CHARACTERIZATION OF BACTERIAL COBAMIDE-DEPENDENT RIBOSWITCHES IN THE HUMAN GUT MICROBIOME

by

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A THESIS

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Vitamin B_{12}, also known as cobalamin (Cbl), is a water-soluble vitamin utilized by bacteria to perform chemically challenging enzymatic functions such as methyl transfer and radical rearrangement. Most gut bacteria are unable to synthesize Cbl themselves and must obtain it from their environment. The B_{12} uptake (Btu) system is a collection of proteins that serves to shuttle Cbl into bacterial cells, including residents of the human gut. *Bacteroides thetaiotaomicron* (*B. theta*) is the most prominent member of the human gut microbiome and the mechanisms by which it trafficks Cbl are vital in dictating its fitness. Some of the most understudied features of the Btu system are its riboswitches, structural elements in RNA that regulate gene expression by binding to small molecules. In *B. theta,* cobamide-dependent riboswitches control the expression of genes that enable the organism to scavenge Cbl from the gut environment and use it for their own cellular processes. To assess the effects of ligand binding and the specificities of the cobalamin-dependent riboswitches from *B. theta,* circular dichroism (CD) spectroscopy was used to assess the changes in the secondary structure of the riboswitches that occurred while titrating in various forms of Cbl. The second riboswitch that lies on locus 1 of *B. theta*'s Btu system was shown to exhibit a significant spectral shift upon addition of aquacobalamin, but not with the addition of 5' deoxyadenosylcobalamin, indicating an upper axial ligand preference for Cbl.

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Introduction

Vitamin B_{12} and Cobalamin-Dependent Enzymes

Vitamin B_{12}, also known as cobalamin (Cbl), is a water-soluble vitamin that plays a vital role in human health, aiding in processes such as DNA synthesis and red blood cell formation (Green et al., 2017). Structurally, it is the most complex natural cofactor and is only synthesized by some prokaryotic organisms in both the presence and absence of oxygen (Fang et al., 2017; Ludwig & Matthews, 1997). In addition to being crucial for humans, cobalamin is equally essential for organisms as small as microbes, including those which reside commensally in the human gut microbiome. Though these microorganisms require vitamin B_{12} for their survival, a majority of them are unable to synthesize it themselves and depend entirely on scavenging strategies as a means of survival. These techniques range from the acquisition and remodeling of various cobamides, molecules within the Cbl family, to the development of alternative enzymatic pathways that do not require cobalamin as a cofactor (Mok et al., 2024).

Chemically, cobalamin is composed of a tetrapyrrole ring held together by a central cobalt atom that is equatorially aligned with an upper and lower ligand (see Fig. 1, Randaccio et al., 2010). The lower ligand, which is most often composed of 5,6-dimethylbenzimidazole (DMB), dictates the molecule's functional specificity, while the upper ligand participates in chemical reactions and is what differentiates the various molecules in the vitamin B_{12} family from one another (Brown, 2005).

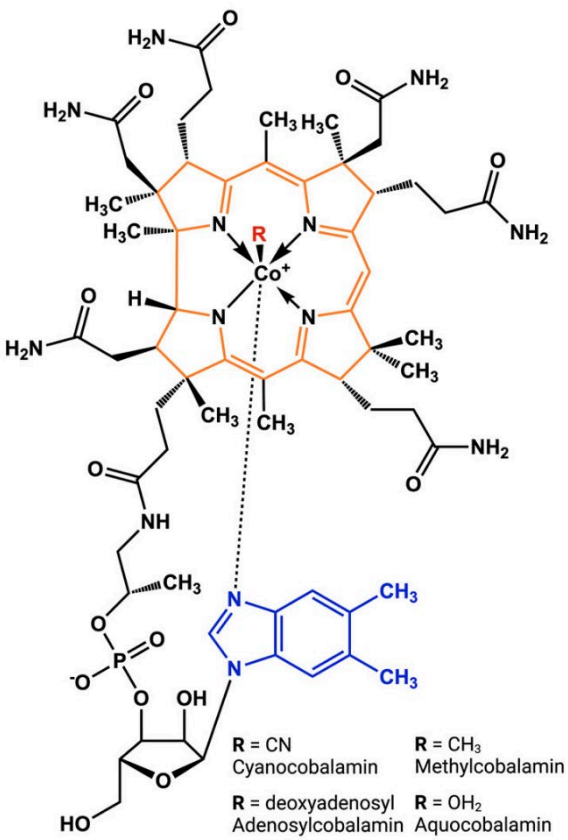

Figure 1: The structure of cobalamin. The tetrapyrrole ring can be seen in orange while the lower axial ligand, occupied by DMB, is seen in blue. 'R' is representative of the upper axial ligand, with its various identities shown in the bottom right corner (Mendoza et al., 2023).

Previous research has found that there are sixteen variants of cobamides that differ in their lower ligand identity, though microbes that are capable of synthesizing cobamides de novo are often only able to produce one or two variants. The range of lower ligands that can be chemically attached to a cobamide has proven to be limited to the specificity, the preference for one ligand over another, of an enzyme known as CobT, which is responsible for catalyzing the addition of the lower ligand during cobamide synthesis (Crofts et al., 2013). Once it is a part of the molecule, the lower ligand can influence the rate at which enzymes that require Cbl can bind to the cobamide, ultimately altering the efficiency of the enzyme-catalyzed reaction (Mok & Taga, 2013).

Several enzymes found in gut bacteria rely on vitamin B_{12} as a cofactor to carry out their specified functions. A cofactor can be defined as a small molecule that is required for an enzyme to carry out the catalysis of a chemical reaction. Methylmalonyl-CoA mutase (MCM), ribonucleotide reductase, and diol dehydratase are all examples of enzymes that rely on cobalamin to carry out their functions (Ludwig & Matthews, 1997). One such B_{12}-dependent enzyme is methionine synthase (MetH), which catalyzes the transfer of a methyl (CH_3) group from 5-methyltetrahydrofolate (5-MTHF) to homocysteine, producing the essential amino acid methionine (see Fig. 2, Banerjee & Matthews, 1990). Some bacterial inhabitants of the gut microbiome, such as *Escherichia coli*, contain genes that encode for a cobalamin-independent methionine synthase (MetE) in addition to a cobalamin-dependent form, enabling them to survive in environments in which vitamin B_{12} is deficient or absent altogether (Weissbach & Brot, 1991). The development of a cobalamin-independent form of enzymes has been an evolutionary strategy used by many bacterial species in an effort to improve the likelihood of survival in a region of low-B_{12} concentration. An alternative strategy involves the overexpression of genes that encode for the proteins that aid bacteria in Cbl uptake from the environment (Mok et al., 2024).

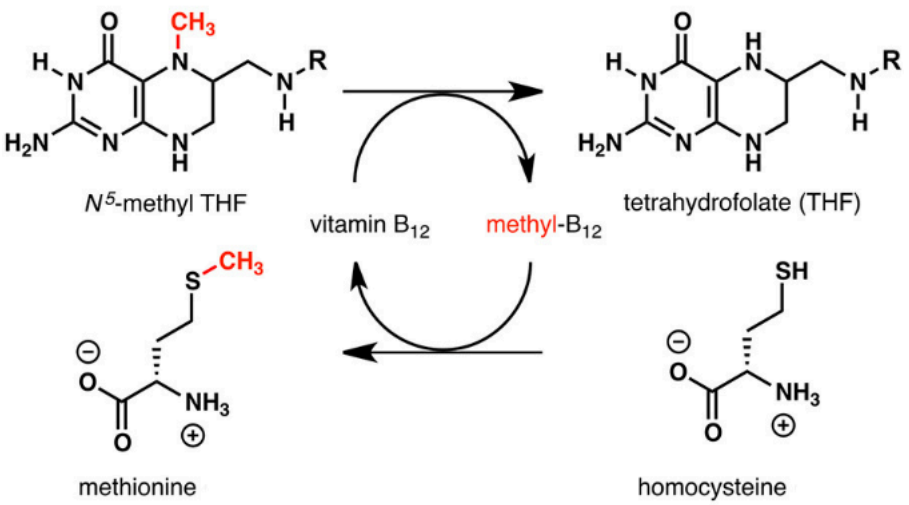

Figure 2: The chemical reaction catalyzed by cobalamin-dependent methionine synthase (MetH). A methyl group, highlighted in red, is transferred from 5-MTHF (top left) to vitamin B_{12}, where it can then be placed onto homocysteine to produce methylcobalamin, labeled above as simply methionine (Lanska, 2019).

Bacteroides thetaiotaomicron in the Gut Microbiome

Approximately 70% of the entire gut microorganism population inhabit the large intestine, a region where concentrations of cobalamin are exceedingly low (Jandyala, 2015). Vitamin B_{12}, like most nutrients, is absorbed in the small intestine, though it must first be bound to a protein produced by the parietal cells of the stomach, intrinsic factor (Shaw & Herzlich, 1989). The minute amount of cobalamin that is not bound to intrinsic factor proceeds through the remainder of the gastrointestinal tract and into the large intestine, where it can be scavenged by the countless microorganisms that reside there (Guetterman et al., 2022). Naturally, this scavenging process becomes highly competitive when taking into account the 35,000 different bacterial species that inhabit the large intestine, many of which do not possess the ability to synthesize their own cobalamin (Jandhyala, 2015).

