mixing Buffer on the day of assay by combining 715 μL Z-buffer, 15 μL Sigma chicken lysozyme, 30 μL NEB DNase I 2U/UL, and 2.65 μL Sigma β-mercaptoethanol (per sample).
3. Prepare Buffer 2 by mixing 50 μL 200 μg/mL MUG (Sigma) and 200 μL Z-buffer (per sample).
4. Harvest an equal number of cells based on the measured optical density (OD) of the culture, according to the equation: (0.375 mL)/OD.
5. Pellet cells by centrifuging at high speed for 1 min. Discard supernatant. Pellet can be frozen at -20 °C, if necessary.
6. Resuspend pellet in 600 μL Mixing Buffer.
8. Incubate at 29 °C for 40 min.
9. Add 400 μL Stop Solution.
10. Centrifuge at high speed for 5 min.
11. Measure fluorescence with Fluoromax®-3 fluorimeter, using “bgal.exp” settings:
   - Excitation: 365 nm
   - Emission: 400-550 nm scan
   - Integration time: 0.250 s
12. Measure fluorescence of 100 μL of—
   - Stop Solution (blank)
   - 10 nM 4-MU
   - 100 nM 4-MU
   - 1000 nM 4-MU
- 10,000 nM 4-MU
- Each incubated cell lysate.

13. Record peak fluorescence value for each.

**Data Manipulation**
1. Subtract fluorescence value of blank from that of each sample.
2. Multiply 4-MU concentrations by 0.1 mL to convert to pmol.
3. Divide by 40 min to obtain Miller Units.
4. Plot fluorescence units of 4-MU samples against corresponding values in Miller Units to generate a standard curve.
5. Use standard curve to convert fluorescence values of cell lysates into Miller Units.

### 2.2. Methods for Quantification of *ykkCD* mRNA Levels

Another approach to quantifying *ykkCD* pump expression levels is to quantify the cellular mRNA coding for the pump, based on the assumption that an elevated mRNA level is associated with elevated protein level. Cell cultures of *B. subtilis* strain NRRL B-765 were grown with tetracycline and its derivates and without antibiotics. The level of *ykkCD* mRNA was quantified using this general outline: a) RNA extraction, b) determination of RNA concentration, c) determination of RNA quality, and d) NASBA or RT-PCR.

**Total RNA Extraction**

In order to quantify *ykkCD* mRNA levels, the total RNA must be extracted from cell cultures and separated from the other cellular components: membrane lipids, proteins, genomic DNA, and so forth. The Qiagen RNeasy® Mini Kit was used to accomplish this. For every trial, one culture was grown without antibiotic and at least one culture was grown with an antibiotic at subinhibitory concentration (SIC). Sometimes more than one antibiotic-grown culture was extracted at a time. The extraction was
performed after approximately 19 hours of growth. Typical yield was 50-300 ng/μL total RNA.

**Materials**

- Qiagen RNeasy® Mini Kit
- Sigma β-mercaptoethanol
- Sigma lysozyme from chicken egg white
- Y1 Buffer:
  - 1 M sorbitol
  - 0.1 M ethylenediaminetetraacetic acid (EDTA)
  - Adjust to pH 7.4.
  - Immediately before use, add to 1 mL (per reaction)—
    - 10 μL β-mercaptoethanol
    - 5 μL Sigma chicken lysozyme

**Protocol**

1. Spin a 3-mL portion of *B. subtilis* cell culture by centrifuging at 5000 rpm for 3-5 min to form pellet.
2. When multiple cultures are prepared, use a proportionally greater volume for those with lower OD to ensure that the same number of cells are processed from each culture.
3. Resuspend pellet in 1 mL of Y1 Buffer.
4. Follow the recommendations of the manufacturer of the RNeasy® Minikit.
5. RNA can be eluted by washing column with two 50-μL aliquots of RNase-free water or with one 50-μL aliquot of RNase-free water passed across the column twice (for higher RNA concentration).
6. Measure RNA concentration by absorbance measurement of 2 μL at 260 nm using Thermo NanoDrop 1000 spectrophotometer.
7. Store mRNA at -80 ºC to retard degradation for up to six months.

**Formaldehyde Denaturing Gel Electrophoresis**

Only high-quality RNA can be used for PCR. *(qPCR Application Guide, p. 17)*

To determine the quality of RNA after extraction, formaldehyde denaturing agarose gel electrophoresis was performed. Roughly 80% of the total cellular RNA is ribosomal
RNA (rRNA), which forms two distinct bands on an agarose gel, representing the 30S and 50S subunits. The quality is judged by the sharpness of these bands and the absence of degradation bands of lower molecular weight. Although we are interested in messenger RNA (mRNA), we presume that the quality of the rRNA is representative of the mRNA quality.

**Materials**

- **10X FA Buffer:**
  - 200 mM 3-(N-morpholino)propanesulfonic acid (MOPS)
  - 50 mM sodium acetate
  - 10 mM EDTA
  - Adjust to pH 7 w. sodium hydroxide

- **FA Running Buffer:**
  - 50 mL 10X FA Buffer
  - 10 mL 37% formaldehyde
  - 440 mL MilliQ water

- **FA Gel Loading Buffer:**
  - 80 µL 500 mM EDTA (pH~8)
  - 720 µL 37% formaldehyde
  - 2 mL 100% glycerol
  - 3.084 mL 100% N,N-dimethyl formamide
  - 16 µL bromophenol blue (BPB)
  - 4 mL 10X FA Buffer
  - MilliQ water to 10 mL
  - Store at room temperature.

- **1% FA Agarose Gel:**
  - Mix 1 g Sigma agarose powder with 100 mL FA Running Buffer.
  - Heat in microwave oven until boiling, approx. 1-2 min.
  - Let solution cool for a few minutes.
  - Add 5 uL ethidium bromide solution.
  - Pour into molds and insert combs. Let sit until solid.
Protocol

1. To 10 μL RNA sample, add 2 μL MilliQ water and 3 μL FA Gel Loading Buffer.
2. Heat in 65 °C water bath for 5 min.
3. Cool on ice for a minute.
5. Load samples into gel.
6. Apply current at 45 V for up to 1 hour.
7. Take UV image w. Bio-Rad Gel-Doc™ XR+ on ethidium bromide setting. Adjust exposure time to make distinct image.

Nucleic Acid Sequence-Based Amplification (NASBA)

Once RNA quality is verified, mRNA levels can be quantified by the isothermal procedure NASBA. This procedure amplifies RNA only, even in the presence of genomic DNA. Using the genome available on the NCBI GenBank, primers had been designed by previous researchers in the lab for ykkC, ykkD, and DNA gyrase B (gyrB) as an internal reference; these primers were then ordered from Integrated DNA Technologies (IDT). The forward primer for each included the T7 promoter. The primer sequences are given in Table 1. The protocol for NASBA is given below.

