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AN INVESTIGATION OF THE EFFECTS OF CORRINOID STRUCTURE ON THE METHANOGENESIS PATHWAY IN *METHANOSARCINA BARKERI* STRAIN *FUSARO*

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To the Graduate Council:

I am submitting herewith a thesis written by Amanda Marie Kennedy entitled "AN INVESTIGATION OF THE EFFECTS OF CORRINOID STRUCTURE ON THE METHANOGENESIS PATHWAY IN *METHANOSARCINA BARKERI* STRAIN *FUSARO*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Frank Loeffler, Major Professor

We have read this thesis and recommend its acceptance:

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Vice Provost and Dean of the Graduate School

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AN INVESTIGATION OF THE EFFECTS OF CORRINOID STRUCTURE ON THE METHANOGENESIS PATHWAY IN *METHANOSARCINA BARKERI* STRAIN *FUSARO*

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ABSTRACT

Methane is responsible for at least 10% of greenhouse gas emissions in the United States alone, and although not the most abundant, it is one of the most dangerously potent greenhouse gases. Methanogens are microbial organisms that require a corrinoid cofactor to synthesize methane.

Previous work in bacteria has demonstrated that corrinoid mediated processes, such as methyl transfers, are directly influenced by the structure of the corrinoid cofactor[1; 2; 3]. Individual organisms show bias or preference for one structure over another, with the sole difference being the lower ligand attached to the corrin ring[4]. Most organisms have a “preferred” corrinoid structure, and when this structure is not available, are forced to use an alternative corrinoid. In some cases, the alternative corrinoid can slow down the organisms metabolic process, while in other cases, the alternative corrinoid cannot be used at all. Availability of corrinoids and their precursors have serious implications on microbial metabolism, and the ability of a microbe to occupy its niche[5].

In methanogenesis, the corrinoid enzyme complex involved in the methyl transfer varies based on the carbon starting material:

In the acetate and CO_2 pathways, the methyl group of methyl-tetrahydramethanopterin (methyl-H_4SPT) is transferred to an 8-subunit transmembrane protein. Subunit A contains the corrinoid prosthetic group.

In the methanol conversion pathway, the corrinoid cofactor is part of a cytoplasmic enzyme complex.

Unlike most organisms requiring corrinoid cofactors, methanogens can synthesize their own lower bases. However, if there are other lower bases present in the environment, they will use those instead. Due to the importance of corrinoid structure to microbial metabolism, we believe that corrinoid structure in methanogens could affect methanogenesis.

Synthetic lower bases could affect the ability of the corrinoid cofactor to properly bind to its enzyme/protein complex, thus affecting the ability for the remaining two steps of the methanogenesis pathway to occur. Finding a way to slow methane production has potential applications in the agricultural industry, which could lower our contributions to global warming.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE INTRODUCTION AND GENERAL INFORMATION

Corrinoid Role in Reductive Dechlorination

Chlorinated solvents (e.g., chlorinated ethenes) are prevalent toxic groundwater contaminants, therefore, research groups have prioritized the remediation of contaminated groundwater sites [6]. The goal of this work was to find the most efficient and cost-effective way to prevent human exposure to contaminated water.

Only *Dehalococcoides mccartyi* (*Dhc*) strains have been documented in complete detoxification of chlorinated ethenes to the harmless product ethene, making *Dhc* an important mediator in bioremediation of chlorinated hydrocarbons[7; 8]. The corrinoid cofactor B12, or a closely related B12 analog, is required for growth in *Dhc,* and is an essential cofactor of reductive dehalogenases, which are the key enzymes catalyzing reductive dehalogenation reactions[9; 10]. A variety of B12 analogs exist, generally differing by the structure of the lower ligand. In B12 the lower ligand is 5,6-dimethylbenzimidazole (DMB) (Figure 2). Corrinoids are composed of three structural units: The upper ligand, the corrin ring, and the lower ligand (Figure 2). The central cobalt atom of the corrin ring is covalently tethered to the lower ligand via a nucleotide loop [11]. The main structural difference between the variety of known corrinoid cofactors is in their lower ligand, and a variety of these are found in nature, such as purines, phenolic compounds and benzamidazole bases [4].

Dhc cannot synthesize its own corrinoid and must rely on other microoganisms in the environment to produce and provide this cofactor[9]. A handful of microbes have the ability to synthesize corrinoids. However, the majority of microbes do not. In the absence of B_{12} synthesizing microorganisms *Dhc* can be supplemented with B_{12} to facilitate reductive dechlorination[12] (Figure 1).