One of the most prominent microbial members of the human gut microbiome, *Bacteroides thetaiotaomicron* (*B. theta*), is unable to synthesize Cbl de novo and depends on

exogenous sources to supply its various vitamin B_{12}-dependent enzymes (Putnam & Goodman, 2020). It resides primarily in the large intestine and belongs to the most common phylum of bacteria found in the human body (Guetterman et al., 2022). The commensal *B. theta* have evolved to emerge as strong competitors in the gut microbiome by developing complex and efficient transport mechanisms to acquire vitamin B_{12}. Composed of a large collection of proteins, the Btu (B_{12} uptake) system serves as a method by which *B. theta* and other related microbes obtain cobamides from the human gut (Putnam et al., 2022).

Vitamin B_{12} Trafficking

The Btu system is a feature of several species of gram-negative bacteria, including *E. coli*, due to its unique ability to transport vitamin B_{12} through both the outer and inner membranes of bacterial cells. However, the complexity of the system varies based on the phylum and species of bacteria and whether the organism is able to adopt alternative mechanisms of acquiring Cbl or function without it. In *E. coli*, which is additionally able to produce the vitamin B_{12}-independent MetE, the entirety of the Btu system is encoded by one region of the bacteria's genomic DNA, known as a locus. However, *B. theta*, which lacks the genes to produce MetE, possesses a Btu system that lies on three different loci and consists of 24 unique proteins, each of which contributes to the organism's acquisition of cobalamin (see Fig. 3, Abellon-Ruiz et al., 2023).

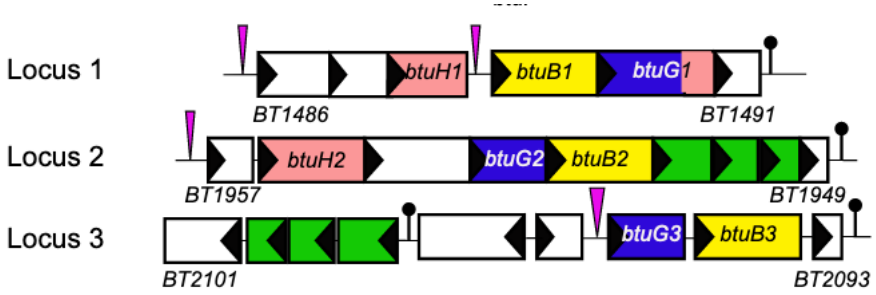

Figure 3: The three loci that make up the Btu system of *B. theta*. Pink triangles are indicative of riboswitches while the black lines with circles are indicative of transcriptional terminators. Between the three loci, *B. theta* produces 24 unique proteins that contribute to cobalamin uptake (Abellon-Ruiz et al., 2023).

To gain entry into the cytoplasm, which is where the vast majority of enzymes are located, Cbl must travel through the outer membrane, the periplasmic space, and the inner membrane (Putnam & Goodman, 2020). The cofactor is known to bind to BtuB, a protein embedded within the outer membrane that actively transports cobalamin into the periplasmic space. Once in the periplasm, vitamin B_{12} binds to BtuF and is transported to the junction between the periplasm and the inner membrane, where it is transported into the cytoplasm by the BtuCD transporter, which hydrolyzes ATP to power this energetically unfavorable process (see Fig. 4, Abellon-Ruiz et al., 2023).

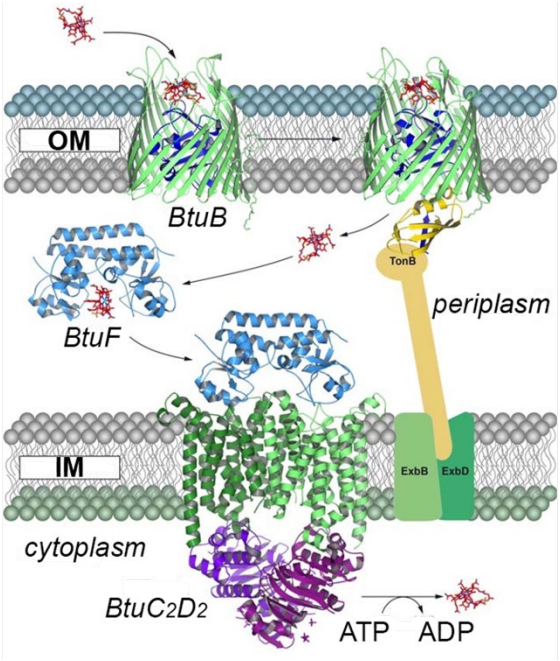

Figure 4: The model for vitamin B_{12} acquisition by *E. coli*'s Btu system. Cobalamin (in red) is taken up by BtuB (in green) in the outer membrane. Aided by the TonB-dependent receptor (in yellow), Cbl is transported into the periplasm, where it is bound by BtuF (in blue). Finally, the $BtuC_2D_2$ complex (in purple) hydrolyzes an ATP to bring Cbl through the inner membrane and into the cell (Krautler and Puffer, 2012).

Cobalamin-Dependent Methionine Synthase

The characterization of interactions that occur between the proteins that compose the Btu system in *B. theta* can provide insight into how vitamin B_{12} is chaperoned to its target enzymes, including the cobalamin-dependent MetH. This enzyme is composed of five domains, three of which are capable of binding and activating various molecules and can fold into multiple distinct conformations depending on the chemical reactions it must catalyze (see Fig. 5).

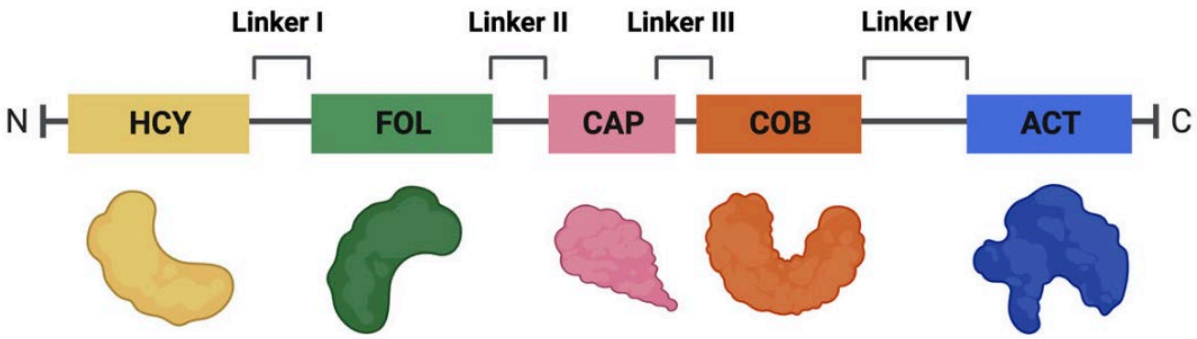

Figure 5: The five domains that compose the cobalamin-dependent methionine synthase (MetH). Each of the domains are connected by a linker region, which allows the enzyme to exhibit a great deal of flexibility and rearrange itself according to the chemical reactions it must catalyze (Mendoza et al., 2023).

Its large, multi-domain structure enables MetH to act as a key regulator of one-carbon metabolism in prokaryotic and eukaryotic organisms alike, linking the folic acid cycle to the biosynthesis of the essential amino acid methionine (Matthews, 2001). It is already known that the cobalamin-dependent form of methionine synthase must bind vitamin B_{12} at its cobalamin binding domain (COB) and must participate in a catalytic cycle in order to successfully yield methionine from folate and homocysteine. However, the mechanisms by which Cbl is loaded onto MetH are largely unknown and it has been hypothesized that cofactor loading is facilitated by a chaperone protein known as BtuR, which has previously been shown to contribute to optimal cobalamin-dependent growth (Mok et al., 2024).

The reaction carried out by MetH is additionally reliant upon the flavodoxin (FldA) and flavodoxin reductase (Fpr) genes, redox partners associated with MetH that act to reduce oxidized cob(II)alamin to cob(I)alamin. Flavodoxin proteins serve as electron donors, facilitating the oxidation and reduction reactions that are required to reduce the oxidized cofactor. Furthermore, flavodoxin reductase serves to reduce flavodoxin, receiving its electrons from the electron carrier NADPH (see Fig. 6).

