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# Elucidating the effect of the antibiotic tetracycline on the regulatory function of the guanidine sensing riboswitch ykkCD

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# Elucidating the Effect of the Antibiotic Tetracycline on the Regulatory Function of the Guanidine-Sensing *ykkCD* Riboswitch

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The primary objective of this research is to develop an assay—one more feasible for undergraduate institutions than currently available methods—that will enable evaluation of the concomitant binding of guanidine and tetracycline to the *ykkCD* bacterial guanidine sensor riboswitch. This noncoding regulatory RNA, in addition to guanidine, recognizes tetracycline, a translation-inhibiting antibiotic, via a high-affinity aptamer domain. The resulting conformational change by this regulator upregulates the expression of genes downstream of it, most of which encode membrane transporters that expel toxins from bacteria. This phenomenon is a major underpinning of the ever-evolving issue of antibiotic resistance in bacteria, further necessitating the viability of the proposed notion that, aside from potentially relying on the multi-generational evolution of completely new tactics, bacteria can quickly adapt with their existing detoxification machinery to expel antibiotics, rendering the therapeutic agents largely ineffective.

A binding assay is planned to see if the two ligands, tetracycline and guanidine, bind to the riboswitch along roughly the same region, to investigate some of the thermodynamics and/or kinetics by which they bind, and to therein distinguish whether or not the binding of tetracycline makes guanidine bind to the aptamer more efficiently than when the antibiotic is unbound. The RNA utilized in this project is from *Bacillus subtilis*. Binding assays currently prescribed in the literature (as of September 2020, to the understanding of this publication's contributors) have called for relatively cumbersome methods, including the use of radioactively labeled RNAs and the tedious, time-consuming running of sequencing gels, but the goal here is to develop an assay that utilizes the more universally accessible method(s) of isothermal titration calorimetry (ITC) and/or surface plasmon resonance (SPR) to measure the efficiency of guanidine binding to the *ykkCD* riboswitch while tetracycline is bound. Due to limitations on in-lab experimentation imposed over this past summer (2020) by the ongoing COVID-19 pandemic, a literature review is the sole component of our research presented here which aids our overall understanding of the project's current standing and provides insight as to how we can benefit from first attempting to apply both ITC and SPR to make the desired measurements. When safe and advisable to do so, we intend to physically collect and analyze assay data in order to closely consider the role of tetracycline binding in the life of the regulator and its effect on guanidine binding.

## Antibiotic resistance continue to pose significant issues for public health care

- Four basic mechanisms of bacterial antibiotic resistance:

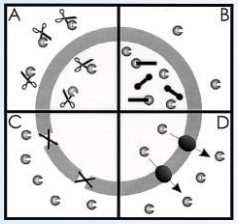
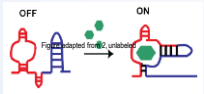



Figure adapted from 2, unlabeled

A) Degradation of antibiotic
B) Modification of antibiotic's target site
C) Prevention of antibiotic from entering cell
D) Toxin removal via efflux pump(s)

- Center for Disease Control (CDC) estimates: antibiotic-resistant infections contracted by at least 2.8 million people in the U.S. annually
- Most recent CDC figures: at least 35,000 annual U.S. fatalities caused by complications with antibiotic resistance
- Inherent resiliency of bacteria as well as overuse and misuse of certain treatments have led to around 70% of infectious hospital-dwelling bacteria developing resistance to at least one common antimicrobial drug (Todar's Online Textbook of Bacteriology).

Roughly 160 nucleotides long, *ykkCD* absent of ligand(s) folds such to adopt transcription terminator (blue); ligand binding causes unfolding such to allow transcription of efflux pump(s); eviction of toxins like tet and guanidine follows.

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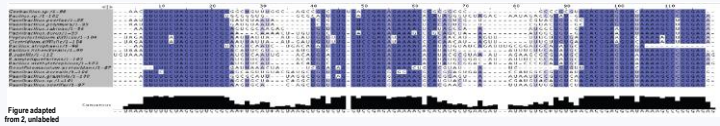
## Tetracycline and several derivatives are recognized by a highly conserved 5' aptamer of the guanidine-sensing *ykkCD* riboswitch, which upregulates expression of an MDR efflux pump in the associated downstream operon

*Multiple sequence alignment (BLAST) of* ykkCD *family aptamers upstream of the associated operon encoding an MDR efflux pump indicates significant conservation.*



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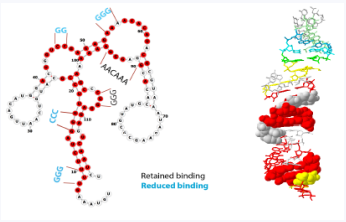
Targeted via site-directed mutagenesis (SDM), the importance of these conserved nucleotides in the recognition of tetracyclines was previously assessed by students and others in the Fernandez lab. Suggested by their apparent clustering together to form an active site, a collective responsibility of binding tetracycline was proposed for conserved segments of the *ykkCD* aptamer module (*2*) .