Materials

- 5X NASBA Buffer:
  - 200 mM Tris-HCl, pH 8.5
  - 65 mM MgCl₂
  - 300 mM KCl

Procedure

1. For each primer set, prepare a Premix by multiplying the following volumes by the number of reactions being set up:
   - 4 μL 5X NASBA Buffer
   - 0.67 μL 12 mM Fermentas dithiothreitol (DTT)
   - 0.5 μL FisherScientific dATP, 100 mM
- 0.5 µL FisherScientific dGTP, 100 mM
- 0.5 µL FisherScientific dTTP, 100 mM
- 0.5 µL FisherScientific dCTP, 100 mM
- 0.5 µL Fermentas dUTP, 100 mM
- 0.5 µL 100 mM Tokyo Chemical Industry Co. inosine 5’-triphosphate (ITP)
- 0.3 µL DMSO
- 1 µL 100 µM Forward primer w. T7 promoter
- 1 µL 100 µM Reverse primer

2. Prepare an Enzyme Mix by multiplying the following volumes by the total number of reactions being set up:
   - 0.32 µL New England Biolabs (NEB) 25 U/µL avian myeloid virus-reverse transcriptase (AMV-RT)
   - 0.16 µL NEB 0.5 U/µL RNase H
   - 0.6 µL NEB 50 U/µL T7 RNA polymerase (T7 RNAP)
   - 3.92 µL MilliQ water

3. Pipet into Cepheid SmartCycler® tube:
   - 9.97 µL Premix
   - x µL total RNA sample, 500 ng
   - (4.53-x) µL MilliQ water
   - (for no-template control, use 4.53 µL MilliQ water)
   - 0.5 µL Lonza 10,000X SYBR® Green II

### Table 1. NASBA Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th># bases</th>
<th>Tm, °C</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ykkC forward primer</td>
<td>56</td>
<td>70.7</td>
<td>AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GCG CGC GTG CGC GGG CTG TTT AGA AA</td>
</tr>
<tr>
<td>ykkC reverse primer</td>
<td>24</td>
<td>59.6</td>
<td>ACA AGC TTC AAC CCG ATT ACA CCG</td>
</tr>
<tr>
<td>ykkD forward primer</td>
<td>51</td>
<td>67.8</td>
<td>AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GGT GCG CGG GCT GTT TAG AAA</td>
</tr>
<tr>
<td>ykkD reverse primer</td>
<td>24</td>
<td>60.0</td>
<td>AAG ATC CGT TTG GCG TCT TTC TGC</td>
</tr>
<tr>
<td>gyrase B forward primer</td>
<td>54</td>
<td>69.0</td>
<td>AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GCG CGC GGT GGT ACA CAT GAG GTA</td>
</tr>
<tr>
<td>gyrase B reverse primer</td>
<td>20</td>
<td>51.2</td>
<td>ACT CTG TAA TTG TTC TCG CT</td>
</tr>
</tbody>
</table>

*Bases in **bold** represent the T7 promoter.
4. Place tubes into Cepheid SmartCycler® II thermal cycler.

5. Select “NASBA New” setting:
   - Denaturation: 65 °C for 6 min
   - Enzyme loading: 41 °C for 6 min
   - Extension: 41 °C for 1 min
   - Fluorescence acquisition: 40 °C for 1 s

   \[ \text{Repeat for 100 cycles} \]

6. Quick spin in centrifuge to deposit mixture at bottom of tube.
7. During enzyme loading step, add 5 µL Enzyme Mix to each tube.
8. Spin briefly in centrifuge.
9. Return to thermal cycler, being careful to put each tube back in correct spot.
10. View log Fluorescence vs. Cycle Number graph to determine Cₜ value.

**DNase I Treatment**

RT-PCR was used as an additional mRNA quantification method. Since it involves the amplification of cDNA, the presence of genomic DNA will result in a false positive. Therefore it is imperative to remove all residual genomic DNA before quantification by RT-PCR. Although the RNeasy® Minikit removes most of the genomic DNA, there may be trace amounts that can be detected by RT-PCR amplification.

Treatment with DNase I removes any remaining genomic DNA.

**Procedure**

1. Mix the following in a micro centrifuge tube:
   - 10 µL NEB 10X DNase Buffer
   - 10 µg RNA extract
   - RNase-free water to 100 µL
   - 1 µL NEB 2 U/µL DNase I

   \[ \text{After DNA was detected by RT-PCR, some samples were treated with 3U or 20U DNase I.} \]

2. Incubate in 37 °C water bath for 10 min.
3. Add 1 µL 0.5 M EDTA (pH 8)
4. Heat inactivate DNase I in 75 °C water bath for 10 min.
5. Cool at room temperature or briefly on ice, then perform phenol-chloroform extraction.

**RT-PCR**

Reverse transcriptase quantitative polymerase chain reaction (RT-PCR or RT-qPCR) was used in addition to NASBA to quantify mRNA levels of \( ykkC, ykkD, \) and \( gyrB \) in cell extracts. Before performing RT-PCR, unique primers were designed. The strain used in this study, \( B. \ subtilis \) NRRL B-765, was only 216 nucleotides different from the genome for \( B. \ subtilis \) subp. Spizizenii strain W23\(^{22} \) available in the NCBI database. The genes \( ykkC, ykkD, \) and \( gyrB \) from this strain were put into the Primer3 software for primer design, publicly available on the Internet at:

[http://primer3.wi.mit.edu/](http://primer3.wi.mit.edu/). The resulting sequences are given in Table 2.

After performing RNA extraction and DNase I treatment and after ensuring good quality RNA by agarose gel electrophoresis, RT-PCR was run with these primers. The following procedure was used, utilizing the Qiagen\(^{®} \) qRT-PCR SYBR\(^{®} \) Green kit QR01000 or Applied Biosystems (Invitrogen) Power SYBR\(^{®} \) Green RNA-to \( CT \) 1-Step Kit 4389986. Both kits had similar set-up protocols. For each sample, appropriate controls were run alongside one another: a no-template control (NTC) to which RNA was not added and a no-RT control to which reverse transcriptase was not added. These checked for exogenous contamination and residual genomic DNA, respectively. During the optimization stage, a positive control using the MasterAmp\(^{®} \) Control Mix was run to verify correct set-up. Since the signal was very consistent, it was omitted in later reactions.
Table 2. Primers for RT-PCR

<table>
<thead>
<tr>
<th>PrimerName</th>
<th># bases</th>
<th>Tm, °C</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ykkC forward primer</td>
<td>20</td>
<td>54.8</td>
<td>ATG GGG ATT AGT CGT GCT TG</td>
</tr>
<tr>
<td>ykkC reverse primer</td>
<td>20</td>
<td>55.4</td>
<td>AAA GAC GGC ATA CAC GGT TC</td>
</tr>
<tr>
<td>ykkD forward primer</td>
<td>20</td>
<td>54.8</td>
<td>TGG GTG CTG TTG ATC ATT GT</td>
</tr>
<tr>
<td>ykkD reverse primer</td>
<td>20</td>
<td>54.9</td>
<td>CGT CTT TCG GCT CCT TGT AA</td>
</tr>
<tr>
<td>gyrase B forward primer</td>
<td>20</td>
<td>55.2</td>
<td>GAG AAA GCC CGT TAC CAC AA</td>
</tr>
<tr>
<td>gyrase B reverse primer</td>
<td>20</td>
<td>55.4</td>
<td>CAC GTT TCC CCT GTT GAA CT</td>
</tr>
</tbody>
</table>

Procedure

1. Prepare a Premix for each primer set by multiplying the following volumes by the number of reactions for that primer set:
   - 12.5 µL 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

2. Place into each Corbett Research reaction tube:
   - 15 µL Premix
   - 50 µg total RNA
   - RNase-free water to 25 µL

3. Prepare a No-Template Control (NTC) for each primer set:
   - 15 µL Premix
   - 10 µL RNase-free water

4. Prepare a No-RT Mix for each primer/sample combination:
   - 12.5 µL 2X SYBR® Green Taq ReadyMix
   - 1 µL 100 µM Forward primer
   - 1 µL 100 µM Reverse primer
   - 50 ng total RNA
   - RNase-free water to 25 µL

5. When a positive control reaction is used, prepare as follows:
   - Master Mix (multiplied by total number of reactions)
     - 12.5 µL 2X SYBR® Green Taq ReadyMix
     - 0.125 µL 200 U/µL M-MLV RT
     - 0.375 µL RNase-free water
o Add to reaction tube—
  i. 13 μL Master Mix
  ii. 0.5 μL Epicentre Control Mix (100 ng/μL RNA + 12.5 μM primers)
  iii. 11.5 μL RNase-free water
  o The remainder of the Master Mix is used to make Premixes for each primer set.

