

Bard

Bard College
Bard Digital Commons

Senior Projects Spring 2020


Bard Undergraduate Senior Projects

Spring 2020

Developing a High-Throughput LacZ Reporter Gene Assay to Evaluate Activation of the xpt-pbuX Guanine Riboswitch by Analogue Ligands

Silvie Hannah Lundgren
Bard College, sl8772@bard.edu

Follow this and additional works at: https://digitalcommons.bard.edu/senproj_s2020

 Part of the [Analytical, Diagnostic and Therapeutic Techniques and Equipment Commons](#), and the [Chemicals and Drugs Commons](#)



This work is licensed under a [Creative Commons Attribution-NonCommercial-No Derivative Works 4.0 License](#).

Recommended Citation

Lundgren, Silvie Hannah, "Developing a High-Throughput LacZ Reporter Gene Assay to Evaluate Activation of the xpt-pbuX Guanine Riboswitch by Analogue Ligands" (2020). *Senior Projects Spring 2020*. 119.
https://digitalcommons.bard.edu/senproj_s2020/119

This Open Access work is protected by copyright and/or related rights. It has been provided to you by Bard College's Stevenson Library with permission from the rights-holder(s). You are free to use this work in any way that is permitted by the copyright and related rights. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself. For more information, please contact digitalcommons@bard.edu.

Bard

Developing a High-Throughput *LacZ* Reporter Gene Assay to Evaluate Activation
of the *xpt-pbuX* Guanine Riboswitch by Analogue Ligands

Senior Project Submitted to
The Division of Science, Math, and Computing
of Bard College

by
Silvie Hannah Lundgren

Annandale-on-Hudson, New York
May 2020

Abstract

Riboswitches are special structures of RNA typically found in bacteria. They respond to the presence of certain molecules, called ligands, within the bacterial cells to regulate expression of certain genes. Genes expression is the process by which information encoded in a segment of an organism's genetic material is used to produce functional molecules utilized by that organism. Riboswitches are currently being researched as a possible new class of antibiotic targets amid the growing public health threat of antibiotic resistance.

In order to compare the effects of guanine (the natural ligand of the riboswitch), guanosine (a biological molecule similar to guanine) and SK4 (a molecule synthesized by previous Bard College seniors based on the structure of guanosine) on the *xpt-pbuX* riboswitch, I used a reporter gene assay in a model organism: a harmless type of bacteria called *Bacillus subtilis*. The structure of the *xpt-pbuX* riboswitch allows it to interact with ligands of shape similar to guanine. For the reporter gene assay, a gene (called *LacZ*) that encodes the enzyme beta-galactosidase is specially inserted into the bacterial genome next to the riboswitch. The production of this enzyme is measure by adding a chemical called ONPG to the bacterial cultures. The enzyme reacts with ONPG to produce a yellow color that can be measured. The less intense the yellow color, the less active enzyme has been produced by the bacteria. Thus, expression of the *LacZ* gene "reports" back on the activation of the riboswitch. If incubating bacterial cultures with a given ligand results in less production of active enzyme as compared to incubation without the ligand, this is presumed to mean that the ligand has activated the riboswitch.

In this project I worked to develop an alternative method for the traditional beta-galactosidase assay, minimizing the quantities of chemicals and bacterial cultures required. Instead of incubating bacterial cultures in vials with the study ligands and using cuvettes of bacterial cultures for the enzyme assay, I incubated very small volumes of bacterial cultures in the wells of 96-well microplates and then performed the assay in 96-well microplates. The intensity of yellow color in these bacterial cultures can be directly measured in the microplates using a plate-reader. I also implemented a kinetic assay in which the changing intensity of yellow color is monitored over time instead of waiting for yellow color to develop and then measuring it, with the hope of minimizing timing error in the assay.

Acknowledgements

This world and the people in it can always benefit from more gratitude. While there are so many more whom I would mention here if space allowed, I especially desire to express my sincere

~ Thanks ~

To all my friends from Bard and my community who did the things that friends do: Talk with me; study with me; have meals with me; sit with me while I cry; have wild, hilarious fun with me; laugh with me; smile at me; check in on me; pray for me; show me love; exchange advice with me; let me stay the night ...

To my biochemistry advisor, Professor Swapan Jain. For welcoming me into your class as a high school student and opening my eyes to how interesting chemistry can be. For all of your support, research mentorship, inspiration, patience and care, and for sharing your family with me. For keeping my best interest in mind and being eager to help me take advantage of opportunities.

To my Russian and Eurasian Studies advisor, Professor Olga Yurievna Voronina. For helping me navigate the requirements for my double major. For teaching me to just keep going, without judgement. For reminding me again and again to rest and take breaks from the academic.

To the Breaker lab at Yale University. For providing our lab with the modified *B. subtilis* bacteria used in this project.

To the entire chemistry faculty. For your instruction and support.

To past and current research students at Bard who made this project possible.

To Professor Emily McLaughlin and Professor Craig Anderson. For being there for me as mentors and friends. For offering me guidance. For all of our hilarious humorous exchanges.

To Professor Chris LaFratta. For emphasizing fun and amazing things about chemistry in your classes and giving us such wise input that helped keep our worried student emotions in check.

To Dr. Rebeca Patsey. For your assistance, advice, and support.

To Dr. Maureen O'Callaghan, Dr. Craig Jude, and Adam Noach. For all your work and assistance.

To my first close college buddy and fellow biochemistry major, Hang Hoang ('18). For being a friend and study partner and for making sure that my shy self had made more friends before you moved away.

To Tamaki Chiba ('17). For being the best lab partner ever in gen chem, and for inspiring me.

To Danielle Santos ('20), Ian McClellan ('21) and Tahmid Siddique ('22) in the chemistry program. For understanding me, spending time with me, and showing me how to live a little.

To Bryan Palma Flores ('22), who assisted me in the lab. For your friendship, for all your help senior year both in and out of the laboratory, and for our miscellaneous candid conversations.

To Rowan Puig ('20, Bard Conservatory '21) and Elana Kellerhouse (SUNY New Paltz '20). For being truly incredible friends and siblings in faith and being such a lifeline after campuses shut down and we all became sequestered from each other.

To so many other friends, classmates, confidants and colleagues whom I cannot list here. For the incredible impact you have made on my life. I am thinking of you!

To Professor James Bagwell. For your warmth.

To David and Rachel Nelson. For your caring and welcoming.

To Professor Matthew Deady. For your incredible kindness and encouragement. For greeting me with a big smile every time you happened by, ever since freshman year. For your stellar responses to my underconfidence.

To Professor Ilka LoMonaco, my voice teacher. For your singing lessons, which were life lessons. For teaching me never to apologize for my existence or undermine my statements. For saying “fear is not a good advisor”—this has stuck with me. For helping me realize what I am capable of accomplishing even when I am nervous.

To Daniel Sullivan, PsyD, from Bard Counselling. For sharing uplifting and cheerful advice and for helping me to see myself differently this last year and find ways to foster my own wellbeing.

To my brother, Noach Lundgren ('16, Hartt School '18), and to my now-sister-in-law, Jerusha (Kellerhouse) Lundgren ('11, Bard MAT '12). For being my magnificent syskon, for helping Bard feel more familiar, and for being able to commiserate with me.

To my parents, who were my first teachers. For always being there for me and supporting me, and for encouraging me to embrace my passions without ever causing it to cross my mind that there could be anything strange about my interest in science.

To my Grandma, Edith Marian Dacks (1935–2016), who saw me enter college and no longer walks this earth. Her support has continued to aid my family and my dreams.

To the College itself, my first school to ever attend, and to its faculty and staff. For being challenging and close-knit. For being an institution that does not tend to rigidly put people and their interests into boxes—where a math and computer science dual major can be a singer and it is not so odd to see a chemistry major toting a musical instrument. For allowing me to study two subjects that enliven me with instructors who inspire me.

To G-d, may He be glorified, who created me—quirks and all—and created this amazing world of chemicals and languages for us to inhabit and explore. It never ceases to wonder me that life exists and that somehow people are capable of communicating with each other.

Contents

1. Introduction	1
1.1 <i>The public health challenge of overcoming antibiotic resistance</i>	
1.2 <i>Genetic regulation in microbes and the discovery of the riboswitch</i>	
1.4 <i>Effects of modified ligands on riboswitch function and gene expression</i>	
1.5 <i>Reporter gene assay to evaluate impact of ligands on riboswitch-function</i>	
1.6 <i>High-throughput and kinetic approaches to the reporter gene assay</i>	
2. Materials and Methods	10
2.1 <i>Individual items and reagents</i>	
2.2 <i>Reagent mixtures and preparations</i>	
2.3 <i>Beta-galactosidase assay</i>	
3. Results and Discussion	23
3.1 <i>Bacterial biofilm production in variations of chemically defined growth media</i>	
3.2 <i>Bacterial solvent tolerance in ligand condition cultures</i>	
3.3 <i>Kinetic and stop-solution beta-galactosidase assays performed in microplates</i>	
4. Conclusion	30
4.1 <i>Riboswitch-mediated gene suppression by analogue ligands</i>	
4.2 <i>Experimental approach for high-throughput and kinetic beta-galactosidase assays</i>	
4.3 <i>Additional features of riboswitch-mediated gene suppression to evaluate</i>	
5. References	32
6. Appendix	35

1. Introduction

1.1 The public health challenge of overcoming antibiotic resistance

Antimicrobial resistance is one of the major public health threats of today. The discovery of antibiotics was an incredible step in modern medicine, but as the years have gone by, more and more antibiotic resistant bacteria have emerged. Few new effective antibiotics have been developed recently, and those that are put into use meet with resistance very quickly. Different tactics such as reducing the prescription of antibiotics and the industrial use of antimicrobials, altering the length of antibiotic courses, combining antibiotics, and treating bacterial infections by the introduction of other bacteria all being attempted both to slow the rapid emergence of “super bugs” and to treat infections by resistant bacteria. Another highly promising approach is phage therapy, the use of bacteriophages (a type of bacteria-targeting virus) to treat infections [1].

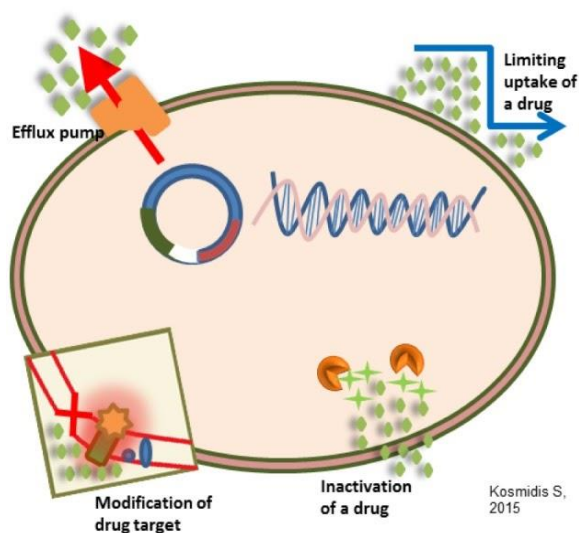


Figure 1. Several major types of antibiotic resistance mechanisms. [2]

Many antibiotics are bacteriocidal, meaning that they induce cell death. Other antibiotics are bacteriostatic, meaning that they halt the growth and reproduction of bacteria but do not necessarily kill them. Bacteriostatic antibiotics most commonly target protein synthesis pathways [3]. One hypothesis is that bacteriostatics may be less likely to trigger the emergence of resistance since they put less selective pressure on the bacteria. However, it has also been posited that bacteriostatics may allow the surviving bacteria to mutate and develop types of resistance that may have been avoided at least in part by using bacteriocidals [4]. If an antibiotic can target something key to successful life and growth of bacteria, something that is involved in the regulation of many genes, this may also make it more difficult or less likely that the bacteria will be able to circumvent the action of the antibiotic. If an antibiotic targets something specific to bacteria that is not present in humans or animals, this could also possibly reduce the toxic effects of antibiotics on the patient being treated with the antibiotic. These are promising aspects of riboswitches [5].