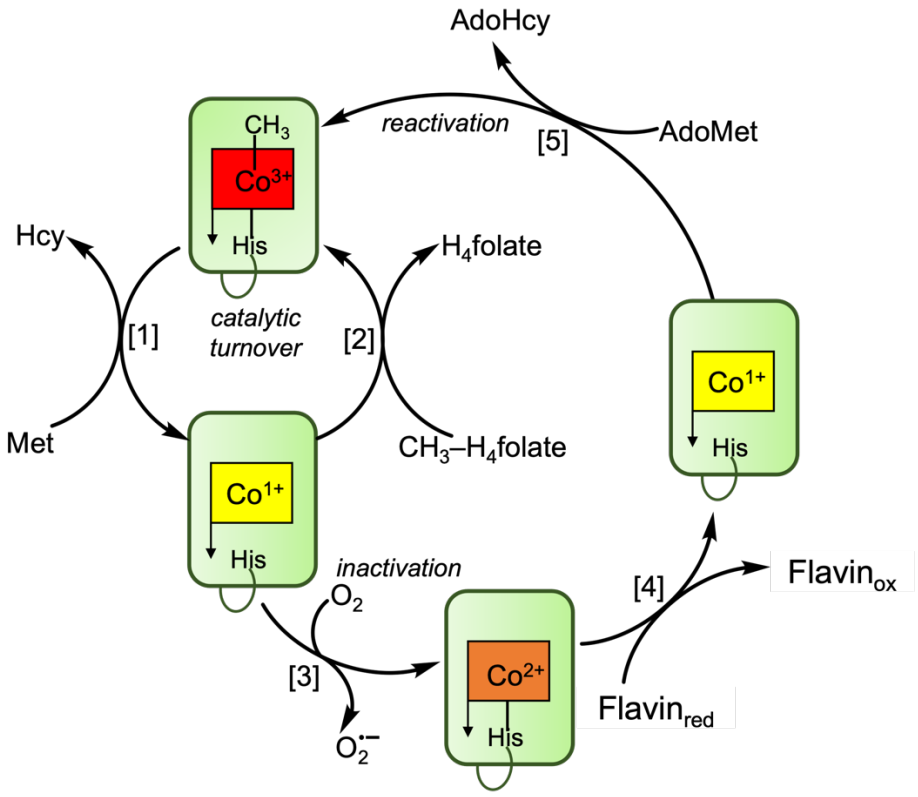

Figure 6: The catalytic cycle of cobalamin-dependent methionine synthase. Highly reactive cob(I)alamin (in yellow) is inactivated to cob(II)alamin (in orange). FldA and Fpr serve to reduce cob(II)alamin back to cob(I)alamin, which is then reactivated via methylation by the activation domain, producing methylcobalamin (in red).

The stepwise movement of electrons from NADPH to Fpr to FldA to MetH is what enables the reactivation of cobalamin, deeming FldA and Fpr necessary elements when considering the entirety of methionine's biosynthesis (Gudim et al., 2018).

Cobalamin-Dependent Riboswitches

Another unique feature of *B. theta*'s Btu system that is not present in other, similar gram-negative bacteria is its cobalamin-dependent riboswitches. When double-stranded DNA is converted into single-stranded messenger RNA (mRNA), a process known as transcription, the resulting mRNA can be divided into two distinct regions: the mRNA that will be further turned into protein through a process known as translation (referred to as the open reading frames

(ORFs)), and the mRNA that will remain untranslated (referred to as the untranslated regions (UTRs)). The UTR that is situated prior to an ORF contains various regulatory elements that dictate how frequently translation of the ORF will occur, and thus, regulated protein expression (see Fig. 6).

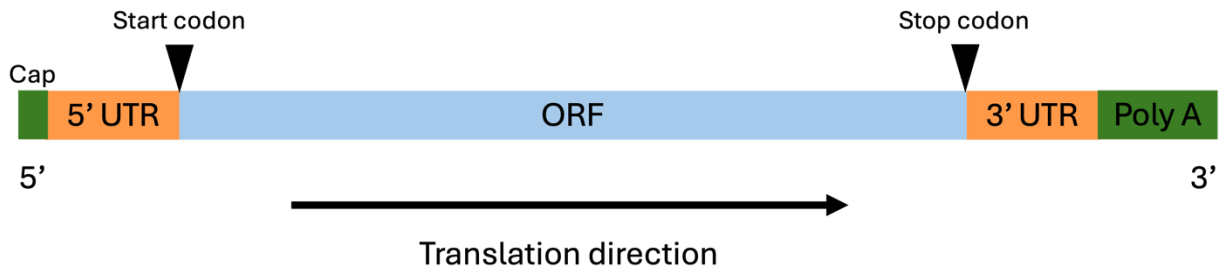

Figure 7: A generic diagram of the structure of messenger RNA (mRNA). In orange, the untranslated regions (UTRs) are where a majority of regulatory elements lie. In blue, the open reading frame (ORF) contains the genetic material that can be made into proteins.

Many of these regulatory elements function by imposing some effect on the ribosome-binding site, also known as the Shine-Delgarno sequence, altering the accessibility of this region which is critical for translation to begin.

Riboswitches are one such regulatory element featured in the UTR of certain mRNA, the vast majority of which is found in prokaryotic cells and relies on the binding of a ligand such as a metal ion or cofactor (Garst et al., 2011). Composed of two general domains, the aptamer (ligand-binding) domain and the expression platform (regulatory region), riboswitches work cohesively to alter rates of translation by undergoing structural changes in response to ligand binding. Such structural changes function to either hide or reveal the ribosome-binding site, which serves to attenuate or enhance translation, respectively (Kavita & Breaker, 2023).

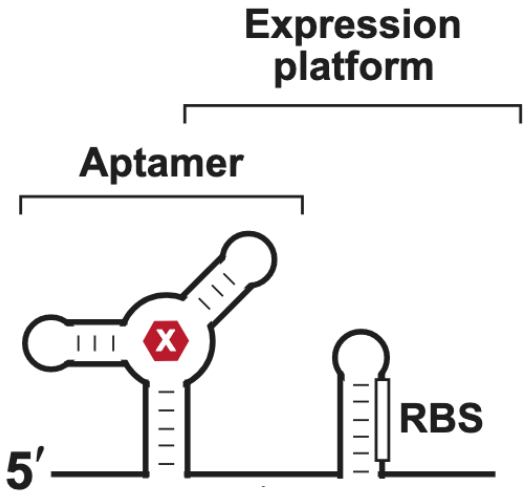

Figure 8: The generic structure of a translation-controlling riboswitch. The aptamer domain binds the ligand (represented by the red X), altering the secondary structure of the expression platform. This conformational change causes the ribosome-binding site (RBS) to become inaccessible to the ribosome, inhibiting translation (Kavita & Breaker, 2023).

Each of the loci in *B. theta*'s Btu system contains at least one cobalamin-dependent riboswitch that binds to the vitamin and either upregulates or downregulates the production of the proteins that enable the bacteria to efficiently acquire Cbl from the environment (Abellon-Ruiz et al., 2023).

B_{12}-Dependent Riboswitches

The first riboswitches discovered were cobalamin-dependent riboswitches that lie within the Btu system of *E. coli* and were first described as RNA-based intracellular vitamin sensors (Nahvi et al., 2002). Though cobalamin-dependent riboswitches are now known to exist in countless bacterial species, the vast majority of those that have been characterized are those found in *E. coli*. These riboswitches can be divided into two classes, known as Cbl-I and Cbl-II, which are distinguished based on the cobamide selectivity that they exhibit as well as their

structural elements that lie beyond the aptamer domain and expression platform. Cbl-I riboswitches are most commonly selective towards adenosylcobalamin (AdoCbl) with a peripheral region that functions to facilitate ligand specificity. Cbl-II riboswitches are further divided into Cbl-IIa and Cbl-IIb riboswitches, with Cbl-IIa being more selective towards smaller cobalamin analogues, such as methylcobalamin (MeCbl) and hydroxycobalamin (HyCbl), and Cbl-IIb being more selective towards AdoCbl (Polaski et al., 2017). To date, the cobalamin-dependent riboswitches within *B. theta* have not been characterized sufficiently to place them into any of the previously described classes, though their structures are likely to mimic those of more well-defined cobalamin riboswitches (see Fig. 9).

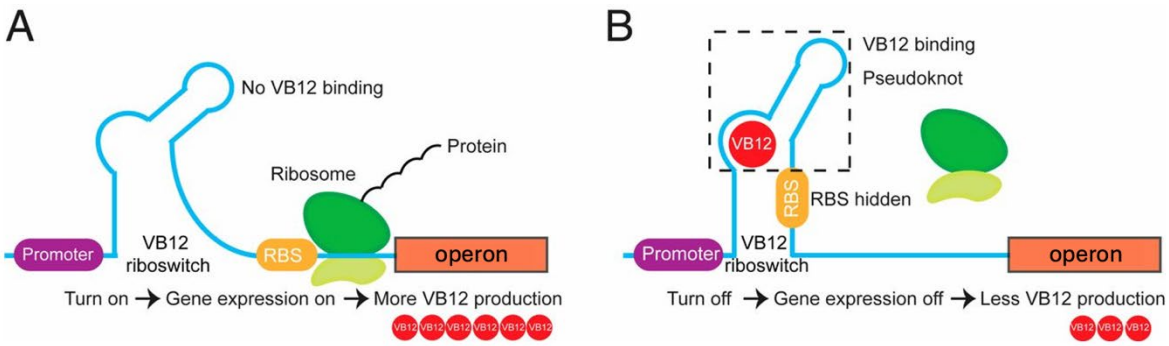

Figure 9: The proposed mechanism for a cobalamin-dependent riboswitch a strain of *Propionibacterium*, a gram-negative bacterium. (A) Gene expression is "on" in the absence of vitamin B_{12}, allowing the bacterium to produce more of the vitamin. (B) Gene expression is "off" in the presence of cobalamin as ligand binding results in the formation of a pseudoknot, which hides the ribosome binding site (RBS) and prevents translation (Li et al., 2019).