6. Load samples into Corbett Research RotorGene RG-3000 with the following cycling conditions:
   o Reverse Transcription: 43 °C for 30 min
   o Initial Denaturation: 95 °C for 2 min
   o Denaturation: 95 °C for 15 s
   o Annealing: 52 °C for 30 s \{ Repeat 40 cycles \}
   o Extension + fluorescence reading: 60 °C for 60 s
   o Melt curve: Ramp at 0.2 °C/s from 45 °C to 95 °C

7. When complete, view results in Quantitation mode.

8. Set threshold at lowest level that permits the software to calculate a C_t value. (Typical threshold range: 0.01-0.02)

<table>
<thead>
<tr>
<th>Table 3. RT-PCR Final Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental Sample</strong></td>
</tr>
<tr>
<td>1X Ready Mix</td>
</tr>
<tr>
<td>1 U/μL M-MLV RT</td>
</tr>
<tr>
<td>4 μM Forward primer</td>
</tr>
<tr>
<td>4 μM Reverse primer</td>
</tr>
<tr>
<td>50 ng RNA</td>
</tr>
</tbody>
</table>

2.3. Other Commonly Used Procedures

The following procedures were routinely used in the course of handling RNA.

**Phenol-Chloroform Extraction**

This technique was used to remove proteins and other contaminants, usually an enzyme, from RNA, such as after DNase I treatment.
• Add 100 µL of phenol/chloroform/isoamyl alcohol (25:24:1).
• Vortex 20 sec.
• Spin for 2 min in benchtop centrifuge.
• Discard lower organic layer.
• Add 100 µL chloroform.
• Vortex 20 sec.
• Spin for 2 min in benchtop centrifuge.
• Discard lower organic layer.
• Perform ethanol precipitation to remove residual phenol/chloroform.

Ethanol Precipitation

Ethanol precipitation was used to removed residual phenol and chloroform or to increase the concentration of RNA if necessary (e.g., NASBA).

• Add NaCl to final concentration of 250 mM.
• Add 2.5 volumes of ice-cold 75% ethanol.
• Store at -20 °C overnight to precipitate.
• Centrifuge full speed at 4 °C for 30 min.
• Discard supernatant liquid.
• Add 50 µL ice-cold 75% ethanol.
• Centrifuge full speed at 4 °C for 5 min.
• Discard supernatant liquid.
• Dry in Labconco® CentriVap Roto-Vac® for 5-10 min.
• Resuspend in 20-50 µL RNase-free water.

2.4. Statistical Methods

General Principles

In a scientific study, a set of sample measurements are used in an attempt to say something about the entire population from which those measurements were obtained. Statistical methods are used to aid in sorting out differences due to random sampling and biological variation from true experimental differences. Each set of measurements can be
described by a standard deviation (SD), which quantifies the scatter about the mean for a
given dataset. SD is defined as:

\[
SD = \left[ \frac{\sum(x - m)^2}{n - 1} \right]^{1/2}
\]

where \( m \) is the mean and \( n \) is the total number of values. The standard error of the mean
(SEM) is defined as:

\[
SEM = \frac{SD}{\sqrt{n}}
\]

This provides a numerical estimation of how far the sample mean is from the true
population mean. From SEM, the 95% confidence interval (CI) can be calculated, which
is the range within which there is a 95% probability that the true population mean lies.

After these values are determined, hypothesis tests such as the t-test can be
performed to compare two sets of data. The t-test begins with the “null hypothesis,”
which states that the populations from which the data were obtained have equal means.
The SEMs are combined and weighted according to the degrees of freedom, that is, \( n - 1 \)
for both samples. A P value is then calculated which gives the probability that the
difference in the means of the samples could be observed through random sampling of
populations with the same mean.\(^{(23)}\) Thus, a P value of 0.62 indicates more similarity
between the samples sets than a P value of 0.10. Typically, the P value of 0.05 is chosen
as the cut-off to reject the null hypothesis; that is, if \( P < 0.05 \), the probability of the
sample means being equal is low enough (less than 5%) that the populations are said to
have different means.
For expression studies using PCR methods, the ΔΔC\text{t} method is traditionally used. In this method, C\text{t} values are referenced to a housekeeping gene and then normalized against a control. For example, in the sample treated with tetracycline, ΔC\text{t} is determined as the C\text{t} of gene X minus the C\text{t} of DNA gyrase (the housekeeping gene). The same is done for the untreated sample. Then ΔC\text{t} of the untreated sample is subtracted from ΔC\text{t} of the treated sample to obtain ΔΔC\text{t}. Since a lower C\text{t} value indicates larger amount of starting material, a negative value means increased expression. The inverse correlation between expression levels and ΔΔC\text{t} values is corrected for by reporting the “fold-change” as: 2^{-ΔΔC\text{t}}. Fold change is greater than 1 for genes that have increased gene expression in the experimental sample and less than 1 for genes that have decreased expression.\textsuperscript{(24)} Although this is a practical and logical method, there is not a satisfactory way to account for the statistical handling of replicates according to this method, nor is there a way to correct for variations in amplification efficiency among all the genes being considered.\textsuperscript{(25)}

As a result, the Relative Expression Software Tool (REST) was employed for data analysis of the RT-PCR results. It uses a randomization technique to calculate 95% confidence intervals and P values without making the assumption of a Gaussian distribution as required for the t-test. The 2009 version of the software has an improvement over previous versions to utilize comparative quantitation data calculated by Rotor-Gene thermal cyclers. The comparative quantitation method takes the second derivative of the real-time amplification curve. Figure 15 compares an example

REST

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amplification curve and second derivative curve for the same sample. The exponential phase is transformed into a peak. The cycle where the curve rises above 20% of the peak height is called the “take-off” (analogous to the C_t value). This method also calculates the efficiency of amplification for each sample. If the polymerase were 100% efficient, the signal would double each cycle. An average efficiency is calculated for each cycle and reported as the “amplification,” on a scale of 1-2. The take-off (TO) and amplification values are entered into REST for the gene of interest (e.g., ykkD) and the reference gene (e.g., gyrB), both for control samples and treated samples.

The software first calculates an efficiency (E) for each gene, using all the data entered, on a scale of 0-1, where 0 is no amplification and 1 is 100% efficiency, or doubling. Second, a pair of sample and control TO values for each gene are matched at random. Third, it calculates a concentration (C) for each gene: 

$$C = E^{TO_{control}} - TO_{sample}$$

Fourth, it calculates a relative concentration by dividing the concentration of the gene of interest by the concentration of the reference gene: 

$$RE = \frac{C_{GOI}}{C_{ref}}$$

This calculation is repeated many more times (2000 by default, but the user can adjust this value) with other randomly selected pairs of data points, creating a range of relative expression values.
Some of these will overestimate the true expression ratio, and some will underestimate it. It is expected that the vast majority will center around the true expression ratio. When these relative expression values are ordered from least to greatest, the 95% confidence interval is determined to be the entire range of values excluding the top and bottom 2.5%. For example, if 2000 randomizations were performed, the 95% confidence interval extends from the value of the 51st point to that of the 1,950th point.