1.2 Genetic regulation in microbes and the discovery of the riboswitch

Incredible discoveries have been made about genetic material and gene regulation in the past few decades. Until recently, it was thought that additional enzymes and cellular machinery were required for gene regulation and that noncoding DNA was simply passive. It was also thought that RNA simply existed to carry information contained in the DNA genome. It has now been shown that noncoding genetic material does play a role in the regulation of gene expression and that some types of RNAs are able to regulate themselves. [6]

Functional RNA structures called riboswitches are an example of this. They are most commonly found in the 5' untranslated regions of bacterial mRNAs. These RNA structures respond to the presence of specific ligands, such as metabolites, to regulate gene expression.

When a ligand binds to the aptamer domain of the riboswitch, this induces a structural change in the expression platform of the riboswitch that can affect transcription and translation of downstream genes without the help of intervening proteins. Riboswitches respond to changes in the cellular environment to adjust protein production and help the cell respond appropriately to changing conditions. [7, 8]

1.4 Effects of modified ligands on riboswitch function and gene expression

Since analogs of natural riboswitch ligands mimic natural bacterial metabolites, they present a possible means of overcoming bacterial resistance pathways. The *xpt-pbuX* guanine riboswitch is involved in bacterial metabolism. When the aptamer domain of the riboswitch is bound by an appropriate ligand, the adjacent expression platform changes conformation (Figure 2).

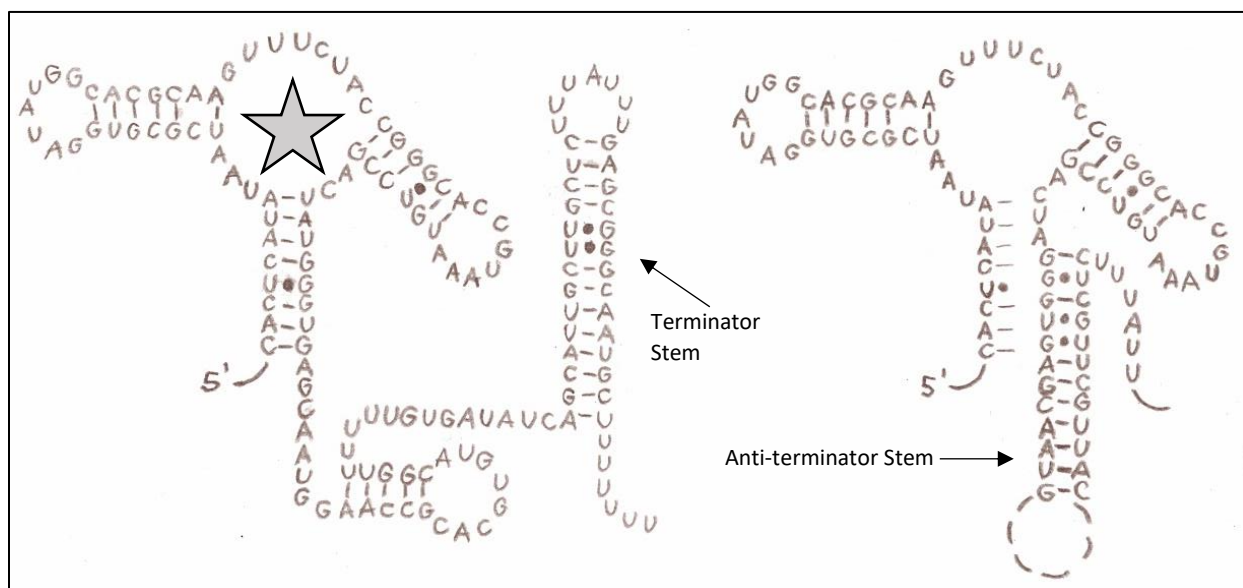


Figure 2. Ligand-bound and ligand-free *xpt-pbuX* riboswitch. The left-hand structure shows the conformation of the riboswitch when bound by its ligand. When the ligand (represented by a star) is bound, the terminator stem prevents the ribosome from binding to the mRNA molecule.

The right-hand structure shows the conformation of the riboswitch when no ligand is bound. In this conformation, the anti-terminator stem allows translation of the mRNA. The binding pocket of the riboswitch is able to accommodate ligands with modified structures [9].

The natural ligand of the *xpt-pbuX* riboswitch is guanine, which has very poor solubility. Guanosine shares the same chemical backbone and is much more soluble than guanine. Previous students synthesized the molecule SK4 (*Figure3*), based on the structure of guanosine, with the goal of creating a riboswitch ligand with greater solubility that also may bind more tightly to the riboswitch because of its functionalization. [10, 9]

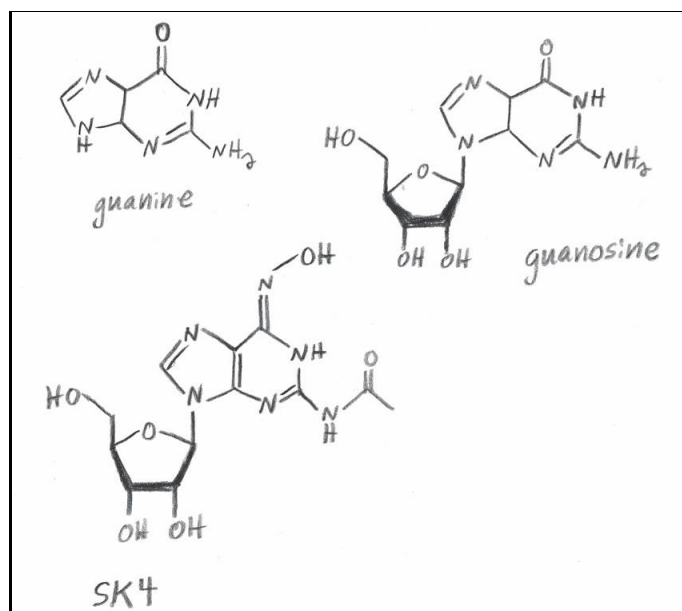


Figure 3. Structures of the three riboswitch ligands compared in this project. Guanine, the natural ligand of the *xpt-pbuX* riboswitch, is a purine nucleobase. Guanosine is the nucleoside of guanine, comprising guanine with a ribose group substituted on the nitrogen at position 9. SK4 is a synthetic analogue of guanosine that has an oxime group at position 6 in place of the carbonyl and an amine substituent at position 2 in place of the amine. [9]

1.5 Reporter gene assay to evaluate the impact of ligands on riboswitch-function

Because the expression of native bacterial genes associated with the *xpt-pbuX* riboswitch cannot be easily monitored, the commonly used *LacZ* reporter gene was artificially fused to the *xpt-pbuX* riboswitch. The *LacZ* reporter gene is used in beta-galactosidase assays, of which

Miller assays are a type. The transformed *B. subtilis* cells used in this project came from a stock received from the Breaker group at Yale University. The parent bacteria is *B. subtilis* 168 and the cells were transformed so that they contain DNA for the *xpt-pbuX* riboswitch in its necessary context fused with the *LacZ* gene from *E. coli*. The *xpt-pbuX-LacZ* fusion is inserted into the *amyE* locus of the bacterial genome via double-crossover recombination involving the homologous recombination of the pDG1661 plasmid (Figure 4).

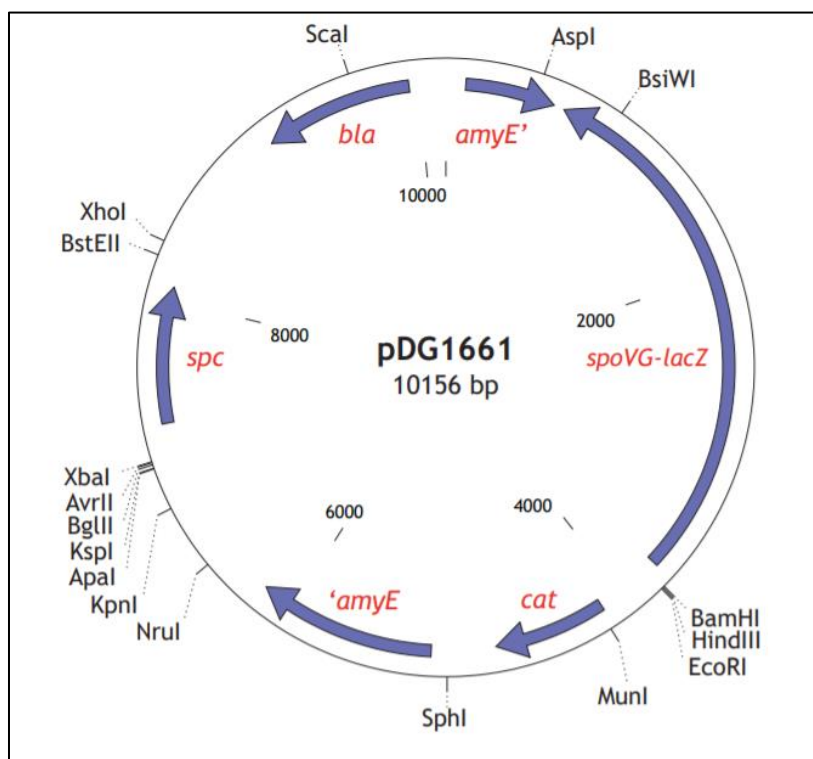


Figure 4. Integration vector map of the pDG1661 plasmid. Successful double-crossover recombination for integration of the *LacZ* construct into the *amyE* locus in *B. subtilis* can be checked by screening the bacteria for tolerance to chloramphenicol 5 ug/mL and sensitivity to spectinomycin 100 ug/mL. [11]

The *LacZ* gene encodes the enzyme beta-galactosidase, which is naturally responsible for catalyzing the transgalactosidation of lactose to allolactose and the hydrolysis of lactose to galactose and glucose [12]. This enzyme can also hydrolyze *o*-nitrophenyl-beta-D-galactopyranoside (ONPG) and produce *o*-nitrophenol (ONP), which has a yellow color and absorbs light in the ~420nm region (Figure 5). Actuation of riboswitches by effective ligands

usually leads to suppressed expression of their associated genes, and this is the case with the *xpt-pbuX* riboswitch. Bacterial cultures that produce less of the active beta-galactosidase enzyme will produce yellow color less quickly. Thus, the level of expression of the *LacZ* gene reports back on the actuation of the riboswitch, and less yellow color indicates more *suppression* of gene expression.