Figures adapted from 2, unlabeled

Reflecting the roles in tetracycline binding of conserved nucleotides in the *ykkCD* aptamer, the Fernandez lab mapped their SDM results on the predicted secondary (conserved regions in red) and tertiary structures of the RNA. Nucleotides to which mutations were shown to diminish the riboswitch's tet-binding ability are shown with the space-filling design in the predicted tertiary structure.




- The *ykkCD* riboswitch upregulates expression of a multidrug-resistant (MDR) efflux pump responsible for expelling antibiotics and other potential toxins from Gram-positive bacterial cells, temporarily negating their therapeutic nature. It was previously shown by Nelson et al. (Breaker lab) that many members of the *ykkC* motif naturally recognize and bind *free* guanidine (otherwise widely featured as the guanidyl moiety in several metabolites), of which the biological importance grows increasingly apparent (*1*, *3*) . It has also been demonstrated that several bacterial gene lines are capable of mitigating the ionic liquid toxicity imposed by the excessive buildup of endogenously-synthesized guanidine, most likely as the cation guanidinium (*3*, *4*, *5*) .
- It was later shown that the *ykkCD* riboswitch RNA recognizes the antibiotic tetracycline (tet) before undergoing conformational change to enable transcription of the efflux pump (*2*) . Complex binding assays and structure-probing techniques had been used across four tet derivatives and twenty *ykkCD* mutants to explore the riboswitch's molecular basis of tet-recognition which suggested, respectively, highly specific recognition via a tet binding site and that allosteric regulation of the associated *YkkCD* (*ykkC*, *ykkD* gene pair) operon via transcriptional attenuation is enacted upon tet binding.

## Applicability of Isothermal Titration Calorimetry (ITC) and Surface Plasmon Resonance (SPR) Methods to Investigate Co-Binding of Guanidine and Tetracycline

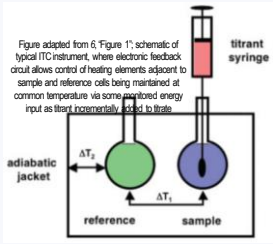
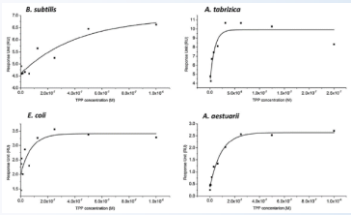



Figure adapted from 6, "Figure 1"; schematic of typical ITC instrument, where electronic feedback circuit allows control of heating elements adjacent to sample and reference cells being maintained at common temperature via some monitored energy input as titrant incrementally added to titrate

- **Isothermal Titration Calorimetry** (*6*)
  - Pros:
    - Ideal for probing RNA-ligand binding thermodynamics.
    - Improved instrument sensitivity; feasible sample requirements for most systems.
    - Versatile thanks to direct probing of reaction heat; avoids perturbations associated with extrinsic labeling.
    - Binding measurable under wide range of solution conditions.
  - Cons, limitations:
    - Measurable binding constant limitation of $K_B \geq 10$ nM → fairly treatable through continuous injection technique.
    - Large background heats, significant error poses issues if inappropriate buffer matching.
    - Titrant, titrate need to be in same buffer system.

Figure adapted from 7, "Figure 6"; SPR-generated kinetic sensorgrams, wherein measured were apparent equilibrium dissociation constants ($K_d$), applied in evaluating TPP- (example ligand) binding affinities among varyingly-sourced TPP riboswitch aptamers




- **Surface Plasmon Resonance** (*7*)
  - Pros:
    - Truly unique measurements accomplishable.
    - Ideal for kinetics analysis of weak macromolecular interactions (the case for guanidine-*ykkCD*), mutants.
    - Reproducibility of equilibrium affinity measurements.
  - Cons, limitations:
    - Ligand immobilization onto sensor surface can be tedious.
    - Potentially significant error in association rate constant $k_{on}$ if too much ligand immobilized.
    - Nonideal for high-throughput assays (time-demanding).

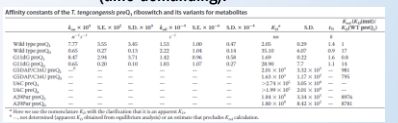


Figure adapted from 7, "Table 3"; example data demonstrating SPR application to measure rates of binding and dissociation of $preQ_1$ and $preQ_0$ metabolites to and from associated riboswitch aptamer i *T. tengcongensis* (and variants introduced through mutagenesis) for determination of binding affinitie

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