The software performs hypothesis testing to estimate the likelihood that the difference between samples is due a true difference and is not random. In REST the Pair Wise Fixed Randomized Reallocation Test is used. The null hypothesis is that if there is no difference in expression between the sample and control sets, the data between the sets could be swapped without increasing the value of the expression ratio. A pair of target and reference data points are randomly swapped between sample and control groups 10,000 times and the percentage of times that the expression value increases is noted. The less frequently this happens, the more likely it is that there is a true underlying cause, not random chance. The software is able to calculate a P value based on this. An example of the REST output is given below.
Relative Expression Report

Notes

3U DNase 30 min, Eliminating--
NoAb-C 29.7, 28.1, 27.7, 29.5
NoAb-D 23.1, 23.3, 22.7
NoAb-gyr 16.4, 15.1, 14.0
Mino-D 23.2, 22.9, 23.5

Assay Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iterations</td>
<td>2000</td>
</tr>
</tbody>
</table>

Results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Reaction Efficiency</th>
<th>Expression</th>
<th>Std. Error</th>
<th>95% C.I.</th>
<th>P(H1)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ykkC</td>
<td>TRG</td>
<td>0.8967</td>
<td>2.521</td>
<td>1.521-5.530</td>
<td>1.222-6.208</td>
<td>0.000</td>
<td>UP</td>
</tr>
<tr>
<td>ykkD</td>
<td>TRG</td>
<td>0.9417</td>
<td>3.394</td>
<td>1.702-7.939</td>
<td>0.922-11.434</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>gyrase</td>
<td>REF</td>
<td>0.925</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.
TRG - Target
REF - Reference

Interpretation

ykkC is UP-regulated in sample group (in comparison to control group) by a mean factor of 2.521 (S.E. range is 1.521 - 5.530).
ykkC sample group is different to control group. P(H1)=0.000

ykkD sample group is not different to control group. P(H1)=0.096

Boxplot
Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

**Non-Normalised Results**

The following results do not have expression values normalised to the selected housekeepers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Reaction Efficiency</th>
<th>Expression</th>
<th>Std. Error</th>
<th>95% C.I.</th>
<th>P(H1)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ykkC</td>
<td>TRG</td>
<td>0.8967</td>
<td>0.711</td>
<td>0.650 - 0.774</td>
<td>0.607 - 0.774</td>
<td>0.048</td>
<td>DOWN</td>
</tr>
<tr>
<td>ykkD</td>
<td>TRG</td>
<td>0.9417</td>
<td>0.957</td>
<td>0.511 - 1.537</td>
<td>0.412 - 1.872</td>
<td>0.805</td>
<td></td>
</tr>
<tr>
<td>gyrase</td>
<td>REF</td>
<td>0.925</td>
<td>0.282</td>
<td>0.136 - 0.456</td>
<td>0.131 - 0.507</td>
<td>0.000</td>
<td>DOWN</td>
</tr>
</tbody>
</table>

Report produced by REST 2009 V2.0.13
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Chapter 3. Results

3.1. Bacillus Strain Validation

In order to determine the level of ykkCD gene expression by the β-galactosidase assay, the ykkCD leader sequence had to be placed in front of the lacZ gene and transformed into B. subtilis. The ykkCD leader was previously cloned into the plasmid vector pDG1661, provided by the Bacillus Genetic Stock Center. It was transformed, as

Figure 16. A successful transformation requires incorporation of the lacZ gene with promoter onto the genome by a double crossover event. The plasmid (top) contains a chloramphenicol-resistance gene (cat) and a spectinomycin-resistance gene (specR). The target, that is, the B. subtilis genome (middle), has an erythromycin-resistance gene within the amyE locus. After the double crossover (bottom), the lacZ and cat genes are integrated into the amyE locus, making the organism chloramphenicol-resistant and erythromycin-sensitive. Since the specR gene is excluded, the transformant is spectinomycin-sensitive.
Table 4. Expected Result of Antibiotic Screening

<table>
<thead>
<tr>
<th></th>
<th>Host</th>
<th>Transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

R: resistance  
S: sensitivity

described in Section 2, into *B. subtilis* strain 1A771, which contains an erythromycin-resistance (ery<sup>R</sup>) gene in the *amyE* locus. The vector pDG1661 is designed to have a double-crossover event with 1A771 at the *amyE* locus, replacing the *ery<sup>R</sup>* gene with a chloramphenicol-resistance gene, the cloned leader sequence, and the *lacZ* gene. A spectinomycin-resistance gene is not incorporated if the double-crossover occurs. The vector without the cloned insert was also transformed as a control.

The colonies that grew on the chloramphenicol plates were validated by antibiotic screening. Successful transformants are expected to grow on chloramphenicol, but not on spectinomycin or erythromycin (Table 4). Colonies from the original vector (control) did not grow on spectinomycin, and only 3 of the 6 grew on erythromycin, resulting in 3 successful transformants (Figure 17). There were many more successful colonies from the

![Figure 17](image_url)  
Figure 17. Untransformed colonies of *B. subtilis* strain 1A771 grown on chloramphenicol, erythromycin, and spectinomycin (left to right). This agrees with the expected result.
transformation with the ykkCD-vector construct. In all, there were 19 successfully transformed colonies that exhibited erythromycin and spectinomycin sensitivity (Figure 18, bottom). Two colonies from each transformation (control and ykkCD-lacZ) were stored with glycerol at -80 °C.

3.2. SIC Values

To quantify how gene expression changes when cells are treated with a given antibiotic, cells have to be grown under conditions where growth rate is reduced, but still substantial. This condition typically has an optical density (OD$_{595\text{nm}}$) that is 30-60% filled compared to that of untreated cells. Under reduced growth conditions the cells are
expected to express protein that allow them to survive the antibiotic such as efflux pumps, antibiotic degrading or modifying enzymes. The antibiotic concentration leading to reduced growth is called the subinhibitory concentration (SIC value). For each antibiotic of interest, the SIC value for using Bacillus subtilis was determined. This was accomplished by growing the cells in a panel of serial dilutions of each antibiotic. The OD$_{595}$ of each was measured after 19 hours growth. The SIC was considered to be the concentration which reduced the OD by approximately 50% compared with untreated culture. Figure 19 provides an example of a typical experiment. At 5 μg/mL of chloramphenicol, the growth is approximately half that of the untreated culture, and so that is considered the SIC. With the assistance of previous and current researchers, the SIC values were determined and are summarized in Table 5.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>SIC, ug/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>12.3±10.6</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>3.2±1.2</td>
</tr>
<tr>
<td>Tetraphenyl phosphonium chloride (TPPC)</td>
<td>85±183</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>20000±23000</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>16.67</td>
</tr>
<tr>
<td>Phosphomycin</td>
<td>3400±4500</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>&lt;0.625</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.313±0.125</td>
</tr>
<tr>
<td>Anhydrotetracycline</td>
<td>0.25±0.22</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>17±2</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.015±0.005</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>1.63±0.55</td>
</tr>
<tr>
<td>Pyronin Y</td>
<td>9.8±1.5</td>
</tr>
<tr>
<td>Proflavin</td>
<td>3.14±0.20</td>
</tr>
</tbody>
</table>

### 3.3. β-galactosidase Assay

To determine whether the ykkCD putative riboswitch upregulates gene expression, β-galactosidase reporter gene assays were conducted. Our initial β-galactosidase experiments used o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, which is broken down into the yellow product o-nitrophenol. However, the sensitivity of this procedure was too low to obtain a useful signal in either untreated or treated cell cultures. As a result, we decided to use a more sensitive, fluorescent assay, in which 4-methylumbelliferyl-β-D-galactopyranoside (MUG) is added as the substrate. Beta-galactosidase converts it into 4-methylumbelliferone (4-MU), which has a maximum emission at 450 nm when excited by 365 nm light. (See Figure 20.) The procedure is given in Section 2.2.