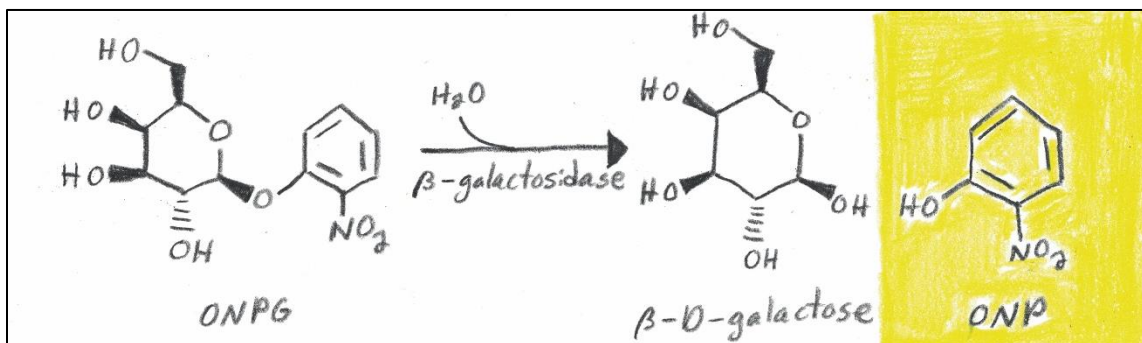


Figure 5. Beta-galactosidase-catalyzed hydrolysis of ONPG to produce the yellow ONP.

The most typical beta-galactosidase assay used with the *LacZ* reporter gene is called the “Miller” assay, after Jeffrey Miller [13]. The bacteria in question are first cultured on agar plates, then in nutrient growth medium, then in chemically defined minimal medium with whatever experimental conditions are being tested. Then aliquots of the cultures are added to reaction buffer, and chloroform and SDS are added and the mixtures are vortexed to permeabilize the cells. Then ONPG substrate is added. Once “sufficient color” has developed, 1 M Na_2CO_3 stop solution is added to stop the reaction (as the ONP production is enzymatic, all samples may eventually reach the same color intensity if left too long).

Beta-galactosidase activity in the assayed cultures is then calculated as “Miller Units” (MU) using the following equation:

$$1 \text{ MU} = \frac{1000 * (OD_{420} - 1.75 * OD_{550})}{\text{volume}}$$

$$t * v * OD600$$

Where t = reaction time, v = volume of culture used (mL), OD600 is the OD600 of the culture before being used in the assay, OD420 is the OD420 of the assay tube at the end, OD550 is the OD550 of the assay tube at the end ($1.75 * OD600$ is an optional term to correct for light scattering due to cell debris). These MU are not absolute enzyme activity units—they are relative units expressing relative beta-galactosidase activity.

Optical density at 600nm (OD600) reflects cell density and is used as a proxy indicator of bacterial growth in liquid media. Beta-galactosidase activity cannot be determined solely on the basis of the intensity of the yellow color and must be calculated with a correction factor for bacterial density (OD600), as differences in the sheer number of bacterial cells in a volume will affect the total amount of beta-galactosidase enzyme produced in that volume. OD600 of liquid cultures must be tested at the beginning and end of incubation periods to ensure the OD600 falls within the desired range, and sometimes must be tested several times throughout the incubation period to track progress. This usually requires the removal of aliquots from the cultures to be tested. One option is to use to fill cuvette with culture to test the OD600 in a spectrophotometer, but this requires the removal of significant volumes of culture over time, drastically increasing the overall volume of culture required for the experiment. Another option is to use the droplet setting of a NanoDrop machine to test the OD600, but this approach is very imprecise and time consuming. The numerous liquid-handling steps involved in the Miller assay are already time consuming, and the time required climbs steeply as the number of tubes to be tested increases. When multiple samples must be assayed, this makes conducting the assay step with accurate timing very difficult.

1.6 High-throughput and kinetic approaches to the reporter gene assay

Various adaptations of the Miller protocol have been made that employ the use of microplates (plastic plates containing multiple wells in the microliter volume range) and plate readers (spectrophotometers designed to take readings through the wells of microplates) in order to reduce the number of liquid handling steps, reduce overall bench time, and allow a large number of samples to be assayed at once. [14, 15]

In this project I attempted to adapt the high-throughput, plate-reader-assisted beta-galactosidase assay presented by Schaefer, Jovanovich, Kotta-Loizou, and Martin in 2016 [16, 17]. In a further effort to reduce the number of liquid-handling steps, they designed a “one-step” approach in which the assay is performed directly on a culture instead of on cell extracts, and cell lysis and the introduction of ONPG substrate occur in one step. The assays are conducted on 200 uL reaction volumes containing 80 uL of bacterial culture and 120 uL of lysis/substrate solution. Instead of using chloroform or toluene for cell lysis, which are difficult to pipette and are hazardous, they used lysozyme and a detergent-based reagent. In addition to performing the assay in a microplate, they also calculate MU based on kinetic data, eliminating the need for making a decision that “sufficient color” has developed, eliminating the OD550 reading, and reducing the impact of timing error on calculated MU.

$$1 \text{ kinetic MU} = \frac{1000 * (\Delta\text{OD}420/\text{min})}{(\text{OD}600 * 2.5) * v}$$

Where $\Delta\text{OD}420/\text{min}$ is the slope of the OD420 signal (read once per minute) in the assay mixture over a linear range, OD600 is the OD600 of the reaction mixture at the first reading, 2.5 is a volume correction for the OD600 since it is read in the reaction mixture instead of in the culture, and v = volume of culture (mL) used in the assay mixture.

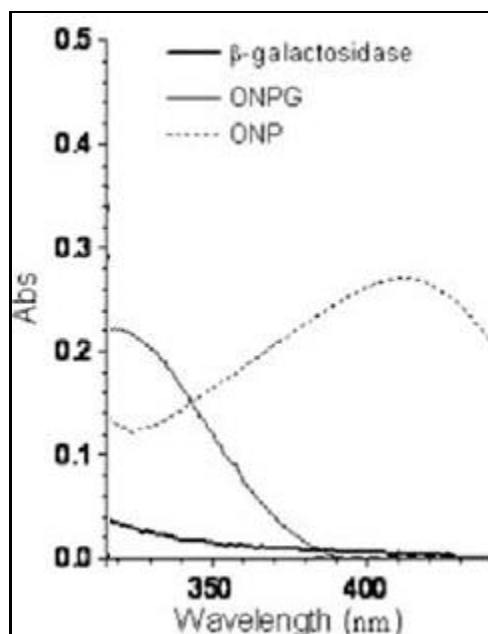


Figure 6. Partial UV-Vis absorbance spectra of ONPG, ONP, and beta-galactosidase, showing the regions of maximum absorbance for ONPG and ONP. [18]

Due to the availability of color filters for our plate reader, the reading for ONP was taken at 410nm rather than 420nm. 410nm is still within the strong absorbance range for ONP and should significantly cross over with signals from other reagents (*Figure 6*). Schaefer et al. used a FLUOstar Omega Microplate Reader (BMG LABTECH) and I used the FLUOstar OPTIMA. Only 3 flashes per well were used in my project (as opposed to the more precise 20 flashes) and the cycle time was 90 seconds (instead of 60 seconds) because of physical limitations of the plate reader that increased the minimum time required to read the entire plate. The slope of the OD410 signal curve was still calculate in terms of minutes.

2. Materials and Methods

2.1 Individual items and reagents

- beta-mercaptoethanol (“BME”) (SIGMA)
 - chloramphenicol (“CHL”)
 - DMSO (ACS Grade, not molecular biology grade) (SIGMA)
 - ONPG
 - PopCulture
 - guanine (“GUANine”)
 - guanosine (“guanOSINE”)
 - SK4 (MW: 340.30 g/mol)
 - L-tryptophan
 - L-lysine (or L-lysine monohydrochloride, which is more shelf stable and soluble—adjust media recipe accordingly)
 - L-methionine
 - L-glutamate
 - glucose
 - LB powder
 - LB with agar (Lennox) powder (SIGMA)
 - $(\text{NH}_4)_2\text{SO}_4$
 - K_2HPO_4
 - KH_2PO_4
 - $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - CaCl_2
 - MgCl_2
 - MnCl_2
 - Na_2HPO_4
 - NaH_2PO_4
 - KCl
 - MgSO_4
 - sodium citrate dibasic sesquihydrate
 - lysozyme from chicken egg white
-
- ❖ sterile reagent reservoirs (“troughs”)
 - ❖ non-sterile reagent reservoirs
 - ❖ disposable glass culture vials and their matching plastic caps
 - ❖ bacterial streaking dowels
 - ❖ 96-well microplates (chimney well, flat-bottom, non-TC treated, clear, sterile, with lids)
 - ❖ AeraSeal gas permeable sterile plate seals
 - ❖ small plastic container with lid (large enough to fit a microplate in it_
-
- ❖ incubator oven at 37 °C
 - ❖ incubator shaker at 37 °C and 200-250 rpm
 - ❖ water bath at 55-60 °C
 - ❖ multichannel pipettor (8-channel)

2.2 Reagent mixtures and preparations

- 1000X CHL stock (0.005 g/L stock)
Dissolve 0.025 g chloramphenicol in 5 mL 200-proof EtOH. Filter-sterilize into aliquot tubes. Store under tinfoil at -20 °C.
- 10 mg/mL lysozyme stock
Reconstitute the entire vial of lysozyme in the appropriate volume of DI-H₂O. Separate into aliquots (preferably enough for one assay at a time) and store at -20 °C. Keep on ice before use.
- LB agar with 5 ug/mL CHL
Dissolve 35 g/L LB agar powder in DI-H₂O in a beaker with a stir bar on a stir-plate, with heat if necessary. Pour the mixture and the stir bar into an autoclavable bottle with thick walls. Put tinfoil over the opening, but not super tightly. Autoclave. After autoclaving, put the bottle in a 55-60°C water bath for at least half an hour to let it cool more but keep the agar from congealing (melting of agar occurs at a much higher temperature than does its gel-formation). Take the bottle out of the water bath and place it on the stir plate. Stir gently (avoid bubble formation) and add CHL to 5 ug/mL as long as the bottle is cool enough to touch (heat destroys antibiotics). Without delay (to avoid the agar congealing prematurely), pour the mixture from the bottle into empty, sterile petri dishes that are prepared. Stack the poured plates to help reduce condensation. Wait about 30 minutes until the agar sets, then flip the plates agar-side up and label. Leave out for several hours to let more excess moisture evaporate. Place in a loosely closes petri dish bag (or partially wrap the stacks with Parafilm) and place in fridge. Using a bottle with thick walls will reduce the likelihood of premature agar gelling.

- lysis/substrate solution

volume of reagent needed in recipe for 10,800 uL:	reagent solution needed	made from
7,200 uL	(2.7 uL BME)/mL solution	BME in Z-buffer
2,700 uL	(4 mg ONPG)/mL solution	ONPG in Z-buffer
720 uL	PopCulture	
180 uL	10 mg/mL lysozyme	

The solutions of BME and ONPG must be mixed fresh.

Dissolving the ONPG may require sonication.