Project Aims

While much research has been done to characterize the protein-protein interactions that enable vitamin B_{12} uptake in bacteria such as *B. theta*, little is known about the mechanisms that are involved in loading cobalamin onto enzymes that require it, such as methionine synthase. Additionally, the riboswitches that control the expression of genes within the Btu system have

not been well characterized and their structures and cobamide-specificities remain unknown. This research focuses on understanding the role of BtuR in loading methionine synthase with cobalamin. In a related study, my research focused on the cobalamin-dependent riboswitches to determine if *B. theta*'s uncharacterized riboswitches exhibit cobamide-specificity.

Implications

As previously stated, the phylum *Bacteroides* makes up an extremely large proportion of the entire bacterial population of the human large intestine, a microbiome which is both impactful and extremely beneficial to human health. Bacteria that reside within the gut microbiome aid in immune defense against foreign pathogens, help to digest dietary fibers and polyphenols, and promote enteric nerve function, assets which can be lost due to changes in the biome's composition (Zhang et al., 2015). Not only does the acquisition of vitamin B_{12} enable *B. theta* and other prominent bacterial species to survive, but it also preserves the fortitude of the microbiome and the healthful advantages that it provides to the human host.

Without the Btu system and related cobalamin transport mechanisms, *B. theta* would be unable to endure the highly competitive, bacteria-rich environment in which it resides. Losing a species as prominent within the gut as *B. theta* would result in a drastic change in the composition of the gut microbiome, altering its functions and leading to diseases including inflammatory bowel disease, obesity, and even cancer (Zhang et al., 2015). Gaining further understanding of the mechanisms of B_{12} acquisition will provide essential molecular detail that enables *B. theta* to outcompete other commensal microbes in the fight for survival, a battle whose outcome impacts the health of microorganisms and their human hosts alike.

Methods

Molecular Cloning of Methionine Synthase

Polymerase Chain Reaction

The cloning of MetH (BT_0180) was carried out using a pET-28a plasmid which holds a kanamycin-resistant gene. For this reason, all cell cultures grown for the cloning and purification of MetH and its activation domain were done in the presence of the antibiotic kanamycin. The gene for *B. thetaiotaomicron*'s MetH was cloned into a pET-28a vector between the *Bam*H1 and *Nde*1 restriction sites to generate an N-terminal 6x His tag construct with a TEV cleavage site. To isolate the DNA encoding for the MetH gene, a polymerase chain reaction (PCR) was performed on the genomic DNA of *B. thetaiotaomicron* using single-stranded primers that would selectively amplify the DNA coding for MetH (see Table 1). After diluting the primers to 10 µM, they were combined with Q5 DNA polymerase, dNTPs, Q5 DNA polymerase buffer, and GC content enhancer corresponding to the polymerase enzyme, and the genomic DNA of *B. thetaiotaomicron*. The reaction mixture was then placed in a MasterCycler and allowed to run for 30 cycles at 58.1ºC and 60.6 ºC. Once the reaction was complete, the resulting PCR product was run on an agarose gel to confirm that the PCR had worked properly and to isolate and purify the DNA. Purification was carried out using the Qiagen Gel Purification kit on the DNA bands excised from the agarose gel. The gel was dissolved in buffer QG, bound to the column, washed, and eluted in 50 µL of de-ionized (DI) water, yielding a final concentration of 56.3 ng/µL.

Restriction Digestion and Ligation

The purified DNA was subsequently digested along with the pET-28a vector using the restriction enzymes listed in Table 1 by combining the insert/plasmid with water, buffer, and the

restriction enzymes, which were added in succession, leaving one hour between additions at 37 ºC. Again, the products were run on an agarose gel to confirm that the digestion had worked and to purify the resulting DNA for further use. The digested insert (MetH) and vector were ligated together by combining them in a microcentrifuge tube with ligation buffer, T4 ligase enzyme, and DI water. Two different ligation reactions were carried out: one with a vector to insert ratio of 1:1, another with a ratio of 1:3.

Transformation

Both reactions were left overnight at 18 ºC and the products were transformed into top 10 cells the following day by combining the products with the cells, allowing them to sit on ice for 30 minutes, heat shocking them at 42 ºC, incubating them in LB media for 45 minutes, and then plating the bacteria on agar plates to incubate at 37 ºC overnight. Only one colony from the 1:3 ligation plate was found to express protein upon being run on an agarose gel and an overnight culture of this colony was created, purified, and sent for sequencing to confirm the successful clone.

Sequencing and Further Cloning

Sequencing results from the cloning of MetH confirmed that the DNA encoding for the protein had been successfully inserted into the plasmid. The cloning process was repeated for the DNA encoding for the MetH activation domain (BT_0249), though the primers and restriction enzymes used differed from those used for MetH (see Table 1). Due to the lack of colonies that grew on the 1:3 ligation plate for MetH, the ligations for the MetH activation domain were carried out at ratios of 1:3 and 1:5. Nearly each of the 21 colonies used for PCR from the 1:5 ligation plate showed appropriate DNA expression on an agarose gel, but the three with the greatest expression levels were purified and sent for sequencing. Lastly, the same molecular

cloning procedure was carried out for the flavodoxin (FldA, BT_0517) and flavodoxin reductase (Fpr, BT_2067) genes associated with the methionine synthase gene, which encode for proteins that aid in the electron transfer that facilitates the activation of MetH. The primers and restriction enzymes used for these clones are found in Table 1.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Restriction Enzymes
MetH	5' ATC ATA TGA TGA AAA AGA CAA TTT CTC AGA TTG TAT C 3'	5' ACG GAT CCT TAA AAT AGG TTT AAC TTA TTT TTC TG 3'	*Bam*H1 and *Nde*1
MetH Act. Domain	5' ATC CAT GGG CAT GAT TTT ATC TTA TAA GAT ACA TAA CGT CGC CCC CTA C 3'	5' ACC TCG AGG GAT TCG TTC AGG TAG AAT TGG GAT TGC AGA TTG GCA GCC AGG AAT TT 3'	*Xho*1 and *Nco*1
FldA	5' ATC ATA TGA ATA AAA TTG GAG TAT TTT ATG G 3'	5' ACC TCG AGT TAG CTG ATT TCT TGT TTT ACT TG 3'	*Xho*1 and *Nde*1
Fpr	5' ATC ATA TGG AAT CTA AAC TTT TCA CCC CTG 3'	5' ACC TCG AGT CAT TGC TTT TCC AGT TTT TC 3'	*Xho*1 and *Nde*1

Table 1: The forward and reverse primer sequences and restriction enzymes used for the molecular cloning of the methionine synthase (MetH) gene, its activation domain (MetH Act. Domain) gene, and its associated flavodoxin (FldA) and flavodoxin reductase (Fpr) genes.

Expression and Purification of BtuR

BtuR was transformed into BL21DE3* cells and an overnight culture was made, and the DNA was purified using the Qiagen Miniprep Kit. 5 liters of Luria Broth (LB) was prepared and autoclaved and cultures of BtuR were created by adding overnight culture to each liter of LB. The cultures were left to incubate at 37ºC until OD reached 0.6 upon which 150 µM of IPTG was added to each flask to induce the expression of BtuR. Cells were pelleted down, harvested, and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM KCl, 10 mM imidazole, 1 mM TCEP, 25 mg lysozyme, and protease inhibitor) and the mixture was sonicated and

centrifuged to ensure cell lysis. The resulting supernatant was run through a Nickel-NTA column, and the product was eluted from the column using a gradient of low-imidazole buffer (400 mL lysis buffer, 3 mL imidazole) and high-imidazole buffer (50 mM Tris-HCl, 300 mM KCl, 400 mM imidazole). The fractions were collected, tested for protein using a Bradford assay, and concentrated prior to overnight thrombin dialysis. Following dialysis and further concentration, the protein was injected onto a Superdex S200 120 mL size exclusion column for further purification and the fractions corresponding to BtuR were combined, concentrated using an Amicon Ultra-15 10 kDa centrifugal filter, and flash frozen in liquid N_2 until further use.

Riboswitch Synthesis and Purification

DNA oligonucleotides were purchased from Integrated DNA Technologies (sequences in Table 2) and four 100 µL PCR reactions were set up for each riboswitch using Q5 High-Fidelity DNA Polymerase (New England Biolabs). The sequence for the forward primer of riboswitch 1 (Rib 1) was 5' GCG CTA ATA CGA CTC ACT ATA GG 3' and the reverse primer was 5' AGT GAA ACG GAT TTT ACA TGG C 3'. The sequence for the forward primer of riboswitch 2 (Rib 2) was 5' GCG CTA ATA CGA CTC ACT ATA GG 3' and the reverse primer was 5' AGT GAA ACG AGT TTG CAT GG 3'. After cleanup with the Qiagen PCR Cleanup Kit, the resulting DNA was pooled according to which riboswitch it encoded for and RNA synthesis reactions were run using T7 RNA Polymerase (New England Biolabs) with DNAse1 enzyme and 0.5 M EDTA being added afterward to remove any remaining DNA and prevent the new RNA from being degraded. A 5% polyacrylamide-urea gel was prepared and the mRNA was resolved with product bands being recovered by electroelution using a BioRad model 422 electro-eluter followed by ethanol precipitation in the presence of 300 mM NaOAc, pH 5.3. The resulting concentrations of Rib 1 and Rib 2 were 4.85 µM and 5.21 µM, respectively.