The β-galactosidase assay was performed on cultures of *B. subtilis* strain 1A771,
Figure 20. Beta-galactosidase breaks down MUG into galactose and the fluorescent product 4-MU.

transformed with the ykkCD-lacZ construct, grown with tetracycline, one of three of its derivatives, or without antibiotic as baseline control. (Antibiotic concentrations are given in Table 5.) Using a standard curve generated alongside the samples as described in Section 2.2, the fluorescence values for each sample were converted into enzyme activity expressed in Miller Units. One Miller Unit is defined as 1 pmol of 4-MU produced per minute. These results are summarized in Figure 21. Some researchers normalize the β-galactosidase activity per microgram of total protein as determined by a Bradford assay. Initial results by this method indicate an increase in β-galactosidase activity due to oxytetracycline compared with the untreated control. The consequences of optimization will be discussed further in Section 4.

3.4. RNA Extraction

Although the β-galactosidase assay is a useful screening test, it has a rather low sensitivity. Consequently, NASBA was used to supplement this data by quantifying the level of ykkCD mRNA. The first step was to extract and purify the total RNA from live cultures of B. subtilis strain NRRL B-765. The cultures were grown without an antibiotic or with an antibiotic at subinhibitory concentration, as before. Using the Qiagen RNeasy Minikit, RNA extraction was streamlined in a convenient, repeatable protocol. Typical RNA concentration following extraction was 50-300 ng/μL. The RNA extracts were
β-Galactosidase Assays

Figure 21. Results of the β-galactosidase experiment, summarizing 28 trials without antibiotic, 9 with tetracycline, 9 with doxycycline, 6 with minocycline, and 4 with oxytetracycline.

analyzed on a denaturing agarose gel prior to NASBA or RT-PCR to verify RNA quality. Although the mRNA is not visible on the gel, we believe that rRNA quality is a good representation of the condition of the mRNA, as this is a common method employed by other laboratories. An example of poor quality, indicative of RNA degradation, and one of good quality RNA are shown in Figure 22.

3.5. NASBA

Each NASBA run included separate reactions with the ykkC and ykkD primers for each sample and with the DNA gyrase B primers as an internal reference. SYBR® Green II, an RNA intercalating dye, was included for detection. Upon interacting with RNA, SYBR® Green II fluoresces, and the signal is detected by the SmartCycler®
Figure 22. Denaturing agarose gel electrophoresis was performed after RNA extraction to verify RNA quality. Left: Good quality RNA. The 23S and 16S rRNA bands are distinct in all three samples. Right: Poor RNA quality. There is significant smearing in all three lanes, which indicates RNA degradation. These samples were eliminated from NASBA or RT-PCR.

thermal cycler and plotted against cycle number. From this plot, the cycle where the fluorescence signal rises above the baseline is determined, which is known as the cycle threshold or Ct value. There is an inverse relationship between Ct value and initial RNA concentration such that a sample with a lower Ct value has a higher starting amount of the RNA template than one with a higher Ct value. Since SYBR® Green II can detect any RNA molecule, it is critical to eliminate sources of contamination. In an effort to reduce sources of exogenous RNA, the following precautions were taken:

- Preparing the reactions inside a sterile hood
- Cleaning the hood’s surface with RNase Away® to remove ribonucleases
- Wiping the pipettors with 75% ethanol
• Using prepackaged aerosol-resistant tips (ART)
• Irradiating everything (including gloves) with UV light for 5 minutes before assay set-up
• Preparing the reactions in the semi-dark to protect the SYBR® Green.

To verify that the reactions were not contaminated, a no-template control (NTC)—containing buffers, nucleotides, forward and reverse primers, and enzymes, but no template RNA—was prepared each time for each primer set.

The raw data is summarized in Table 6. The variability as indicated by the standard deviation shows that there was little reproducibility between runs. This included some of the early runs during which the technique was being optimized. The last month’s worth of reactions is summarized in the right hand side of Table 6. By this point there should have been reduced contamination by the experimenter, and yet the reproducibility had not improved much, especially for the ykkC primers. The ykkD and gyrase B primers have better standard deviations, but their values closely resemble those of the NTC reactions. This suggested that there was consistent contamination in all the samples.

<table>
<thead>
<tr>
<th></th>
<th>All Reactions</th>
<th></th>
<th>Final Month of Reactions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># rxns</td>
<td>Average Ct</td>
<td>St.Dev.</td>
<td># rxns</td>
</tr>
<tr>
<td>NTC C</td>
<td>11</td>
<td>53.0</td>
<td>19.9</td>
<td>4</td>
</tr>
<tr>
<td>NTC D</td>
<td>9</td>
<td>44.0</td>
<td>17.1</td>
<td>3</td>
</tr>
<tr>
<td>NTC gyrase</td>
<td>9</td>
<td>38.8</td>
<td>4.9</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline: C</td>
<td>9</td>
<td>46.1</td>
<td>20.1</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline: D</td>
<td>9</td>
<td>39.6</td>
<td>13.6</td>
<td>3</td>
</tr>
<tr>
<td>Tetracycline: gyrase</td>
<td>11</td>
<td>37.6</td>
<td>10.9</td>
<td>4</td>
</tr>
<tr>
<td>No Antibiotic: C</td>
<td>6</td>
<td>35.3</td>
<td>8.1</td>
<td>2</td>
</tr>
<tr>
<td>No Antibiotic: D</td>
<td>6</td>
<td>45.7</td>
<td>26.8</td>
<td>0</td>
</tr>
<tr>
<td>No Antibiotic: gyrase</td>
<td>10</td>
<td>39.1</td>
<td>11.8</td>
<td>3</td>
</tr>
</tbody>
</table>
Since new enzymes had been periodically ordered and new buffers had been prepared, the next likely source of contamination was the primers.

The primers that I had been using up to this point were used by a previous researcher and had been in storage for at least two years. It stood to reason that they might have degraded or become contaminated. To test this idea, I set up a series of No-Template reactions. Some of the samples included one of the primer sets (C1/2, D1/2, G1/2), some had the enzymes but no primers (NP), and one reaction had neither primers nor enzymes, just buffers, nucleotides, and SYBR® Green II. The result is shown in Figure 23, and the C_t values are listed in Table 7. Since these reactions did not contain mRNA, there should not have been a signal in any of them. Signals in the ykkC and gyrase samples were similar to the previous results, indicating that the original primers were contaminated. Thus, all primers were reordered at this time; their sequences are given in Table 1 in Chapter 2.

The primers were reconstituted at 100 μM in water. The ensuing reactions were much cleaner. However, many of the fluorescence signals were trailing off toward

<table>
<thead>
<tr>
<th>Table 7. No-Template Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>NE</td>
</tr>
<tr>
<td>NP1</td>
</tr>
<tr>
<td>NP2</td>
</tr>
<tr>
<td>C1</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>D1</td>
</tr>
<tr>
<td>D2</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>G2</td>
</tr>
</tbody>
</table>

Figure 23. NASBA without template. NE=no enzymes. NP=no primers. C1 & C2=primers for pump C. D1 & D2=primers for pump D. G1 & G2=primers for DNA.
negative infinity, leaving only 6 usable C\textsubscript{T} values out of 21 reaction set-ups. These signals, rather than having the expected sigmoidal amplification curve, had a gradual increase above the baseline and a distinctive “saw-tooth” pattern. (See Figure 24)

According to the user manual for the SmartCycler, a potential cause of the “saw-tooth” pattern was too much SYBR\textsuperscript{®} Green. Up until this point I had been using 2 μL of 10,000X SYBR\textsuperscript{®} Green II per reaction. To find the optimal SYBR Green concentration, I set up a series of reactions with 2 μL, 1μL, and 0.5 μL of SYBR\textsuperscript{®} Green II per reaction. (See Figure 25.) The lineforms became more clear as the amount of SYBR\textsuperscript{®} Green was...
Figure 26. Left: plot of Figure 25. NASBA is expected to produce a regression line with a negative slope, so using 1 μL SYBR Green still has too much non-specific detection. Right: summary of four NASBA experiments using 0.5 μL SYBR Green per reaction.

reduced, so apparently the sample had been flooded with SYBR® Green previously.