- Z-Buffer

REAGENT	FINAL CONCENTRATION
Na ₂ HPO ₄	60 mM
NaH ₂ PO ₄	40 mM
KCl	10 mM
MgSO ₄	1 mM
add DI-H ₂ O to volume	

➤ Chemically Defined Medium—Variation 1 (CDM-1), based on [19]

REAGENT	~ FINAL CONCENTRATION IN THE MEDIUM	NOTE
• $(\text{NH}_4)_2\text{SO}_4$ (A)	2 g/L	
• K_2HPO_4	14 g/L	
• KH_2PO_4	6 g/L	
• $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g/L	
• CaCl_2	0.5 mM	
• MgCl_2	2.5 mM	
• MnCl_2	5 μM	use a 100x stock
• sodium citrate dibasic sesquihydrate	0.895 g/L	(MW: 263.1 g/mol)
• glucose (B)	0.5% w/v = 5 g/L	use a 100x (filter-sterilize) stock
• tryptophan	50 $\mu\text{g}/\text{mL}$	use a 200x (filter-sterilize) stock

Dissolve/mix the reagents in section (A) in the final volume of DI- H_2O , then autoclave.

After autoclaving, aseptically add the reagents in section (B).

Store the medium in the fridge.

Add antibiotic to individual aliquots of medium at time of use.
(CHL)

➤ Chemically Defined Medium—Variation 2 (CDM-2), based on [20]

REAGENT	~ FINAL CONCENTRATION IN MEDIUM	NOTE
• $(\text{NH}_4)_2\text{SO}_4$ (A)	20 g/L	
• K_2HPO_4	19.16 g/L	
• KH_2PO_4	6 g/L	
• $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g/L	
• sodium citrate sesquihydrate	0.895 g/L	
• glucose (B)	0.4% w/v = 4 g/L	each of these reagents must be prepared as a 100x filter-sterilized stock solution
• tryptophan	50 $\mu\text{g}/\text{mL}$	
• methionine	50 $\mu\text{g}/\text{mL}$	
• lysine	50 $\mu\text{g}/\text{mL}$	
• glutamate	0.2% w/v = 2 g/L	

Dissolve the reagents in section (A) in the necessary volume of DI- H_2O , accounting for the fact that reagents in solution form will be added later, then autoclave.

After autoclaving, aseptically add the reagents in section (B).

Store the medium in the fridge.

Add antibiotic to individual aliquots of medium at time of use.
(CHL)

➤ Chemically Defined Medium—Variation 3 (CDM-3), based on [21]

REAGENT	~ FINAL CONCENTRATION IN MEDIUM	NOTE
<ul style="list-style-type: none"> • $(\text{NH}_4)_2\text{SO}_4$ (A) • K_2HPO_4 • KH_2PO_4 • $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ • MnCl_2 • CaCl_2 • sodium citrate sesquihydrate 	<ul style="list-style-type: none"> 2 g/L 14 g/L 6 g/L 0.7 g/L 5 μM 5 μM 0.895 g/L 	<ul style="list-style-type: none"> use a 100x MnCl_2 stock use a 100x CaCl_2 stock
<ul style="list-style-type: none"> • glucose (B) • tryptophan • methionine • lysine 	<ul style="list-style-type: none"> 0.5% w/v = 5 g/L 50 $\mu\text{g}/\text{mL}$ 50 $\mu\text{g}/\text{mL}$ 50 $\mu\text{g}/\text{mL}$ 	<ul style="list-style-type: none"> use a 100x stock use a 200x stock use a 200x stock use a 200x stock

Mix the reagents in section (A) with an amount of DI- H_2O equating the final volume minus the volumes of any reagents required that are in stock-solution form, then autoclave. After autoclaving, aseptically add the reagents in section (B). Store the medium in the fridge. Add antibiotic to individual aliquots of medium at time of use.
(CHL)

All growth media used in the beta-galactosidase assay procedure for cultures and blanks were supplemented with 1 μL of filter-sterilized 1000X CHL per mL of growth medium.

Some reagents cannot withstand the heat of the autoclave or may have dangerous reactions with each other or with metal in the machine. When working with a new reagent or mixture of reagents, do not autoclave without making sure that it is okay to do so.

Antibiotic, amino acid, and glucose solutions were filter-sterilized into sterile tubes using disposable syringes with 0.22 μm sterile syringe filters.

DMSO solvent and ligand solutions were not filter-sterilized, as the acetate membrane syringe filters that were available are not designed to withstand solvents such as DMSO. DMSO is not a hospitable medium for most typical bacteria and fungi, so this at least reduces the risk of the DMSO as a significant source of microbial contamination.

Ligand stocks of various concentrations were not made via serial dilution, as I was conserving the SK4. Each ligand was made in an initial stock solution of 1 mM. Each diluted stock was made by adding an aliquot of 1 mM stock to a tube containing additional DMSO.

Ligand stocks and DMSO were stored at room temperature under tinfoil to protect them from light.

The specific strain of *B. subtilis* 168 used in this project is either 1A40 or 1A1. Based on the ID and year labelling on the cryoculture stock vials in the box, the literature cited by previous

students, and screening experiments I conducted (see Appendix), I believe it is 1A40, but I cannot absolutely verify this. 1A40 is the wild-type riboswitch strain mentioned in a senior project from 2013, wherein the mutant riboswitch strain identified conversely was 1A1 [22]. There appears to have been some confusion in projects within the last seven years regarding the true identity of bacteria used. The most recent cryostock subculture made and used by previous students is dated 2018 and had been used under the impression that it was correct, but antibiotic screening conducted during the course of this project (see Appendix) showed that the bacteria did not have a profile consistent with the proper transformants intended for use in the riboswitch beta-galactosidase assays.

2.3 Beta-galactosidase assay

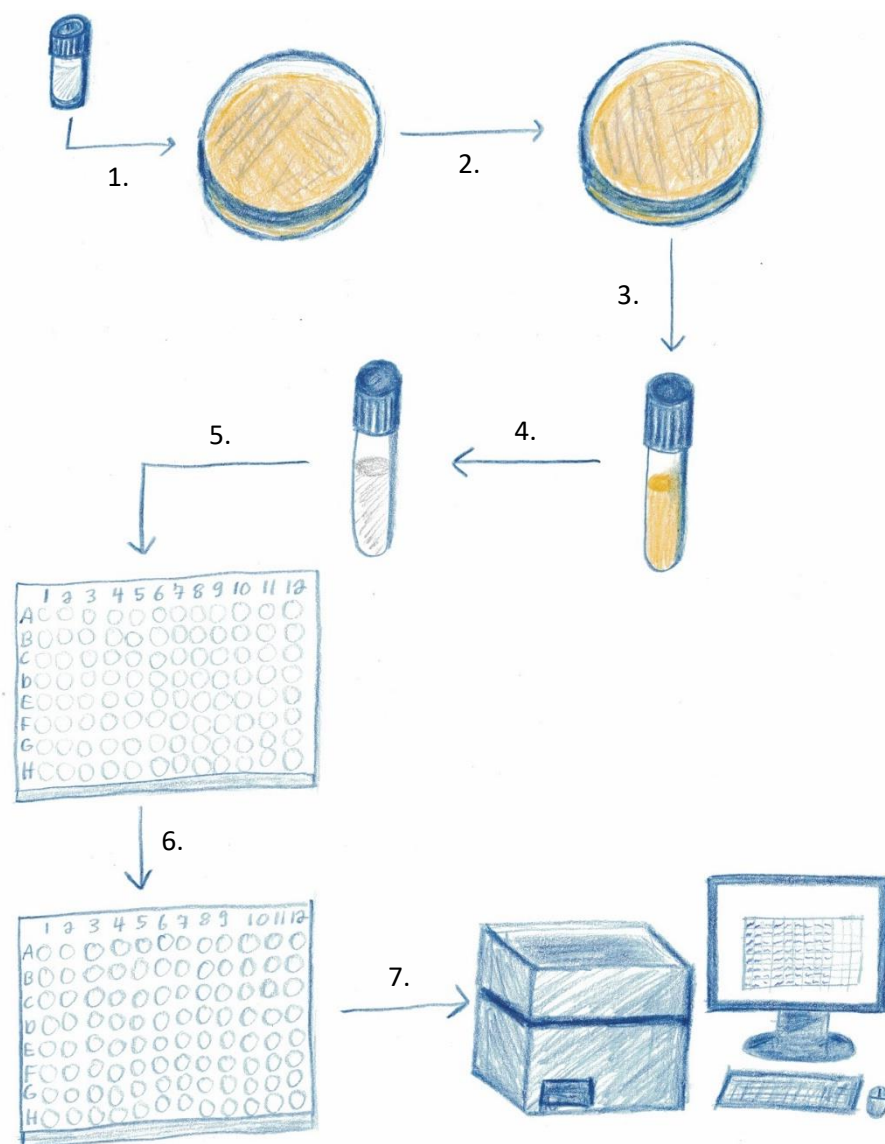


Figure 7. General Scheme of the Plate-Read Beta-Galactosidase Assay Procedure.

Plate-Read Beta-Galactosidase Kinetic Assay—Description of General Scheme (*Figure 7*)

(The progression of steps #1.-#4. leading up to the assay takes 5-6 days.)

Basic considerations before attempting the procedure:

- All steps requiring contamination-prevention were performed using “quasi-aseptic technique” (see next section).
- All steps involving microbial work, even when contamination of the work was not a concern, were conducted in such a way as to prevent contamination of the general lab space or of anyone else’s work and followed by disinfection.
- Assume that incubators and shakers are covered in bacteria.
- Communicate with other biochemistry students about who needs to use what equipment at what times with what settings. Label equipment such as the shaker very obviously with the settings you need it to have and your name and contact information—putting a paper sign on the lid and over the settings buttons seemed to be the most effective means of preventing unintended multi-user interference.
- Be conscious of whatever you may need to touch after touching something that may have contaminated your gloves, and be sure to disinfect your gloves in between steps.
- Always include a negative control in any step involving culturing bacteria. Only proceed to the next step if the intended bacterial culture show growth and the negative control shows no growth.
- Always include an appropriate blank when testing OD.
- Ensure that the plate-reader is on and warmed up, that the computer software is functioning, and that the settings and plate-layout in the software are prepared ahead of time for any step involving the plate-reader.
- Continually restock any necessary sterile materials (sterile culture tubes and streaking sticks, for example). You will need a continual supply of autoclaved yellow pipette tips.
- Check OD₆₀₀ at the beginning and end of liquid incubation steps (I used 100 uL aliquots in a microplate in triplicate against triplicate blanks for most steps).
- Make sure to mix up a culture each time before you remove an aliquot from it or measure its OD.
- Separate blanks for each of the ligand conditions must be used, as the ligands affect OD differently.
- Media used in negative control and blank wells must be the proper ratio of LB and CDM media according to the ratio in which cultures from the incubation steps have been mixed with fresh media.
- Pipetting bacterial cultures and lysis/substrate solution (which contains detergent from the PopCulture) without blowing bubbles is difficult and may take practice.
- BME is a volatile irritant and has a strong odor.
- Viability of stock cultures decreases with every freeze-thaw cycle. Avoid letting the cryoculture stock thaw.
- Do not touch the bottom of the microplates or allow them to touch dirty or rough surfaces. Smudges and scratching will affect OD readings.