	Gene Block Sequence
Rib 1 (219 bp)	5' GCG CTA ATA CGA CTC ACT ATA GGC CGC ATT GGT TTG CGA CTC TCA TCA CGA GGG AAG CGA TTA AAA GGG AAT CAG GTG TAA ATC CTG AAC AGT CCC GCT GCT GTA AGT TCC ATA GAT ATG TTG CGA GCA ATC TAC TCA AAG CCA CTG GAA AGA AAA TTC CGG GAA GGC GCC CGC AAC AGG AAT AAG TCA GAA GAC CTG CCA TGT AAA ATC CGT TTC ACT 3'
Rib 2 (219 bp)	5' GCG CTA ATA CGA CTC ACT ATA GGG CGC ATT GGT TTG CAA CTC TCA TGC ACG AGG GAA GCA ATT AAA AGG GAA TCA GGT GAA AGT CCT GAA CAG TCC CGC TGC TGT AAG TTC ATT TTT TGG TTG CGA GCA ATC GAC TTT ATA GCC ACT GGA AAG CAA ATT CCG GGA AGG CGT TCG CAA CAG GAA CAA GTC AGA AGA CCT ACC ATG CAA ACT CGT TTC ACT 3'

Table 2: The gene block sequences of Rib1 and Rib2 purchased from Integrated DNA Technologies. These sequences were amplified and subsequently used to synthesize the RNA that composes the two riboswitches present on locus 1 of *B. thetaiotaomicron*'s Btu system.

Circular Dichroism Spectroscopy

All spectra were recorded at 20ºC with the riboswitch sample in a buffer consisting of 50 mM HEPES and 100 mM KCl. The samples were heated to 80ºC for 2 min and then cooled to room temperature for 10 min, with 1 mM $MgCl_2$ being added after cooling was complete. CD spectra were recorded on a Jasco J-1500 spectrometer with samples in a 1 cm path-length cuvette with the riboswitch at a concentration of 200 nM. Cobalamin was titrated in, beginning with a concentration of 25 nM, and ending at a concentration of 1 mM, with scans being performed from 220 to 350 nm at a rate of 20 nm per minute with an integration time of 2 s and an excitation bandwidth of 2 nm. Two sequential scans were averaged together and subsequently background corrected by subtracting a buffer scan with the same concentration of cobalamin.

Native-PAGE Gel Analysis of Various Annealing Conditions

Following the initial spectral measurements of Rib 1 and Rib 2 with adenosylcobalamin (AdoCbl) and aquacobalamin (AqCbl), emphasis was placed on the interaction exhibited

between Rib 2 and AqCbl. In an effort to optimize the conditions for binding of Rib 2 to AqCbl, various heating and cooling conditions were conducted, and the cooled riboswitch was run on a Native-PAGE gel to determine which condition yielded the most homogeneous solution of folded mRNA. Rib 2 was annealed using 11 different methods, outlined in Table 3, and 6 µL of each sample were combined with 6 µL of loading buffer to be loaded into the Native-PAGE gel. A 10% polyacrylamide gel was made, and all samples of annealed RNA were run at 4ºC for approximately 90 minutes. Though, this percentage of gel was not sufficient to resolve such a large riboswitch while ensuring proper separation. As such, the same 11 samples were run on a 6% polyacrylamide gel at 4ºC for approximately 135 minutes to provide ample time for the separation of differentially folded RNA. Each of the acrylamide gels was imaged with a Typhoon Biomolecular Imager and was analyzed in the ImageJ software.

Sample Number	Annealing Conditions
1	Denatured at 90ºC for 2 mins, cooled at room temperature, 1 mM $MgCl_2$ added after 10 mins of cooling
2	Denatured at 90ºC for 2 mins, crash cooled on ice for 10 mins, 1 mM $MgCl_2$ added after cooling
3	Denatured at 80ºC for 2 mins, cooled at room temperature, 1 mM $MgCl_2$ added 30 secs into cooling
4	Denatured at 90ºC for 2 mins, controlled gradient cool from 90ºC to 20ºC over 30 mins, 1 mM $MgCl_2$ added after cooling
5	Denatured at 80ºC with 200 nM AqCbl, cooled at room temperature, 1 mM $MgCl_2$ added after 10 mins of cooling
6	Denatured at 80ºC, cooled at room temperature, no $MgCl_2$ added
7	Denatured at 90ºC, controlled gradient cool from 90ºC to 20ºC over 30 mins, no $MgCl_2$ added
8	Denatured at 90ºC, cooled at room temperature, no $MgCl_2$ added
9	Replaced 50 mM HEPES in buffer with 50 mM Tris-HCl pH 8.0, denatured at 80ºC, cooled at room temperature, 1 mM $MgCl_2$ added after 10 mins of cooling
10	Replaced 50 mM HEPES in buffer with 50 mM Tris-HCl pH 8.0, denatured at 90ºC, cooled at room temperature, no $MgCl_2$ added
11	Denatured at 90ºC, crash cooled on ice for 10 mins, no $MgCl_2$ added

Table 3: The various annealing conditions performed on Rib 2 for the purpose of running on a Native-PAGE gel to determine which would yield the most homogeneous solution of RNA. Where not noted, the same spectroscopy buffer (50 mM HEPES, 100 mM KCl) was used. All gradient cools were performed on a MasterCycler at a rate of 0.2ºC cooled per second.

Results

Molecular Cloning of MetH and its Associated Proteins

Each of the molecular clones, once purified, were sent to Plasmidsaurus to be sequenced to confirm that the genes had been successfully cloned. The results suggested that each of the genes had been successfully inserted their respective vectors, meaning that each protein could be later purified and utilized for further experiments to assess the interprotein interactions that likely occur within *B. thetaiotaomicron* (see Figure 8).

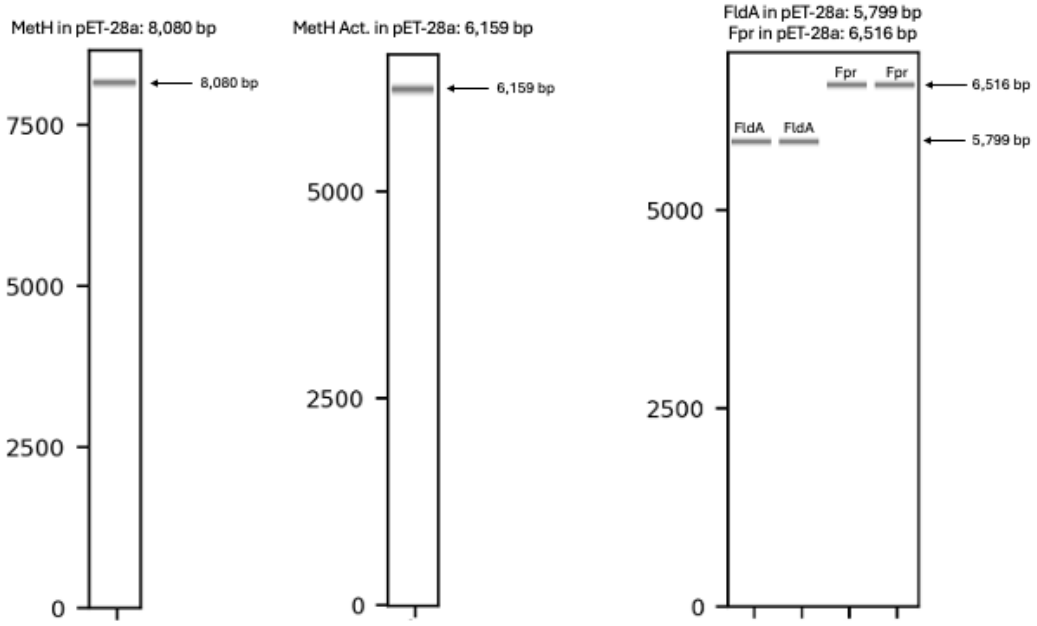

Figure 10: The sequencing results of MetH (left), MetH activation domain (middle), and FldA and Fpr (right). Each vector and insert was expected to be a certain number of base pairs (bps) had the cloning been successful, and the size of each sequenced plasmid confirms that each of the genes had been successfully cloned into pET-28a vectors.