Since *Dhc* is incapable of B_{12} synthesis, it must acquire corrinoids produced by other organisms. However, previous research demonstrates that corrinoid structure significantly impacts dechlorination activity[2; 12; 13]. *Dhc* shows a specific preference for the B_{12} type corrinoid, and demonstrates reduced rates or incomplete dechlorination activity when supplemented with corrinoids containing a lower base other than DMB[3; 13]. (Figure 3).

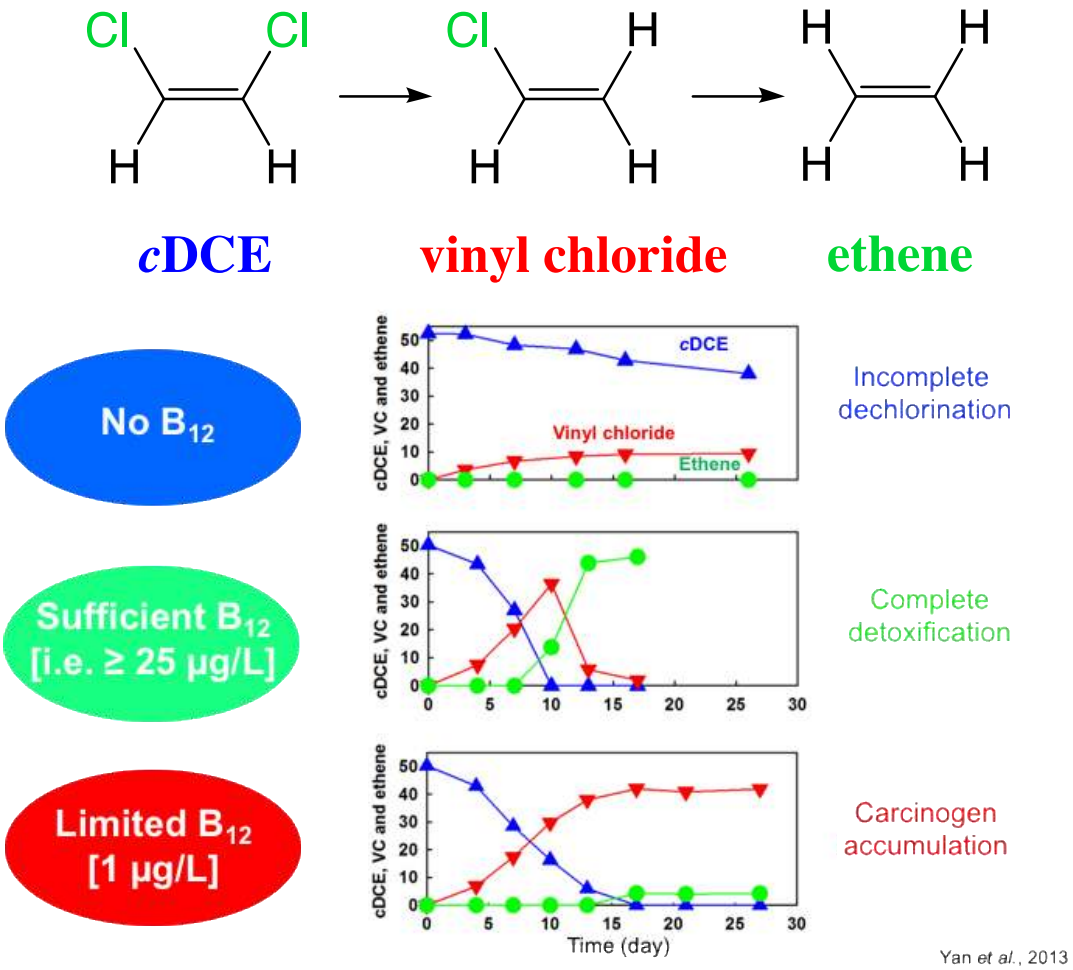


Figure 1. Dhc Corrinoid requirement.

Dhc **requires corrinoids (ie. Vitamin B_{12}) in order to perform reductive dechlorination of cis-dichloroethene (cDCE) to ethene. When there is no B_{12}, there is no dechlorination. In the presence of sufficient (>25 µg/L) quantities of B_{12}, cDCE is completely dechlorinated to non-toxic ethene. However, when B_{12} is present, but not in a sufficient quantity(<25µg/L), there is incomplete dechlorination, leading to a toxic accumulation of vinyl chloride.**

Figure 2. General Corrinoid Structure.