Procedure:

1. Revival of cryoculture on LB agar plate

- a. Prewarm two LB agar plates (agar-side-up) for 30 minutes in the 37°C incubator, then put them on your disinfected work area.
- b. Prepare a small bowl of dry ice.
- c. Wearing gloves, take the intended vial of *B. subtilis* cryoculture out of its box in the -80°C freezer and put it into the dry ice.
- d. Put the box back in the freezer and shut the door.
- e. Wipe down the outside of the vial with 70% EtOH. Be careful to remove clumps of melting ice that may cause slipping or drippage.
- f. Flip one of the agar plates lid-side-up.
- g. Loosen the lid of the cryoculture vial. Be very careful not to let the vial tip over.
- h. Use a sterile streaking stick (dowl) to scrape a small amount of frozen cryoculture off of the surface in the vial. It will melt quickly--be swift.
- i. Immediately streak the cryoculture-end of the stick across one third of one of the LB agar plates while keeping the lid of the plate “clam-shelled” over it to prevent contamination. Put the used stick bacteria-side down into a “used stick” beaker and immediately tighten the cap of the cryovial.
- j. Disinfect your hands and the outside of the vial. Put the vial back into its place in the box in the -80 °C freezer.
- k. Use fresh sterile sticks to continue the streaking process. (Please refer to plate-streaking instructions available from many sources elsewhere.)
- l. Wait a few minutes. Return the streaked agar plate (“cryoculture revival plate”) to an agar-side up orientation and label the bottom of the plate with the strain ID, your initials, and the date.
- m. Turn the other agar plate lid-side-up. Use sterile sticks to streak the plate, but without any bacterial culture. Return the plate to its agar-side-up orientation and label the bottom as a negative control with your initials and the date.
- n. Place both agar plates agar-side-up in the 37 °C incubator and incubate for up to 24 hours.

2. Restreak of single colony onto LB agar plate

- a. Prewarm two LB agar plates.
- b. Use a sterile streaking stick to select a single colony from the cryoculture revival plate and streak it on one of the fresh, prewarmed plates (“restreak plate”). Use generic plate-streaking methods as before. Set up a new negative control streak plate as well.
- c. Incubate the restreak plate and the new negative control plate at 37 °C for up to 24 hours.
- d. Wrap the cryoculture revival plate in Parafilm and store in fridge. It can be saved for use for up to 1 week.

3. Inoculation of LB broth in a culture tube with single colony from the restreak plate

- a. Prewarm two blue-cap culture tubes containing 5 mL of LB broth medium in the shaker.
- b. Use a sterile stick to retrieve a single colony from the restreak plate and dip it into one of the tubes.
- c. Use another sterile stick without bacteria to dip into the other tube without, as a negative control.

- d. Incubate culture tubes in shaker (37°C, 200-250 rpm) for ~24 hours, to an OD600 of ~0.7.
 - e. Wrap the restreak plate in Parafilm and store in fridge. It can be saved for use to up to 1 week.
 - 4. Inoculation of chemically defined broth medium (“CDM broth”) in a culture tube (“Phase 1 Culture”) with an aliquot of the LB broth culture**
 - a. Take the LB broth tubes out of the shaker and allow them to cool for a few minutes to minimize evaporation.
 - b. Check the LB broth negative control for visible growth. Test the OD600 of the LB broth culture.
 - c. Prewarm green-cap culture tubes containing 8 mL of CDM broth.
 - d. Pipette 2 mL of LB culture into one tube to initiate a Phase 1 Culture.
 - e. Pipette only air into the other tube as a negative control.
 - f. Incubate the tubes in the shaker (37 °C, 250 rpm) for ~24 hours, by which time the culture OD600 should be in the 0.7-1.1 range.
 - 5. Inoculation of CDM broth in 96-well microplate (“Phase 2 Incubation Plate”, see example layout below) wells containing ligand solution or solvent control with aliquots from Phase 1 Culture**
 - a. Add fresh CDM medium to each well of the Phase 2 Incubation Plate
 - b. Add ligand solutions, solvent control, and control media to the appropriate wells of the plate as indicted by the layout. Record any errors.
 - c. Pour the Phase 1 Culture into a sterile trough and mix back and forth
 - d. Use the multichannel pipettor to transfer aliquots of Phase 1 Culture into each appropriate well of the Phase 2 Incubation Plate, changing pipette tips and agitating the trough gently between columns.
 - e. Press sterile, gas permeable plate seal over the microplate, make sure it adheres to the chimneys of each well.
 - f. Wet some paper towels and place in the plastic container. Set the microplate on top of its lid (to protect the bottom) on the paper towels. Close the plastic container and tape it down in the shaker. Incubate in shaker (37 °C, 250 rpm) for ~24 hours, by which time OD600 should be in the 0.7-1.1 range. The container with wet paper towels is necessary to prevent the cultures in the incubation plate from drying out. Clearly post a notice on the shaker that you have cultures incubating in the container, as other people may not realize anything is in it.
 - 6. Transfer of Phase 2 Incubation Plate culture aliquots into new 96-well microplate (“Assay Plate”, see example layout below). Addition of lysis/substrate solution to Assay Plate**
 - a. Take the Phase 2 Incubation Plate off of the shaker and let sit for a few minutes to minimize evaporation. Then remove the seal from the plate.
 - b. Add the appropriate blanking mixtures to the appropriate wells in the Phase 2 Incubation Plate, 200 uL per well.
 - c. Use the multichannel pipettor to gently stir up each well by gently pumping up and down. Change pipette tips between each column. Take an OD600 reading of the plate.
 - d. Use the multichannel pipettor to transfer 80 uL from each well of the Phase 2 Incubation Plate into the appropriate well of the Assay Plate.

- e. Pour the lysis/substrate solution into a trough. Use the multichannel pipettor to transfer 120 uL lysis/substrate solution into each occupied well of the Assay Plate, changing pipette tips between columns.
 - f. Check for large bubbles. Pop with fresh pipette tips of possible. Take a picture of the plate as a record.
- 7. Kinetic readings of the Assay Plate (plate reader software must be set up well ahead of time with proper plate layout and settings for wavelengths, shaking, timing, movement pattern, etc.)**
- a. Immediately insert the Assay Plate into the plate reader and initiate the kinetic reading protocol.
 - b. Make sure the protocol is running properly and check on the assay progress by viewing the current state signal curves.
 - c. At the end of the assay, take another picture of the plate as a record.
 - d. Export data to a spreadsheet.

Plate reader settings used for the kinetic assay in this project:

Plate mode flying settings		
No. of cycles:	50	
Cycle time [s]:	90	
No. of flashes per well:	3	
Optic settings		
No.	Excitation	Emission
1	600	empty
2	410	empty
Shaking settings		
Shaking width [mm]:	2	
Shaking mode:	double orbital	
Additional shaking time:	26s before each cycle	
General settings		
Bottom optic used		
Reading direction:	unidirectional, horizontal left to right, top to bottom	
Target temperature [°C]:	30	
Absorbance path length correction volume [µl]:	200	

Sample layouts of “Phase 2 Incubation Plate” and “Assay Plate”:

PHASE 2 INCUBATION PLATE LAYOUT [condition blanks will be added to the shaded wells in rows G and H *after* the Phase 2 Incubation period]

	1	2	3	4	5	6	7	8	9	10	11	12
A	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM	(-) GUANine 50 uM	(-) GUANine 50 uM
B	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM	(-) guanOSINE 50 uM	(-) guanOSINE 50 uM
C	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM	(-) SK4 50 uM	(-) SK4 50 uM
D	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM	(-) DMSO	(-) DMSO
E	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
F	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
G												
H												

- 140 uL fresh CDM per text-labelled well.
- 20 uL 10X ligand solution or DMSO solvent control per appropriate well (i.e., wells A through F in column 4 and wells D11 and D12).
- 40 uL Phase 1 Incubation culture per text-labelled well (but substitute with 40 uL of LB:CDM 1:4 media mixture in the (-) control wells) (the (-) control wells can serve as a blank for the beginning of incubation but not the end)

ASSAY PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
B	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
C	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
D	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
E	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
F	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
G	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		
H	GUANine 25 uM	guanOSINE 25 uM	SK4 25 uM	DMSO	GUANine 50 uM	guanOSINE 50 uM	SK4 50 uM	GUANine 100 uM	guanOSINE 100 uM	SK4 100 uM		

- 80 uL Phase 2 Incubation culture per well for wells A1 through F10 from the corresponding well of the Phase 2 Incubation Plate.
- 80 uL of the appropriate condition blanking mixture per well for wells G1 through H10. Blanks have all the same contents as the sample wells, except LB and CDM media mixed in the proper ratios are used in place of bacterial culture.

“Quasi-Aseptic Technique”

- Make sure your hair is tied back (without any dangling in front of you—this is less about keeping something from getting on your hair and more about keeping any debris from your hair from falling into your work and contaminating it) and that none of your clothing can trail down if you lean or reach for something.
- Make sure your workspace is clean. Wash if necessary. Minimize airflow and dust (dust carries microbial contaminants).
- Wash hands before beginning work.
- Put on gloves and wipe them with 70% EtOH before working with any sterile materials or microbial cultures.
- Do not breathe on your work. Do not talk, whistle, or sing while performing sterile work.
- Wipe down with 70% EtOH the surface you will work on immediately before conducting any microbial work or work with sterile reagents and materials.
- Wipe down with 70% EtOH all tools and the outsides of all containers, packages and bottles you will be using or handling before you put them in your sterile area.
- Wipe down with 70% EtOH the cap area of bottles and tubes before opening them. (The one incident I had with a bottle of liquid growth media becoming obviously contaminated occurred after I had opened it without specifically wiping down the cap/neck area of the bottle.
- Always check growth media for signs of contamination before using them for any culture or blank.
- Do not reach into a bag of sterile supplies.
- Do not put anything non-sterile into a sterile reagent.
- Only open bottles, sterile supplies, plates and culture vials just long enough to do what you need to do. Close them again immediately.
- Never reach over an open container of sterile material or a over a culture.
- Never set down the lid of a container. If you must, place it open-side down on a cleaned surface.
- Do not pour out of sterile reagent bottles. Use a sterile pipet to work with liquids, and use each pipet only once. Do not put the same pipet back into a bottle.
- Disinfect your gloves and pipettor with 70% EtOH after using it with a culture and before moving on to any other culture or reagent! Note that evaporating microbial cultures also contaminate areas of the pipettor that never directly touched the liquid.
- Use standard, careful pipetting methods. Use the smallest possible size pipettor and pipette tips for any step (e.g., use a P100 to pipet 100 uL, not a P1000). Depress the pipettor plunger to the “first stop” before placing the tip into the liquid. Avoid injecting air into the liquid. Aspirate liquids into the pipette very slowly. Dispense liquid from the pipette tip very slowly to avoid aerosolization and bubbling.
- Label your sterile reagents and materials so that others know that they are not to be used without aseptic technique.
- Make sure that other colleagues know not to disturb required supplies or workspace.
- Keep a supply of necessary sterile materials (such as autoclaved pipette tips) at hand in your specific workspace.