Characterization of Cobalamin-Dependent Riboswitch Binding

Though both riboswitches from locus 1 of *B. thetaiotaomicron*'s Btu system were observed with CD spectroscopy, only the second riboswitch, Rib 2, exhibited a significant

spectral shift upon addition of cobalamin. The spectral changes seen in Rib 2 with the addition of cobalamin were particularly notable with the addition of aquacobalamin (AqCbl) and such changes were not able to be replicated with Rib 1 (see Figure 9).

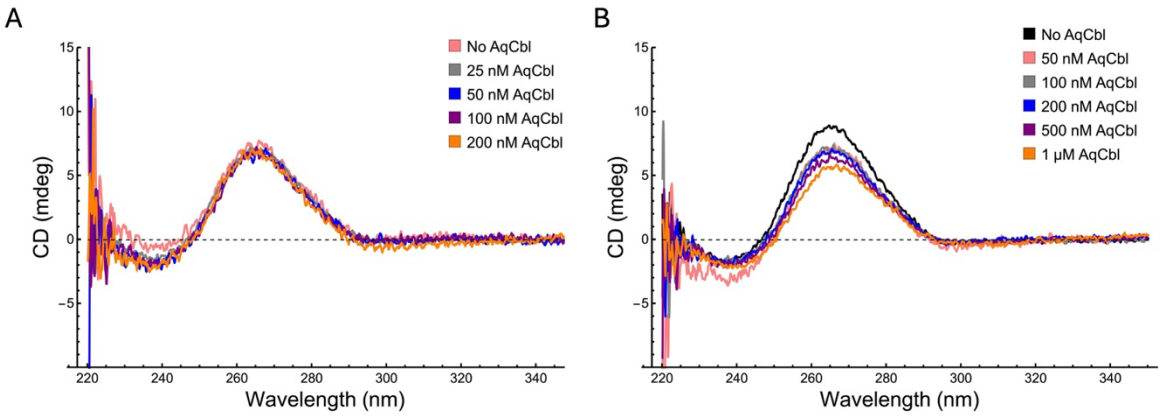

Figure 11: The CD spectra of both locus 1 riboswitches with titration of AqCbl. (A) The CD spectrum of Rib 1 with AqCbl, beginning with no cobalamin and ending at a concentration of 200 nM. The lack of change in CD with increasing concentrations of AqCbl implies that no ligand is binding to the RNA. (B) The CD spectrum of Rib 2 with AqCbl, beginning with no cobalamin and ending at a concentration of 1000 nM. The significant spectral change can be attributed to ligand binding to the riboswitch.

Moreover, neither riboswitch demonstrated a significant spectral change upon addition of adenosylcobalamin (AdoCbl), with Rib 2 exhibiting a slightly larger change than Rib 1, but not one that was comparable to that seen with the addition of AqCbl (see Figure 10).

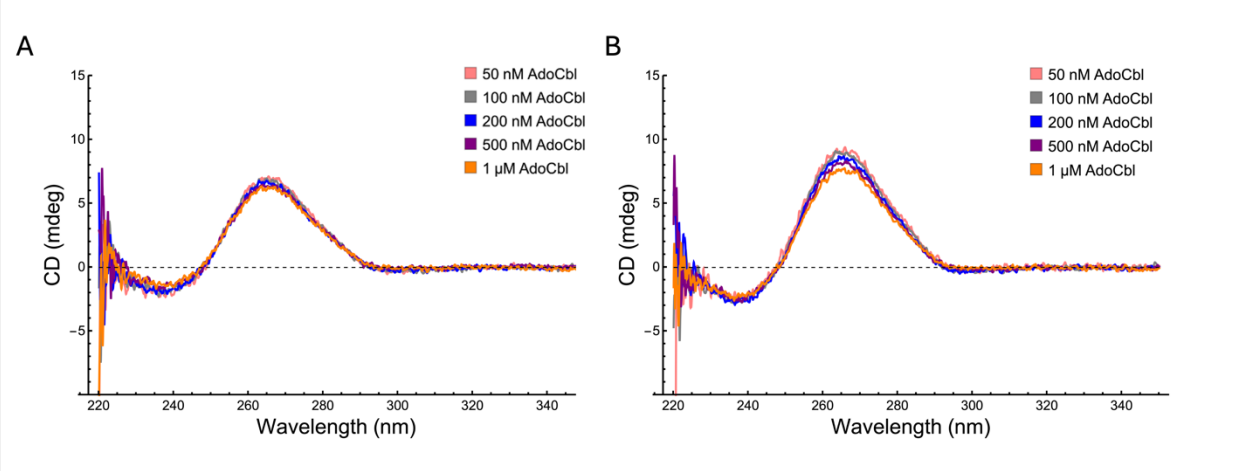

Figure 12: The CD spectra of both locus 1 riboswitches with titration of AdoCbl. (A) The CD spectrum of Rib 1 with AdoCbl, beginning at a concentration of 50 nM and ending at a concentration of 1000 nM. The lack of change in CD with increasing concentrations of AdoCbl implies that no ligand is binding to the RNA. (B) The CD spectrum of Rib 2 with AdoCbl, beginning and ending at the same concentrations. There is a greater change in CD with increasing concentrations of AdoCbl, though it is not as significant as the change seen with the addition of AqCbl.

Much of the remaining spectroscopy was thus conducted on Rib 2, due to the apparent lack of binding of AdoCbl or AqCbl to Rib 1, though spectra of Rib 1 were repeated to confirm the absence of activity upon addition of cobalamin. As such, the remainder of this project focuses primarily on Rib 2, including the running of various Native-PAGE gels to determine which annealing condition would yield the most homogeneous solution of mRNA for future spectroscopic experiments.

Riboswitch Homogeneity Under Various Annealing Conditions

Initially, each of the 11 annealing conditions were run on a 10% acrylamide gel for approximately 90 minutes, which was not sufficient time for the mRNA to migrate through the gel (see Figure 11).

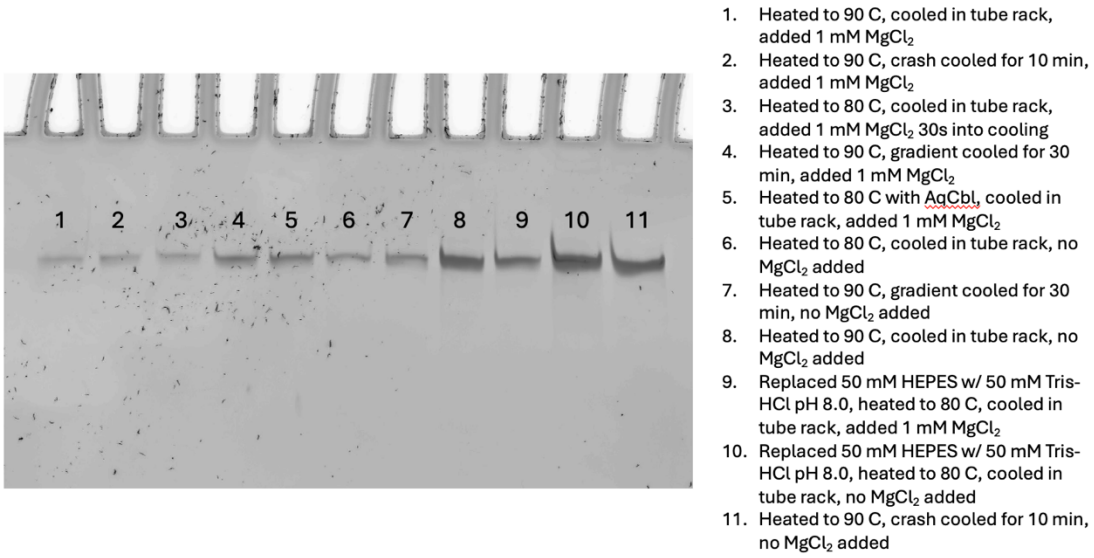

Figure 13: The image of the 10% acrylamide gel, which was run at 4°C for approximately 90 minutes. Each well was loaded with 12 µL of a solution composed of 50% annealed RNA and 50% loading buffer. All of the annealing conditions seemed to yield similar levels of homogeneity, though some of the first wells appeared much lighter than the last wells, indicating lower concentrations of RNA.

Due to the small distance travelled, it was inferred that a lower percentage acrylamide gel would be needed to ensure that the mRNA could move further into the gel, allowing more time and distance for separation of any heterogeneous solutions. Thus, the same 11 annealing conditions were run on a 6% acrylamide gel (see Figure 12).