Plotting the C\textsubscript{T} values against the log of total RNA concentration (Figure 26, left), a regression line with a positive slope was generated for the experiment using 1 μL SYBR® Green. A negative slope would be expected, since there is an inverse relationship between RNA concentration and C\textsubscript{T} value. Thus [SYBR Green] was lowered. The summary of four experiments using 0.5 μL SYBR® Green per assay is plotted in the right-hand graph of Figure 26. Even though there is scatter, the lineform has a slight negative slope (as expected), and so the optimization stage was considered complete. All future reactions after this followed the procedure given in Chapter 2.

RNA isolated from *B. subtilis* cultures grown in subinhibitory concentrations of tetracycline, doxycycline, and anhydrotetracycline was quantified by NASBA. In some cases, the sample C\textsubscript{T} value was greater than that of the NTC, and in others, the fluorescence signal fell below 0. These points were eliminated, leaving the results in Table 8. The C\textsubscript{T} value of DNA gyrase was close for all four culture types, which means that these antibiotics did not affect the levels of total mRNA. Thus a direct comparison
Table 8. NASBA Results

<table>
<thead>
<tr>
<th>Antibiotic/primer</th>
<th># assays</th>
<th>Ct, mean ±st.dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None/ykkC</td>
<td>3</td>
<td>26.6±15.3</td>
</tr>
<tr>
<td>Tetracycline/ykkC</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Doxycycline/ykkC</td>
<td>2</td>
<td>23.0±14.1</td>
</tr>
<tr>
<td>Anhydrotetracycline/ykkC</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None/ykkD</td>
<td>3</td>
<td>31.3±1.5</td>
</tr>
<tr>
<td>Tetracycline/ykkD</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Doxycycline/ykkD</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>Anhydrotetracycline/ykkD</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None/gyrase</td>
<td>2</td>
<td>30.0±1.4</td>
</tr>
<tr>
<td>Tetracycline/gyrase</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Doxycycline/gyrase</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>Anhydrotetracycline/gyrase</td>
<td>1</td>
<td>29</td>
</tr>
</tbody>
</table>

within each primer set is possible. Unfortunately, there are not enough data to draw any reliable conclusions. All the data in the table was from 14 NASBA reactions out of 51 set up, which is an inefficient and high-cost method of generating a complete data set. It is also possible that under the mild reaction conditions of NASBA (41 °C), the primers may not be selective enough, considering that we are trying to selectively amplify the mRNA of one of the pump subunits (ykkC or ykkD) in the presence of the other. These mRNAs have significant sequence similarities and long AT-rich regions. At 41 °C any duplex with $T_m < 48$ °C, even nonperfect ones, are stable, leading to nonspecific amplification of the mRNA. Therefore, we decided to utilize an alternative method of quantifying mRNA levels with a higher annealing temperature, RT-qPCR.
3.6. RT-PCR

Before performing RT-PCR, it was noted that the NASBA primers were not exact matches when aligned with *B. subtilis* subp. *Spizizenii* strain W23 on the NCBI GenBank. The forward *ykkC* and forward *ykkD* primers each had several mismatches, the forward *gyrB* primer had 5 mismatches, and the reverse *gyrB* primer had 7 mismatches. (Incidentally, the NASBA gyrase primers were exact matches for the DNA gyrase B gene of several other *Bacillus* species, including *B. cereus*, but not for any *B. subtilis* strain.) These primers might be useful for NASBA due to its lower reaction temperature, but they were not expected to achieve selective amplification at the more stringent conditions of RT-PCR. Consequently, new primers were designed to match our genome as described in Section 2.2, with the sequences given in Table 2.

RT-PCR performed with the Sigma QR0100 kit on RNA treated with DNase I quickly produced consistent and believable data. Figure 27 demonstrates the quantitative ability and sensitivity of this technique. The amount in each reaction was actually the total RNA, of which the mRNA is 10-20%, of which the target mRNA is a small percentage. This shows that RT-PCR is able to detect RNA in picogram amounts. The regression lines for *ykkC* and gyrase B were nearly parallel but not exact, indicating dependence on [RNA]. Based on these graphs, we concluded that 50 ng total RNA is optimal because it had good separation of C<sub>t</sub> values between pump *ykkC* and DNA gyrase and helped us conserve sample.

Since mRNA is converted to DNA in RT-PCR, melting curve analysis was used to evaluate each reaction. DNA fragments of various lengths and/or GC content can have
Figure 27. RT-PCR was performed on an RNA sample extracted from cells grown with tetracycline at SIC, using a range of amounts of total RNA. This was repeated with ykkC and gyrase primers. N=4 for all points except 1 ng (N=1).

different melting temperatures ($T_m$). Thus the identity of each amplicon in an RT-PCR reaction can be determined, making it possible to identify cross-contamination. The principal ykkC amplicon melted at 83.5-84°C, the principal ykkD amplicon melted around 85.5-86°C, and the principal gyrase amplicon melted at 83-83.5°C.

For RNA extracted from each treatment type—none, tetracycline, doxycycline, minocycline, oxytetracycline and anhydrotetracycline—RT-PCR was performed for the genes $ykkC$, $ykkD$, and DNA gyrase. In spite of the early success, a number of samples produced a positive No-template control (NTC), especially the earlier runs, and a number produced a positive signal in the No-RT control. In the course of optimizing the protocol, a lot of RT-PCR data was collected. They were categorized according to the following reaction conditions—
- RT-PCR kit: Sigma or Invitrogen brand of RT-PCR kit was used depending on availability. Different kits use slightly different genetically modified reverse-transcriptase, buffer conditions, and additives, making C_t values differ between kits. Thus data collected from the two kits has to be analyzed separately.

- Contamination: If No-template or No-RT controls are close to sample C_t values it indicates contamination, degradation, or insufficient removal of genomic DNA. Any samples that were relatively close to the No-template and No-RT controls run at the same time were excluded from analysis.

- Freshness of sample: Judging mRNA quality by gel electrophoresis uses the assumption that as long as rRNAs are intact, the mRNA is also intact. Since rRNA is much more structured and resistant to degradation than mRNA, judging mRNA quality based on rRNA quality has limitations. Particularly for the Invitrogen kit, only samples that were subjected to RT-PCR within two weeks of RNA extraction were kept for further analysis to ensure that the mRNA was still intact.

- DNase treatment: Using the Invitrogen kit, the amount of DNase I per sample was increased from 2 to 3 and even 20 U. Empirically, using 3 U DNase yielded the cleanest agarose gels and PCR amplification curves, so only these were included in the analysis.

There are two ways of analyzing RT-PCR data. The first is by a direct comparison of the C_t values. This method assumes that the amplification efficiency for each gene is
the same and is valid only if the reference gene produces the same C\textit{t} or take-off (TO) value for each sample. Table 9 summarizes the comparative quantitation results from the clean RT-PCR runs using the Sigma kit. (See Section 2.4 for a description of comparative quantitation.) For each gene, the average TO value ± standard deviation and the average amplification efficiency ± standard deviation are shown for each treatment. For DNA gyrase, the TO is not the same for each sample. In particular, it is 18 for doxycycline versus 15 for the untreated sample. Since PCR is an exponential amplification process, this represents nearly an order of magnitude difference, preventing a direct comparison of the sample results. Antibiotics are known to affect mRNA halflife, so it is possible that this is what has occurred. In addition, the efficiency varies not only according to the gene but also according to the treatment used. Consequently, we cannot draw any reliable conclusions from a direct comparisons of the TO values, so we used a higher-order algorithm of data analysis known as the Relative Expression Software Tool (REST).