Notes on ethanol use:

- Do not spray ethanol near open containers, exposed microbial cultures, or computers.
- Do not use ethanol near heat sources or flame.
- Make sure that surfaces wiped with ethanol have dried before you continue. This is in order to ensure disinfection and to prevent the evaporating ethanol from affecting your cultures and reagents.
- To avoid the labels on bottles and vials getting washed off by the ethanol, put clear tape over your labels. Be mindful of labels that may become obscured when disinfecting containers.

3. Results and Discussion

3.1 Bacterial biofilm production in variations of chemically defined growth media

Difficulties performing the beta-galactosidase assay have been reported in the past due to excessive biofilm formation in the steps requiring chemically defined medium (CDM). Biofilm formation in the liquid medium leads to non-homogenous cultures and to viscous globs that plug pipette tips. I cultured the *B. subtilis* bacteria in three major variations of chemically defined media—CDM-1, CDM-2, and CDM-3—whose recipes are listed in the materials section and qualitatively compared the extent of biofilm formation in each type of medium (previous work may refer to these media as “GMM”). Cultures grown in CDM-3 broth showed little to no problematic biofilm formation in most attempts, whereas cultures grown in CDM-1 broth or CDM-2 broth regularly contained major biofilm globs. The difference in biofilm production in these media may be due to the presence of MnCl₂ and MgCl₂. These are not variables that I was able to independently assess. I believe that CDM-1 (which contains only one amino acid: tryptophan) is designed for use with *B. subtilis* strain 1A1 while the other two variants of CDM (which contain multiple amino acids) are designed for use with strain 1A40, a the *Bacillus* Genetic Stock Center lists strain 1A1 with a *trpC2* description and 1A40 with a *lys-3 metB10 trpC2* description [23].

3.2 Bacterial solvent tolerance in ligand condition cultures

Trials were first attempted with the DMSO ligand solutions or solvent control making up 1 fifth the volume of each Phase 2 Incubation well, in order to make it possible to incubate cultures with ligand concentrations up to 200uM (The solubility of guanine in DMSO is ~ 1mM). This meant a final DMSO concentration of 20% in the cultures. The bacteria were not able to

grow at this concentration of DMSO, and a laboratory staff member indicated that even 10% DMSO is harsh for most bacteria. In order to keep the DMSO concentration in the cultures at 10%, DMSO ligand solutions were added as 1 tenth the volume of each Phase 1 Incubation well, making that the maximum ligand concentration in the test cultures 100 μM .

3.3 Kinetic and stop-solution beta-galactosidase assays performed in microplates

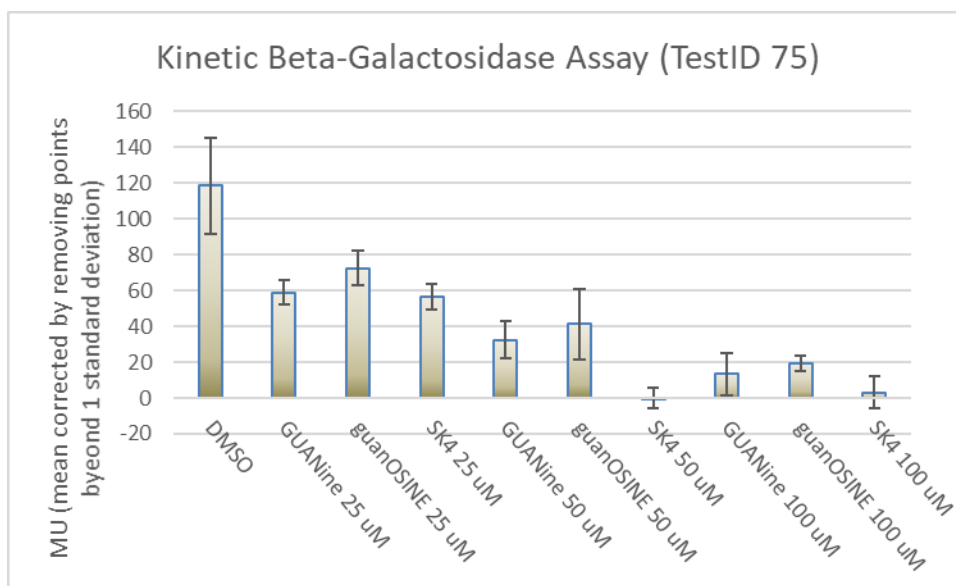


Figure 8. Kinetic MU calculated for bacteria incubated with GUANine, guanOSINE, or SK4, each at concentrations of 25 μM , 50 μM , and 100 μM . DMSO is solvent control. The slopes of the OD410 curves were calculated using the data from minutes 45 to 60.

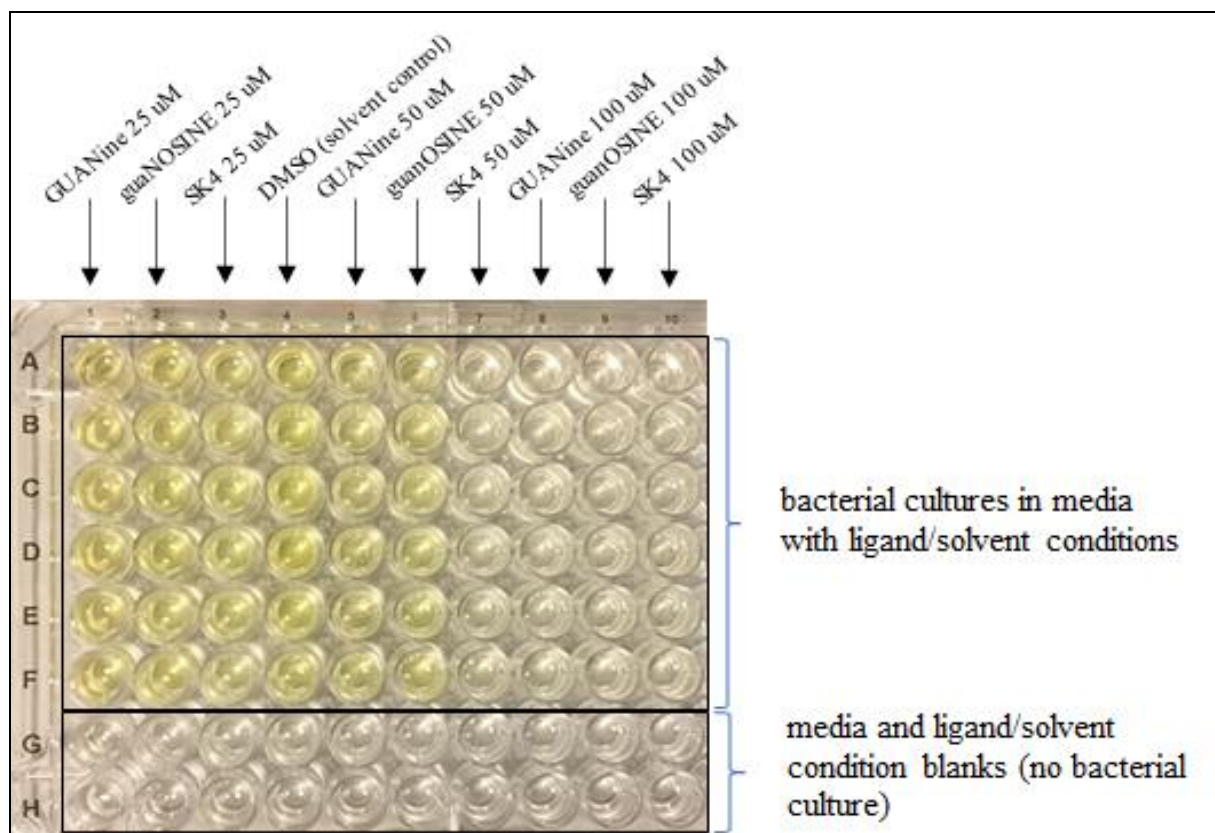


Figure 9. Visual appearance of assay plate at end of kinetic analysis (TestID 75).

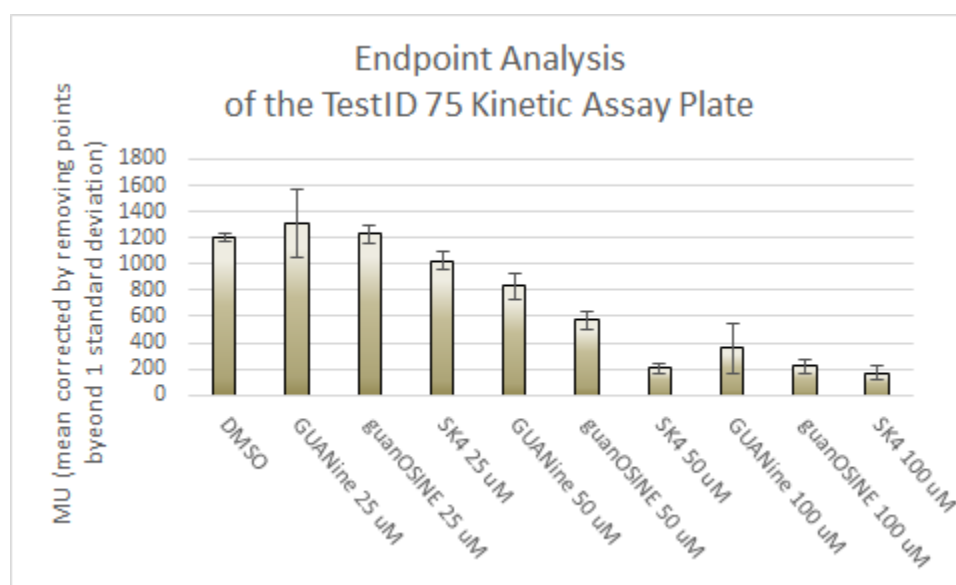


Figure 10. Endpoint analysis of the assay plate used in the TestID 75 kinetic assay (Figure 8). These MU were calculated using OD410 and OD550 readings taken at the end of the kinetic assay ($t = 80$ min) and the OD600 reading taken at $t = 0$.

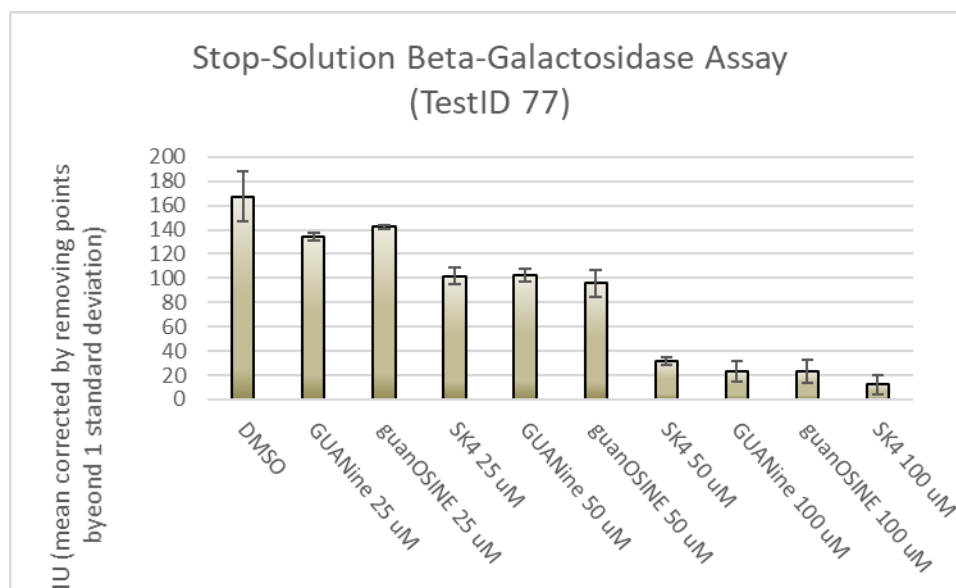


Figure 11. Stop-solution assay performed with aliquots from the same Phase 2 Incubation Plate as the TestID 75 Kinetic assay (Figure 8). 80 uL cell culture were added to the wells of a microplate. 120 uL lysis/substrate solution was added per well as usual. The plate was incubated at 30 °C for 1 hour and then 75 uL of 1 M Na₂CO₃ was added per well before taking OD410 and OD550 readings.