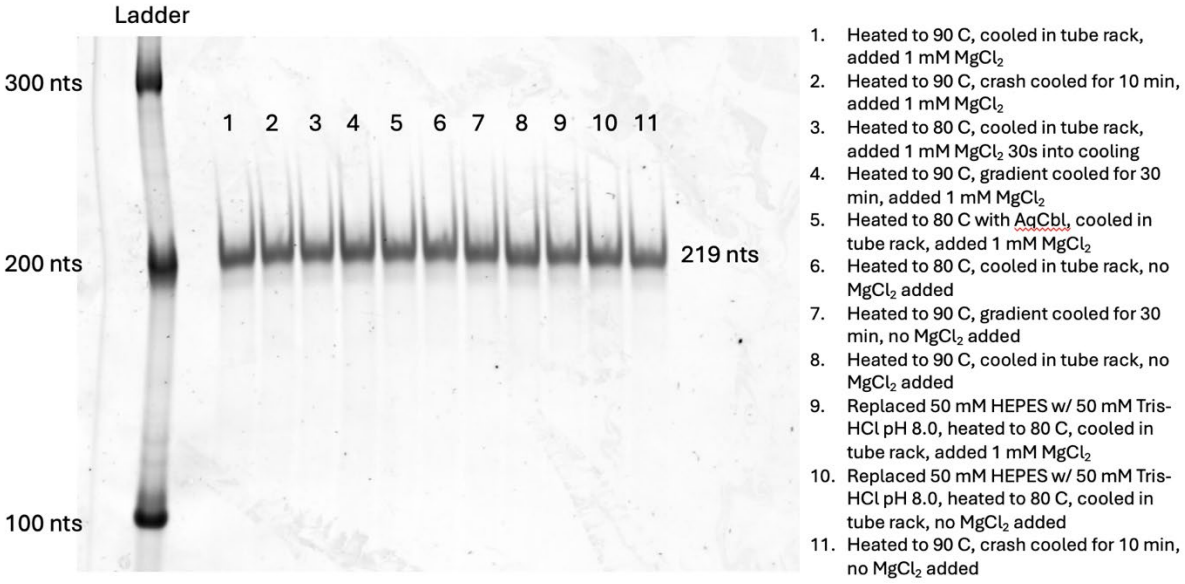

Figure 14: The image of the 6% acrylamide gel, which was run at 4ºC for approximately 135 minutes. The annealing procedures for each sample are identical to those used for the previous 10% gel and, once again, each condition appeared to yield an almost identical level of homogeneity.

Discussion

The initial spectra of Rib 1 and Rib 2 with the titration of AqCbl were immediately likely to be indicative of ligand binding in one riboswitch, Rib 2, but not the other, Rib 1 (see Figure 9). Though minor, the changes in CD, measured in millidegrees (mdeg), suggested that the secondary structure of Rib 2 was altered by the binding of AqCbl and that this conformational change was significant enough to impact the degree to which the circularly polarized light was perturbed by the RNA. The lack of any change in CD, seen in Figure 9A, indicated that Rib 1 was not binding to the AqCbl that was being titrated into the solution, regardless of how high the concentration was. Repeated scans of Rib 1 with AqCbl with buffer and baseline subtractions confirmed that the absence of a response to the ligand was likely a result of no interaction occurring between AqCbl and the riboswitch. Similarly, repeated scans of Rib 2 with the same subtractions confirmed that the changes in CD seen upon addition of AqCbl were more than likely due to binding of the ligand to the riboswitch, inducing a change in secondary structure.

When the same spectra were taken with the titration of AdoCbl in place of AqCbl, the results differed slightly, revealing that the ligand was likely not binding to either of the riboswitches tested (see Figure 10). The slight changes in the CD of Rib 2 with higher concentrations of AdoCbl, seen in Figure 10B, may have been indicative of ligand binding, though the changes were not nearly as significant as those induced by addition of AqCbl seen in Figure 9B. This data, combined with that acquired from the titration of AqCbl, suggests that Rib 2 may exhibit selectivity for the upper axial ligand of cobalamin, with a preference for aquacobalamin's small water molecule over adenosylcobalamin's bulky adenosyl group (see Figure 13).

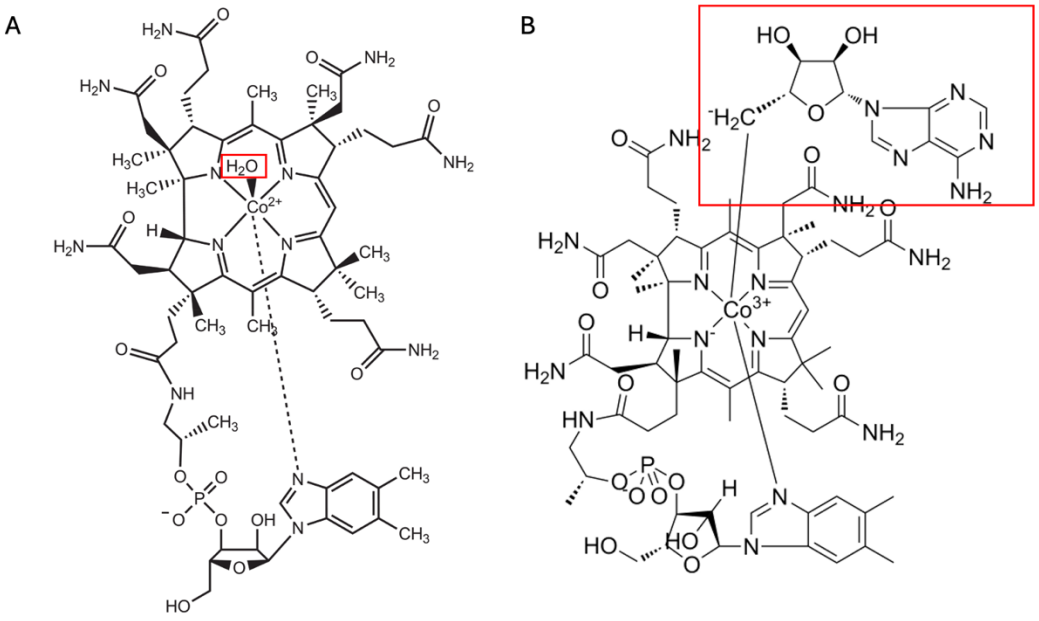

Figure 15: The differences in the upper axial ligand identities of AqCbl (A) and AdoCbl (B). Though the entire cobalamin molecules are shown, the red boxes are indicative of the upper axial ligand, demonstrating the drastic size difference between that of AqCbl and of AdoCbl (Derevenkov et al., 2020).

Due to the apparent lack of ligand binding to Rib 1, the remainder of the characterization of *B. thetaiotaomicron*'s riboswitches focused on Rib 2, which repeatedly demonstrated behavior indicative of ligand binding, particularly with AqCbl. As such, both Native-PAGE gels tested the various annealing conditions outlined in Table 3 to determine which would yield a solution with the highest level of homogeneity. An inhomogeneous solution of mRNA is one which includes the same molecule that has been folded into numerous different conformations, only one of which is biologically relevant. As a result, any spectroscopic data gathered using a heterogeneous solution is more likely to produce incorrect spectra or data that does not accurately represent the true biological structure of the molecule. Both the 6% and 10% acrylamide Native-PAGE gels showed similar results, with the results of the 6% gel seeming

more reliable due to the fact that the samples were unable to move very far through the 10% gel as a result of the high percentage of acrylamide and the shorter runtime. Nevertheless, the 6% gel demonstrated uniformity across all 11 samples, which seemed to suggest that the annealing condition of the mRNA did not impact its folding. This result was unexpected, as the variables that were manipulated during annealing (denaturing temperature, method of cooling, and presence or absence of $MgCl_2$) are all factors that ordinarily have an impact on the folding of mRNA. In particular, the presence of magnesium ions during annealing functions to stabilize the tertiary structure of RNA by neutralizing the negatively charged phosphate backbone (Leipply & Draper, 2010). The 1 mM concentration of $MgCl_2$ added into solution was intended to mimic the cellular concentrations of Mg^{2+} ions that assist with mRNA folding, meaning that the absence of $MgCl_2$ should have had a more significant impact on the homogeneity of the riboswitch.

In an effort to further investigate the affects, or lack thereof, of magnesium ions on the folding of Rib 2, further spectra were taken of the riboswitch with titration of AqCbl. Since this ligand is known to bind to the riboswitch, the presence or absence of similar spectral changes would indicate whether the absence of $MgCl_2$ truly had no impact on the structure of Rib 2. The same protocol was followed, with the exception of the addition of $MgCl_2$, and the results indicated that AqCbl was no longer binding to the riboswitch when it was annealed in the absence of magnesium (see Figure 14).

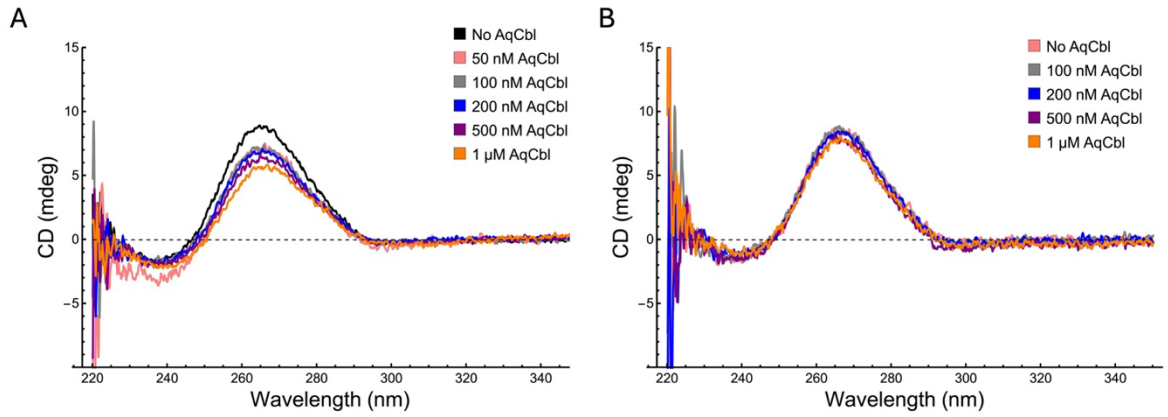

Figure 16: The CD spectra of riboswitch 2 (Rib 2) in the presence and absence of $MgCl_2$. (A) The change in CD exhibited by Rib 2 with the addition of AqCbl in the presence of $MgCl_2$ implies that the ligand is binding to the mRNA. (B) The CD exhibited by Rib 2 upon titration of AqCbl in the absence of $MgCl_2$, suggesting its necessity for proper ligand binding.