The theory and basic features of the REST software are described in Section 2.4. It takes into account the variation of both the TO values and amplification efficiencies among the samples and calculates P values to establish the reliability of the results. The

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<tbody>
<tr>
<td>No antibiotic</td>
<td>10.8±1.1</td>
<td>1.80±0.02</td>
<td>10.2±1.7</td>
<td>1.75±0.08</td>
<td>15.3±1.8</td>
<td>1.81±0.07</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>11.3±1.3</td>
<td>1.82±0.02</td>
<td>10.4±1.9</td>
<td>1.77±0.11</td>
<td>16.9±1.2</td>
<td>1.82±0.08</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>8.7±1.8</td>
<td>1.72±0.11</td>
<td>8.5±1.6</td>
<td>1.64±0.18</td>
<td>18.0±1.7</td>
<td>1.87±0.08</td>
</tr>
<tr>
<td>Minocycline</td>
<td>NA</td>
<td>NA</td>
<td>10.4±4.3</td>
<td>1.68±0.27</td>
<td>15.2±1.8</td>
<td>1.82±0.05</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>NA</td>
<td>NA</td>
<td>7.6</td>
<td>1.7</td>
<td>15.0±0.9</td>
<td>1.86±0.10</td>
</tr>
<tr>
<td>Anhydrotetracycline</td>
<td>9.7±0.0</td>
<td>1.75±0.01</td>
<td>11.7±1.3</td>
<td>1.81±0.07</td>
<td>14.4±0.6</td>
<td>1.83±0.06</td>
</tr>
</tbody>
</table>
data was limited to the RT-PCR runs with the Invitrogen kit that had been treated with 3 U DNase.

**Tetracycline.** For tetracycline, no difference was noticed when comparing all the Sigma values or all the Invitrogen values with untreated values of the same category. However, when restricting the analysis to samples that had received 3U DNase treatment, ykkC was overexpressed by a factor of 4 (p=0.011*), and ykkD had a modest increase in expression of 1.7 (p=0.181). Thus, in the presence of tetracycline, the mRNA expression of ykkC is upregulated. These data are diagramed as whisker plots in Figure 28.

**Doxycycline.** Examining the results obtained with the Sigma kit suggests that doxycycline results in overexpression of ykkC and ykkD. Interestingly, when all the data from the Sigma kit was analyzed with REST, a 6X increase in ykkD was found (p=0.002) with a 95% confidence interval of 0.009-1309! However, limiting the REST analysis to fresh doxycycline samples that had been treated with 3 U DNase and tested with the

![Image](image.png)

**Figure 28.** Tetracycline relative expression, using 3U DNase-treated samples.
Figure 29. Whisker plot of doxycycline relative expression, using 3U DNase-treated samples.

Invitrogen kit, we observed essentially no difference from the untreated samples (Figure 29). *YkkC* had a 1.08X increase (p=0.960) and *ykkD* had a 1.05X increase (p=0.914). We can confidently say that doxycycline treatment does not result in overexpression of either *ykkC* or *ykkD* mRNA.

Figure 30. Whisker plot of minocycline relative expression.
Minocycline. Using the samples that received 3U DNase treatment, there is an observable upregulation of ykkC of about 2.5X (p=0.000*) For ykkD there was a 3.4X increase (p=0.096) See Figure 30 for whisker plot.

Oxytetracycline. Preliminary results for oxytetracycline indicate down-regulation. For ykkC the relative expression is 0.4 (p=0.185), and for ykkD, the relative expression is 0.2 of untreated (p=0.000*). However, this was based on triplicate RT-PCR runs of one extraction sample, so it would be beneficial to run additional replicates to verify this finding.

Anhydrotetracycline. A small number of RT-PCR experiments have been performed on mRNA from cells grown with anhydrotetracycline, but not enough to permit analysis. According to Table 9, there may be a slight decrease in mRNA levels, but this was only based on one RNA extraction. Future research plans include performing additional experiments with the Invitrogen kit.
Chapter 4. Discussion

4.1. Comparison of NASBA and RT-PCR

Two methods of quantifying mRNA levels were utilized in this study, NASBA and qRT-PCR. NASBA was attractive because of its sensitivity and its ability to detect mRNA in the presence of genomic DNA. However, NASBA kits are available through only one supplier who owns the patent, and using them for optimization would have been cost prohibitive. Our efforts to prepare the appropriate reaction conditions in the lab demonstrated the high likelihood of contamination. As a result, we chose to do qRT-PCR.

Kits for qRT-PCR are readily available from many suppliers, including Sigma and Invitrogen, for a reasonable price. Primers can be designed and produced quickly for the genes of interest. The procedure is readily adaptable to various primers by adjusting the annealing temperature. RT-qPCR achieves real-time detection through SYBR Green® fluorescence, allowing for relative quantification. Alternatively, fluorescent probes to the gene of interest can be designed to achieve real-time detection. Referencing to a bacterial housekeeping gene, such as DNA gyrase B, accounts for potential fluctuations in RNA levels due to inefficiency of the RNA extraction or changes in RNA half-life caused by the antibiotic itself. Although RT-PCR has great sensitivity, it requires highly trained personnel in order to avoid contamination and to interpret the results. Genomic DNA carry-over is a potential source of error, because after the mRNA is converted to cDNA
in the reverse transcription step, there is not a way to distinguish it from residual genomic DNA in the PCR step. Another disadvantage is that RT-PCR only quantifies mRNA and assumes that changes in protein expression levels correlate with changes in the amount of mRNA in the cell.

4.2. Reporter assay

In order to correlate an increase in mRNA levels to an increase in protein levels, we used the β-galactosidase reporter gene assay. By cloning the putative riboswitch in front of the lacZ gene and transforming the construct into B. subtilis, regulation by tetracycline or other potential ligands results in changes in β-galactosidase levels instead of ykkCD pump levels. Changes in β-galactosidase levels can be quantified through a colorimetric or fluorescent assay. The initial steps of cloning and strain validation of transformants take time and require more highly trained personnel. After that, running the individual assays is cheap, amenable to high-throughput, and can be carried out by less trained personnel. This makes it a useful screening assay, although its sensitivity is somewhat low.

Another advantage that the β-galactosidase assay has over other methods is that it can be used to study mutants of the sensor in vivo. Mutants have been designed and tested by the binding assays with tetracycline to find which bases of the riboswitch interact with the ligand. The most realistic method to investigate the in vivo effects of these mutants is through the β-galactosidase assay. The mutated sensor, rather than the native sensor, is cloned in front of the lacZ gene and transformed into B. subtilis. Beta-galactosidase activity is then determined for cell extracts as described above. The results of this
4.3. Comparison with binding affinities

When comparing the \textit{in vivo} mRNA expression studies with the \textit{in vitro} binding assays, a correlation is noted. The K\textsubscript{D} of tetracycline with the RNA was determined to be 10.7±4.5 nM. The tetracycline concentration at which the cultures were grown was 27.7 μM. It is reasonable to assume that the cellular concentration is within an order of magnitude of this value. The \textit{ykkCD} mRNA should be fully saturated with tetracycline at these cellular concentrations (cf. Figure 32) and is expected to increase expression of the \textit{ykkCD} gene. An increase in \textit{ykkC} and \textit{ykkD} mRNA is in fact seen with RT-PCR (Figure 32).

![Binding affinity of tetracycline derivatives](image)

Figure 32. K\textsubscript{D} of tetracycline derivatives as determined by binding assays. Doxycycline and oxytetracycline showed no pattern of binding.\textsuperscript{(26)}

experiment could be correlated with those of the mutant binding assays to map out the effective interactions of the \textit{ykkCD} sensor with tetracycline.
28), and so this supports the premise that tetracycline increases the expression of \( ykkCD \) mRNA.