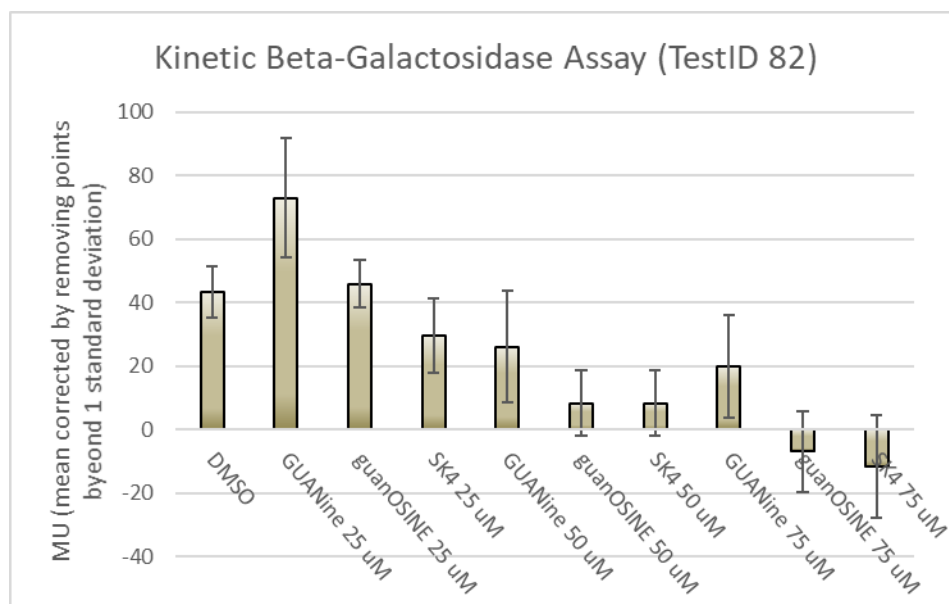


Figure 12. Kinetic MU calculated for bacteria incubated with GUANine, guanOSINE, or SK4, each at concentrations of 25 uM, 50 uM, and 75 uM. DMSO is solvent control. The slopes of the OD410 curves were calculated using the data from minutes 45 to 60.

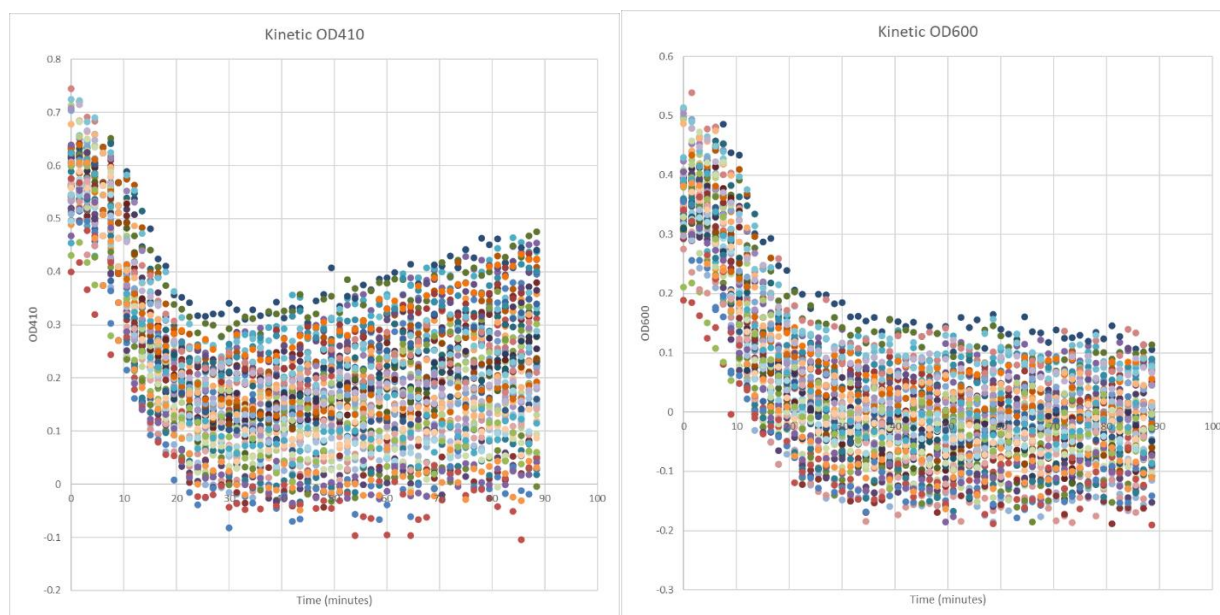


Figure 13. Representative OD410 and OD600 signal curves from one of kinetic beta-galactosidase assay trials.

The only reasonable conclusion I can make from these data about the effects of the ligands on endogenous beta-galactosidase enzyme activity in the bacterial cultures is that all three test ligands (GUANine, guanOSINE, and SK4) each cause an increased reduction in enzyme activity with increasing concentration of the ligand from 25 μ M to 100 μ M. Whether the three ligands have significantly different effects on gene expression in these experiments is not clear. Of the few assays successfully brought to completion, only the results using bacteria from one assay appeared to indicate a significant drop in beta-galactosidase activity in the cultures incubate with SK4 as compared to with guanine or guanosine. The effects of guanine and guanosine in those assays relative to each other were very close. In the final kinetic assay trial conducted (TestID 82), many negative Miller Unit values were yielded, and the spread of values for each condition was very large. The data from that trial cannot be interpreted. Several other attempted trials were rendered unusable due to excessive bubbles in the assay plate wells or aborted due to closure of the labs at several points.

Miller Unit calculations are not meant to meaningfully accommodate negative signals, especially not negative OD410 slopes in the kinetic assays. Further assays would need to be performed in order to clarify any significant difference in effect between the ligands, possibly with denser bacterial cultures. It is possible that the apparently low production of active beta-galactosidase in the bacteria cultured lead to signals too close to the lower detection limit and in too narrow an intensity range for adequate resolution. I surmise that the generally low OD410 signals in all of the assay cultures are due to low baseline enzyme activity in the bacteria used. Towards the beginning of this project, I performed X-Gal blue/white and antibiotic screening tests on several different cryocultures belonging to the lab that had been unclearly labelled. The cryoculture that I ended up selecting for use in my experiments had the appropriate antibiotic susceptibility and tolerance profile, but it had very little production of blue color (blue color is the indicator of beta-galactosidase production in the X-Gal test). It is likely, over years of storage, that the expression of *LacZ* in the stock culture of modified bacteria diminished.

Schaefer et al. indicated that lysis did not significantly affect OD600 in their assays, and that the OD420 signals began to rise immediately from the beginning of the beta-galactosidase assay upon the addition of the lysis/substrate solution (called “BGal mix” in their text). The trials in this project, however, showed a steep decrease in both OD600 and OD410 for approximately 45 minutes, which is a quite long period of time, followed by relatively steady OD600 readings and rising OD410 readings (in cases when beta-galactosidase detectable by this means was present, with regard to the OD410). The only time I saw an immediate rise in OD410 was when I performed a test run of the apparatus with stock beta-galactosidase enzyme and with some leftover Phase 1 Incubation liquid culture (not shown). I wonder whether the significant drop of OD600 in my cultures could be due to a lower initial growth density, although it was within the

range used by Schaefer. The main problematic differences observed between my kinetic assay signal curves and the signal curves in Schaefer's work is the prolonged lysis period, as "slow permeabilization [can] reduce ONPG availability within the cell and have adverse effects on OD420 production and Miller units" [16].

It is important to note that the Schaefer protocol is designed for use with *E. coli*, which is Gram-negative, as are most beta-galactosidase assays found in the literature. The cell walls of Gram-positive bacteria are stronger and more elastic than the cell walls of Gram negative bacteria, and this renders Gram-positive bacteria more difficult to lyse [24]. Typical beta-galactosidase assays of either Gram-negative or Gram-positive bacteria usually involve performing enzymatic assays on cell extracts that have been separated by centrifugation from cells previously lysed by mechanical or chemical means [25]. Another high-throughput option found in the literature proposed by Griffith and Wolf in 2002 involves performing the assay on cultures where the cells were permeabilized by toluene or chloroform and SDS, without extracting the lysate, but this approach requires the use of polypropylene 96-well blocks for the lysis stage, as toluene and chloroform can both damage plastic microplates [26].

4. Conclusion

4.1 Riboswitch-mediated gene suppression by analogue ligands

The guanine, guanosine, and SK4 ligands each cause a decrease in endogenous beta-galactosidase activity in exposed cultures of model bacteria containing the *xpt-pbuX-LacZ* fusion. Over the 25 uM to 100 uM ligand concentration range tested, an increasing ligand concentration led to further decrease in beta-galactosidase activity, indicating more powerful suppression of the riboswitch-mediated *LacZ* gene expression. Cultures from one batch showed more powerful gene suppression by SK4 than by guanine or guanosine across the tested range of concentrations, but more testing would be necessary to confirm or disqualify these results.

4.2 Experimental approach for high-throughput and kinetic beta-galactosidase assays

While the “one-step” beta-galactosidase method described for use in *E. coli* by Schaefer offered hope for decreasing the number of liquid handling steps, increasing the time-efficiency of the assay process and reducing the quantity of reagents required, cell lysis in the *B. subtilis* used in this project was not rapid enough for the sake of evaluating beta-galactosidase activity via the colorimetric evaluation of its hydrolysis product, ONP. With the awareness (informed both my own experience and the reports of previous students) that any otherwise-functional assay can become infeasible if it involves an impractical number of handling steps, quantity of materials or amount of bench time, especially for an undergraduate researcher, I encourage future students to pursue a beta-galactosidase assay that resembles the one proposed by Griffith and Wolf and is ultimately analyzed using a plate reader but involves a more rigorous, separate lysis step than incorporated by Schaefer. Hopefully, the basic outline of my procedure could be retained up through step , with the exception of adjustments to volumes and the cell-culture vessels used. It should be noted that the high-throughput scheme presented by Griffith and Wolf is intended for screening the beta-galactosidase production of multiple strains of bacteria, not for screening the same type of bacteria subjected to multiple experimental conditions. Because of this, I would recommend working with a batch culture as much as possible up until the ligand-incubation step, rather

than starting off by separately inoculating multiple polypropylene wells. The aliquots of bacterial culture introduced to the wells of the of the Phase 1 Incubation plate (or block) should all have the same origin and have experienced the same manipulations and environment as each other leading up to this step. Another potential option could be to simply conduct the lysis step separately by adding lysis solution to the assay cultures, waiting a sufficient amount of time, and then introducing substrate solution, as done by Robinson et al. in 2014 [27].