The lack of a spectral change upon addition of AqCbl up to concentrations of 1 μM resembles that seen in Figures 9A and 10A and is indicative of no structural changes due to ligand binding. In combination with the data extrapolated from the Native-PAGE gel, it is inferred that the absence of $MgCl_2$ during annealing induces a slight conformational change that, while not large enough to be distinguishable on a gel, is significant enough to prevent the ligand from accessing the aptamer domain despite its small upper axial ligand. Due to the breadth of the Native-PAGE gel, it is unlikely that a minute conformational change would be sufficient to separate the incorrectly folded from the correctly folded riboswitch by size, providing an explanation as to why no additional bands were seen when samples were annealed without $MgCl_2$.

The crystal structures of Rib 1 and Rib 2 are currently unknown, though their true structures would be exceedingly beneficial in determining the structural limitations that prevent AdoCbl from binding to both riboswitches and prevent AqCbl from binding to Rib 1. Moreover, these crystal structures would allow for closer examination of the aptamer domain and would facilitate further investigations into the conformational changes induced by the presence and

absence of $MgCl_2$ that impacts ligand binding. Future studies would likely involve the examination of possible conformational changes induced by other cobamide variants, such as cyanocobalamin (CNCbl), a synthetic corrinoid often found in dietary supplements with an upper axial ligand similar in size to that of AqCbl. Furthermore, future research into the kinetics of cobalamin binding would provide further insight into the binding affinity of *B. thetaiotaomicron*'s riboswitches for various cobamides, supplying further detail into how readily the mRNA binds to cobalamin in a biological setting.

The aim of this thesis was to begin the characterization of previously under-researched aspects of the Btu system in *B. thetaiotaomicron*, a member of the human gut microbiome whose importance cannot be overstated. From the data acquired, it can be inferred that, while Rib 1 exhibits little to no affinity for two of the most biologically relevant forms of cobalamin, Rib 2 appears to demonstrate a preference for AqCbl, likely due to its small upper axial ligand. It was also shown that, although various annealing conditions appeared to have no impact on the folding of Rib 2, the absence of $MgCl_2$ in solution results in an inability for the riboswitch to bind to AqCbl, implying that the presence of magnesium induces a structural change that is slight but significant for ligand binding.

Glossary

Agarose gel: a porous gel used to separate DNA or RNA fragments based on their size by inducing an electric field that attracts negatively charged molecules toward the positive electrode.

Anneal: the process by which single-stranded RNA molecules form molecular interactions, facilitated by a drop in temperature after heating.

Aptamer domain: the region of a riboswitch that binds to a ligand, leading to a change in the structure of the entire riboswitch.

ATP: adenosine triphosphate. A molecule which stores and releases energy used for cellular processes. Often referred to as the energy currency of the cell.

Catalytic cycle: a series of chemical reactions in which a catalyst is regenerated, allowing it to repeatedly participate in the same reaction.

Chaperone protein: a protein that assists with the folding, refolding, and translocation of another protein within the cell.

Circularly polarized light: light in which the electric field vector rotates in a circular path as the light moves. Either clockwise or counterclockwise.

Cleavage site: the specific region in a molecule recognized by an enzyme that serves to cleave or cut the molecule into two or more parts.

Cobamide: a family of molecules characterized by a ring enclosing a cobalt ion, the most well-known of which is cobalamin.

Cofactor: a compound whose presence is essential for the activity of an enzyme, though it is not used up in the chemical reaction.

Commensal: an organism associated with another organism, in which one benefits and the other derives neither benefit nor harm.

Corrinoid: a family of cyclic compounds whose structure is derived from a central corrin ring, the most well-known being cobalamin.

Cytoplasm: the liquid that composes the inside of a cell.

Denature: disruption of the characteristic structural properties of a molecule.

De novo synthesis: the process of building complex molecules from the much simpler building blocks found within the cells of living organisms.

Dialysis: a step in protein purification in which a semi-permeable membrane is used to separate large macromolecules (such as proteins) from much smaller molecules.

Electroelution: a technique used to remove DNA or RNA from a gel after electrophoresis through further application of an electric field that drives the molecule out of the gel entirely.

Encode: provide instructions for creating a specific protein or part of a protein.

Enteric nerve: a neural network within the gastrointestinal tract that regulates digestion and absorption of nutrients.

Essential amino acid: an amino acid which cannot be synthesized by an organism but is required for its survival.

Eukaryote: single-celled or multicellular organisms characterized by the possession of a nucleus and membrane-bound organelles.

Expression platform: the region of a riboswitch that changes its structural conformation upon the binding of a ligand to the associated aptamer domain.

Folic acid cycle: a cycle by which one-carbon units are transferred between biomolecules. Requires folate and plays a vital role in cellular processes such as energy production and protein synthesis.

Genomic DNA: the total genetic material comprising an organism.

Gram-negative: bacteria characterized by the color that they turn upon Gram staining. Indicative of an inner and an outer cell membrane surrounding the entire cell.

Heterogeneous: diverse in content, containing several molecules of various identities or conformations of the same molecule.

Homogeneous: uniform in content, containing one molecule in the same conformation.

Hydrolysis: the process by which ATP (adenosine triphosphate) is broken down into ADP (adenosine diphosphate) and an inorganic phosphate molecule, releasing energy that can be used for cellular processes.

Intrinsic factor: a protein produced by cells lining the stomach that is required for the absorption of vitamin B_{12} in the small intestine.

Ligand: a molecule that binds to another (usually larger) molecule.

Ligation: the joining of two DNA fragments.

Lipoprotein: proteins that are anchored to the outer membrane of a cell.

Locus: a specific location of a gene on a chromosome.

NADPH: an essential electron donor present in all living organisms. Provides power for anabolic reactions.

Native-PAGE gel: a technique used to separate proteins based on size while maintaining their native, folded structures. Uses an applied electric field similar to that used for agarose gels.

Open reading frame: the portion of a DNA sequence that will be transcribed into RNA and further translated into protein.

Oxidation reaction: a reaction in which a molecule loses electrons. Always coupled with a reduction reaction.

Parietal cells: cells within the stomach lining that produce intrinsic factor and HCl to maintain the low pH of the stomach.

Periplasmic space: the space between the outer membrane and the inner membrane in gram-negative bacteria.

Phosphate backbone: the structural framework of DNA and RNA composed of sugar and phosphate groups. Negatively charged.

Plasmid: small, circular DNA strands that can replicate independently of chromosomes in a bacterium. Commonly found in bacteria and protozoa.

Polyacrylamide-urea gel: an acrylamide gel similar to that used in a Native-PAGE gel, with the exception of the inclusion of urea, which unfolds the DNA or RNA, disrupting their native structures.

Polymerase chain reaction: a technique used to rapidly amplify a specific DNA sequence, creating millions or billions of copies from small amounts of DNA.

Polyphenol: a family of naturally occurring compounds containing multiple phenol rings. Found in fruits, vegetables, and herbs.

Primer: a short, single-stranded DNA sequence that provides a starting point for the synthesis of DNA.

Prokaryote: a single-celled organism characterized by its lack of a nucleus and distinct, membrane-bound organelles.

Reduction reaction: a reaction in which a molecule gains electrons. Always coupled with an oxidation reaction.

Restriction digestion: a process by which specialized enzymes cut DNA molecules at designated sites, producing fragments with defined ends that can be ligated together in the future.

Ribosome binding site: a sequence on a strand of mRNA that is detected and bound by ribosomes, which initiate the production of protein.

Specificity: an enzyme's ability to bind to one molecule over another, preventing unwanted reactions.

Tertiary structure: the overall, three-dimensional shape of an RNA molecule, characterized by long-range interactions between various regions of the molecule.

Tetrapyrrole ring: a chemical structure consisting of four pyrrole rings linked together in a cyclic manner.

Transcription: the process by which DNA is turned into mRNA by the enzyme RNA polymerase.

Transformation: a process by which a cell takes up and incorporates foreign DNA, typically from a plasmid, into its own genome. Can occur naturally or be induced in a laboratory setting.

Translation: the process by which mRNA is turned into protein by ribosomes.

Untranslated region: portions of mRNA that are not translated into protein. Serve to regulate expression of the genes that will be translated.

Vector: an engineered DNA molecule used to carry a specific DNA segment into a host cell.

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