The binding assays have shown that minocycline has an elevated \( K_D \) compared with tetracycline, 97.4±17.2 nM (see Figure 32); that is, it has reduced binding affinity for the \( ykkCD \) RNA. \textit{In vivo}, minocycline has a very low SIC value. The concentration used for RT-PCR analysis was 10 ng/mL, or 22 nM. Although this is below the saturation point based on analysis of the binding curve (Figure 33), the \( ykkCD \) mRNA is expected to be 28% saturated with minocycline. This level of saturation should not trigger gene expression to the level tetracycline does but some increase in gene expression is expected. Using RT-PCR, overexpression of \( ykkC \) by 2.5 times was seen with minocycline treatment, which is somewhat surprising, and a less noticeable effect was seen on \( ykkD \) expression. However, this result was obtained from one RNA extraction. It would be advantageous to repeat RT-PCR in order to draw more definitive conclusions.

Anhydrotetracycline shows tight binding with the mRNA according to the binding assays, with a \( K_D \) of 24.5±9.2 nM. The \textit{in vivo} concentration in this study was 648 nM, far above the saturation level, and so an elevated level of \( ykkCD \) mRNA would be

![Figure 33. The minocycline binding curve, from the binding assay performed by Delores James.](image-url)
expected. The data collected so far for anhydrotetracycline came from just one extraction and is rather inconsistent. It has yet to be repeated with the Invitrogen kit, and so it would be informative to perform this analysis to determine the effect of anhydrotetracycline with more confidence.

The doxycycline results of the binding assay were ambiguous. Saturation was eventually achieved, but the data points were too scattered to determine a $K_D$ value. We typically classify doxycycline as a non-binder. The RT-PCR results agree with this, with relative expression straddling the value of 1. The concentration at which the cultures were grown was about 1.6 μM. At this concentration, we would not expect any upregulation due to doxycycline since we cannot reach appropriate levels of doxycycline-$ykkCD$ complex formation. The cultures do not survive at higher concentrations and so an in vivo method could not be used evaluate pump regulation.

Oxytetracycline clearly does not bind to the $ykkCD$ RNA, even at very high concentrations. $B. subtilis$, however, tolerates rather high concentrations of oxytetracycline, for the cultures were grown with 40.3 μM oxytetracycline. At this concentration it is conceivable that a non-specific interaction may be formed between the $ykkCD$ mRNA and oxytetracycline. Our initial RT-PCR result shows a potential downregulation of $ykkCD$ mRNA, which can be explained by a lack of interaction with the mRNA. Since a small sample size was used, we intend to repeat this experiment to verify its repeatability. From a therapeutic point of view, it is encouraging that a compound may have been found that evades this mechanism of resistance.
4.4. Regulation of ykkCD

For *B. subtilis* cultures grown with three of the antibiotics, an interesting pattern was noted. The relative expression of *ykkC* mRNA was greater than that of *ykkD* for tetracycline and oxytetracycline, and although the mean of *ykkC* with minocycline was lower, the confidence interval did not extend as low as *ykkD*. A similar disparity in mRNA levels for the two genes was detected in the original NASBA screening assay performed by Ambar Rana. Comparing Figure 28 (RT-PCR on tetracycline) with Figure 3 (NASBA on a variety of compounds), the increase in *ykkC* expression due to tetracycline is pronounced but that of *ykkD* is less dramatic. Although the newer method of mRNA quantification is validated, a question arises as to the biological rationale for this disparity. The YkkCD efflux pump cannot function unless both subunits are expressed.\(^5\) (It is presumed that the pump functions as a dimer, though it could be a tetramer, etc.) If both are necessary, why is one mRNA increased more than the other in the presence of the ligand? Perhaps regulating *ykkC* is sufficient for regulating protein expression. Another possibility is that both mRNAs are transcribed on the same transcript, but since *ykkC* comes first it is more likely than *ykkD* to be read through before the RNA polymerase falls off the DNA.

4.5. Does an increase in cellular mRNA levels mean an increase in protein levels?

The β-galactosidase assay has produced differing results from the binding assays and RT-PCR. Of the four antibiotics investigated so far, oxytetracycline produced the greatest increase in β-galactosidase levels, although RT-PCR indicated a decrease in mRNA levels. Tetracycline and minocycline produced a slight decrease in β-
galactosidase, even though they produced an increase in ykkC mRNA levels. Although it would be a waste of the cell’s energy, it is conceivable that mRNA levels do not trigger increased protein production or that ykkCD pump production is under translational control. It would be beneficial to answer this question by performing a Western blot using the antibiotics that had produced an increase in ykkCD mRNA levels to determine whether there was a corresponding increase in ykkCD protein levels.

On the other hand, the β-galactosidase assay may need further optimization. Since it is an absolute quantification method, bias can be introduced when unequal sample sizes are used. Thus, each time the β-galactosidase assay is performed, the same number of cells is used, normalized to an OD of 0.375. The problem may come from the fact that the cultures are not homogenous and the same cell count may not mean the same total protein. Other groups have normalized β-galactosidase activity to total protein by means of a Bradford assay. Using this method will be considered for future assays.

4.6. Conclusion

In this study, three methods of in vivo quantification have been optimized and evaluated. Each has its advantages and its limitations, and so when used together a more or less complete picture of the effects of antibiotics on ykkCD expression can be determined. These methods should prove valuable in discovering and evaluating other potential ligands of the ykkCD riboswitch. At this point, a potential correlation has been found between in vitro binding of the ykkCD mRNA with certain tetracycline antibiotics and the mRNA expression levels in vivo. Further investigation would be beneficial to establish these findings with more confidence. The procedures have been optimized so that this can be accomplished with relative ease.
Figure A. 1. Sequence and predicted secondary structure of putative ykkCD riboswitch, aptamer domain and expression platform.
Figure A. 2. Output of Primer3, giving alignment of ykkC primers with ykkC gene. Amplicon is 172 bp.

Figure A. 3. Output of Primer3, giving alignment of ykkD primers with ykkD gene. Amplicon is 160 bp.
Figure A. 4. Scatterplot of RT-PCR results: no antibiotic/ykkC. In this and the following figures, the dashed circles indicate the values used for Table 9, and the solid circles indicate the values used in the REST analysis.

Figure A. 5. Scatterplot of RT-PCR results: no antibiotic/ykkD
Figure A. 6. Scatterplot of RT-PCR results: no antibiotic/gyrase

Figure A. 7. Scatterplot of RT-PCR results: tetracycline/ykkC
Figure A. 8. Scatterplot of RT-PCR results: tetracycline/ykkD

Figure A. 9. Scatterplot of RT-PCR results: tetracycline/gyrase
Figure A. 10. Scatterplot of RT-PCR results: doxycycline/ykkC

Figure A. 11. Scatterplot of RT-PCR results: doxycycline/ykkD
Figure A. 12. Scatterplot of RT-PCR results: doxycycline/gyrase

Figure A. 13. Scatterplot of RT-PCR results: minocycline/ykkC
Figure A. 14. Scatterplot of RT-PCR results: minocycline/ykkD

Figure A. 15. Scatterplot of RT-PCR results: minocycline/gyrase
Figure A. 16. Scatterplot of RT-PCR results: oxytetracycline/ykkC

Figure A. 17. Scatterplot of RT-PCR results: oxytetracycline/ykkD
Oxytetracycline: gyrase

Figure A. 18. Scatterplot of RT-PCR results: oxytetracycline/gyrase

Anhydrotetracycline: ykkC

Figure A. 19. Scatterplot of RT-PCR results: anhydrotetracycline/ykkC
Figure A. 20. Scatterplot of RT-PCR results: anhydrotetracycline/ykkD

Figure A. 21. Scatterplot of RT-PCR results: anhydrotetracycline/gyrase