4.3 Additional features of riboswitch-mediated gene suppression to evaluate

Increased gene suppression due to the presence of a ligand can be due to multiple possible factors, including solubility, ability of the ligand to enter the cell, and binding affinity of the ligand to the riboswitch [28]. Once a feasible beta-galactosidase assay is implemented, there are additional experiments key to evaluating the interactions between the riboswitch and the ligands and any relevance this may have to antibiotic utility.

- Inhibitory concentration test (MIC) and Kirby-Bauer disc diffusion assay to evaluate effects of ligands on bacterial growth
- Control test with bacteria containing a riboswitch inactivated by a mutation to evaluate whether effects on the bacteria are due to riboswitch activation or other possible factors
- Isothermal titration calorimetry (ITC) analysis of the aptamer domain of the riboswitch (synthesized in house) to characterize the kinetics and thermodynamics of isolated riboswitch-ligand binding *in vitro*.

Growth inhibition tests should be performed to show whether the ligands have an appreciable effect on the growth of the bacteria. Beta-galactosidase assays should be performed with bacteria that have an inactivated riboswitch, to show whether gene suppression by the ligands is, in fact, mediated by the riboswitch. ITC analysis of the riboswitch aptamer domain with the ligands could be performed in order to examine their binding affinity and kinetics to explore why certain ligands have more prominent effects on riboswitch-mediated gene regulation.

5. References

- [1] Principi, N., Silvestri, E., Esposito, S. Advantages and Limitations of Bacteriophages for the Treatment of Bacterial Infections. *Front. Pharmacol.* **2019**, <https://doi.org/10.3389/fphar.2019.00513>
- [2] Reygaert, W.C., An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* **2018**, 4 (3), 482-501. doi: 10.3934/microbiol.2018.3.482
- [3] Loree J, Lappin SL. Bacteriostatic Antibiotics. [Updated 2020 Mar 29]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK547678/>
- [4] Stratton, C.W., Dead Bugs Don't Mutate: Susceptibility Issues in the Emergence of Bacterial Resistance. *Emerge Infect Dis* **2003**, 9 (1), 10-16. doi: 10.3201/eid0901.020172
- [5] Machtel, P., Bąkowska-Żywicka, K., & Żywicki, M. Emerging applications of riboswitches - from antibacterial targets to molecular tools. *Journal of applied genetics* **2016**, 57 (4), 531–541. <https://doi.org/10.1007/s13353-016-0341-x>
- [6] Mauger, D.M., Siegfried, N.A., Weeks, K.M. The genetic code as expressed through relationships between mRNA structure and protein function. *FEBS Letters* **2013**, 587, 1180-1188.
- [7] Garst, A. D., Edwards, A. L., & Batey, R. T. Riboswitches: structures and mechanisms. *Cold Spring Harbor perspectives in biology* **2011**, 3(6), a003533. <https://doi.org/10.1101/cshperspect.a003533><https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3098680/>
- [8] Barrick, J. E., & Breaker, R. R. The distributions, mechanisms, and structures of metabolite-binding riboswitches. *Genome biology* **2007**, 8(11), R239. <https://doi.org/10.1186/gb-2007-8-11-r239>
- [9] McDonough, Brian, "On the Use of Nucleosides as Ligand Analogues in Interactions with the pbuX-xpt Purine Riboswitch" (2015). Senior Projects Spring 2015. 284.
- [10] Gindinova, Katherina, "Synthesis of Potential Antibacterial Compounds Using Guanosine as a Structural Scaffold" (2016). Senior Projects Spring 2016. 19.
- [11] *Bacillus* Genetic Stock Center Catalog of Strains, Seventh Edition, Volume 4: Integration Vectors for Gram-Positive Organisms http://www.bgsc.org/_catalogs/Catpart4.pdf
- [12] Cold Spring Harbor Protoc; doi:10.1101/pdb.top096198
- [13] Miller, Jeffrey H. *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, 1992. 72-74.

- [14] Thibodeau, S.A., Fang, R., Joung, J.K. High-throughput β -galactosidase assay for bacterial cell-based reporter systems. *BioTechniques* **2004**, *36*, 410-415.
- [15] Li, W., Zhao, X., Zou, S., Ma, Y. Zhang, K., Zhang, M. Scanning Assay of β -Galactosidase Activity. *Applied Biochemistry and Microbiology* **2012**, *48* (6), 603-607.
- [16] Schaefer, J., Jovanovich, G., Kotta-Loizou, I. Buck, M. Single-step method for β -galactosidase assays in *Escherichia coli* using a 96-well microplate reader. *Analytical Biochemistry* **2016**, *503*. <https://doi.org/10.1016/j.ab.2016.03.017>
- [17] Schaefer, J., Jovanovich, G., Kotta-Loizou, I. Buck, M. A data comparison between a traditional and the single-step β -galactosidase assay. *Data in Brief* **2016**, *8*. <https://doi.org/10.1016/j.dib.2016.05.063>
- [18] Zhang, H.; Wang, L.; Shen, Q.; Wu, B.; Gao, P. A Novel Approach for Estimating the Relationship between the Kinetics and Thermodynamics of Glycoside Hydrolases. *Acta Biochimica et Biophysica Sinica* **2011**, *43* (5), 409–417. DOI: 10.1093/abbs/gmr014
- [19] Kim, Jane N., et al. “Design and Antimicrobial Action of Purine Analogues That Bind Guanine Riboswitches.” *ACS Chemical Biology*, vol. 4, no. 11, 2009, pp. 915–927., doi:10.1021/cb900146k.
- [20] Mandal, M., Boese, B., Barrick, E.J., Winkler, W.C., Breaker, R.R. Riboswitches Control Fundamental Biochemical Pathways in *Bacillus subtilis* and Other Bacteria. *Cell* **2003**, *113*, 577-586.
- [21] Sudarsan, N., Wickiser, J.K., Nakamura, S., Ebert, M.S., Breaker, R.R. An mRNA structure in bacteria that controls gene expression by binding lysine. *Genes & Development* **2003**.
- [22] Wang, Weiqing, "Gene Expression Analysis of a Guanine-Binding Riboswitch in *Bacillus Subtilis*" (2013). Senior Projects Spring 2013. 381.
- [23] *Bacillus* Genetic Stock Center Catalog of Strains, Seventh Edition, Volume 1: *Bacillus subtilis* 168 (2000). <http://www.bgsc.org/catalogs/Catpart1.pdf>
- [24] Mahalanabis, M.; Al-Muayad, H.; Kulinski, M. D.; Altman, D.; Klapperich, C. M. Cell Lysis and DNA Extraction of Gram-Positive and Gram-Negative Bacteria from Whole Blood in a Disposable Microfluidic Chip. *Lab on a Chip* **2009**, *9* (19), 2811. DOI: 10.1039/B905065P
- [25] Placier, Gaël; Watzlawick, H.; Rabiller, C.; Mattes, R. Evolved β -Galactosidases from *Geobacillus Stearothermophilus* with Improved Transgalactosylation Yield for Galacto-Oligosaccharide Production. *Applied and Environmental Microbiology* **2009**, *75* (19), 6312–6321. DOI: 10.1128/AEM.00714-09

[26] Griffith, K.L., and Wolf, R.E. Measuring β -Galactosidase Activity in Bacteria: Cell Growth, Permeabilization, and Enzyme Assays in 96-Well Arrays. *Biochemical and Biophysical Research Communications*, vol. 290, no. 1, 2002, pp. 397–402., doi:10.1006/bbrc.2001.6152.

[27] Robinson, C. J.; Vincent, H. A.; Wu, M.-C.; Lowe, P. T.; Dunstan, M. S.; Leys, D.; Micklefield, J. Modular Riboswitch Toolsets for Synthetic Genetic Control in Diverse Bacterial Species. *Journal of the American Chemical Society* **2014**, *136* (30), 10615–10624. DOI: 10.1021/ja502873j

[28] Jain, SS. Structural Insights into the Interactions of the xpt Riboswitch with Novel Guanine Analogues: A Molecular Dynamics Simulation Study. *Journal of Biomolecular Structure and Dynamics* **2015**, *33* (2), 234-243.

6. Appendix

X-Gal and antibiotic screening LB agar streak plates, Nov-21-2019

slot in freezer box	label on vial	no antibiotic	SPT 100 ug/mL	SPT 100 ug/mL + CHL 5 ug/mL	CHL 5 ug/mL	CHL 50 ug/mL
A9	[NO LABEL]	BLUE	BLUE	BLUE	BLUE	BLUE
A10	[NO LABEL]	BLUE	BLUE	BLUE	BLUE	BLUE
B8	B. subtilis Glycerol stock 7/26/18 LSAF	BLUE	BLUE	BLUE	BLUE	BLUE
C5	B. sub lacZ 11/12/11	WHITE bit of blue?	[no growth]	[no growth]	WHITE + BLUE	[no growth]
C6	lacZ B. subtilis #?/11/11	WHITE (dark blue possible contamination dots)	[no growth] (with exception of a dot of contamination?)	[no growth]	white- bluish	[no growth]
E1	B Sub lacZ 7.15.14	BLUE	BLUE	BLUE	BLUE	BLUE
E2	B Sub lacZ 7.15.14	BLUE	BLUE	BLUE	BLUE	BLUE
E3	7/27/12 B. subtilis lac-Z	WHITE + BLUE	tinest blue dots	[no growth]	WHITE + bluish	[no growth]
E4	1A40 B. sub 6/24/12	WHITE	[no growth]	[no growth]	[no growth]	[no growth]
E5	(G?? can barely read label) MG/SJ 12-1-16	BLUE	BLUE (dots)	BLUE (dots)	BLUE	[no growth]
E6	B. sub WT lacZ 12/7/12	WHITE bluish	[no growth]	[no growth]	white- bluish	[no growth]
E7	B. sub, spec, PSG1154 C?B 6/11/10	WHITE	WHITE	[no growth]	[no growth]	[no growth]
E8	B. subtilis 1A1 6/13/12	WHITE	[no growth]	[no growth]	[no growth]	[no growth]
E9	B. subt M6 10/4/12	BLUE	BLUE	BLUE	light bluish	BLUE
E10	B. sub WT10 lacZ 2/20/13 WW	white-bluish	[no growth]	[no growth]	white	[no growth]

(-)	[no growth]	[no growth]	[no growth]	[no growth]	[no growth]
-----	-------------	-------------	-------------	-------------	-------------

SPT = spectinomycin, CHL = chloramphenicol

This is what I observed on the plates as I had them. This is not absolute information. Some cross contamination (such as by dripping, which appears to have happened) is possible. Blue showing up in white could be due to later development, a mixture of lacZ transformed and non-transformed cells of the same culture/vial, and/or a mix of cultures/vials. (Did I check all the actual tube labels as I took them out and not just go by slot number? I know I did for some.)

The cultures from slots E4 and E8 appear to be untransformed parent strains (1A40 and 1A1, respectively). Both are dated June 2012.

The cryoculture stock I selected to use in my project came from slot E6, in the vial marked "B. sub WT lacZ 12/7/12".