The general corrinoid structure comprises of three main components: (1) the corrin ring with a cobalt center, (2) the upper ligand (green X), and (3) and the lower base (blue). There are 16 naturally occurring lower bases, and their structures are shown in the shaded boxes.

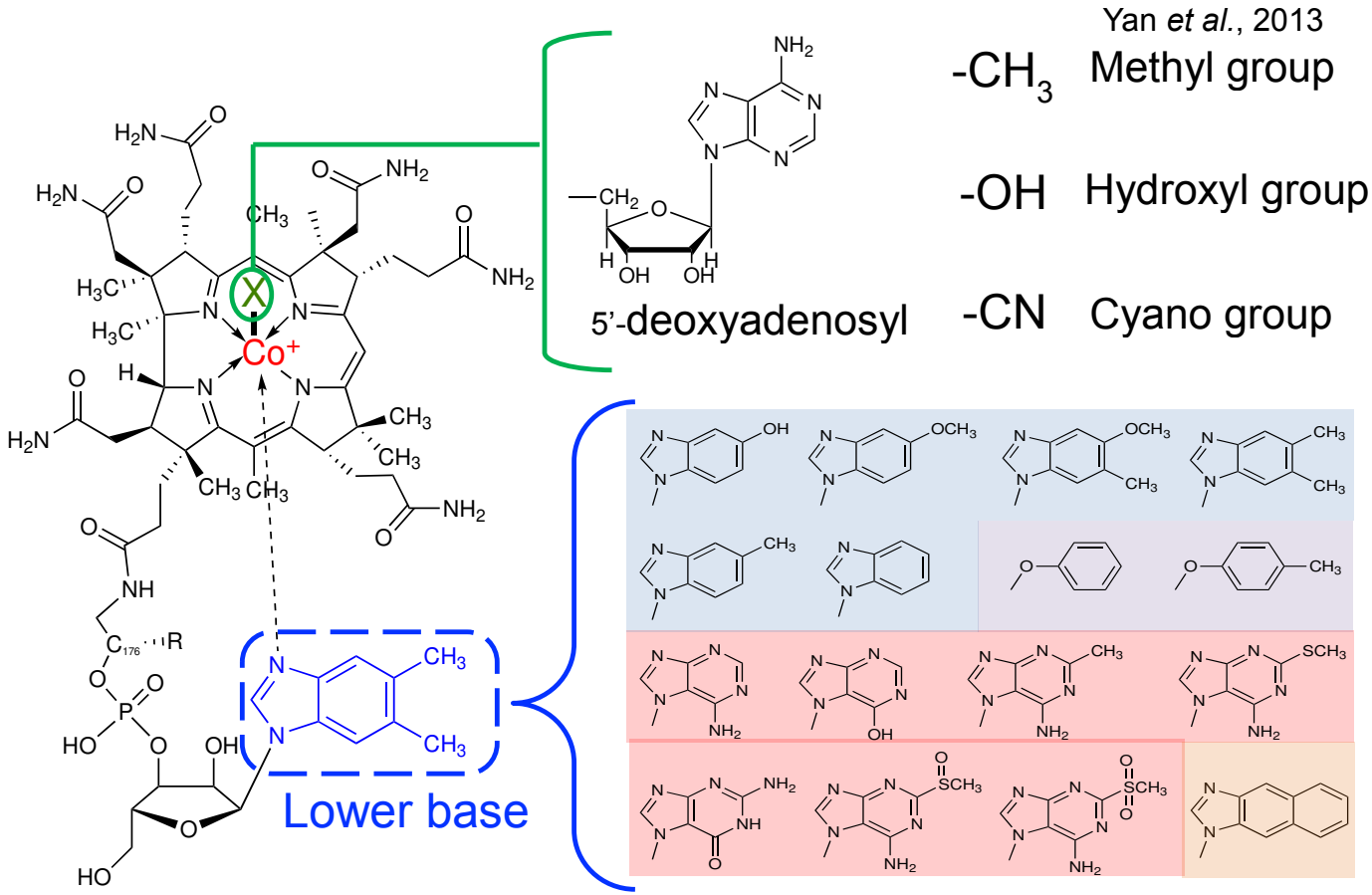
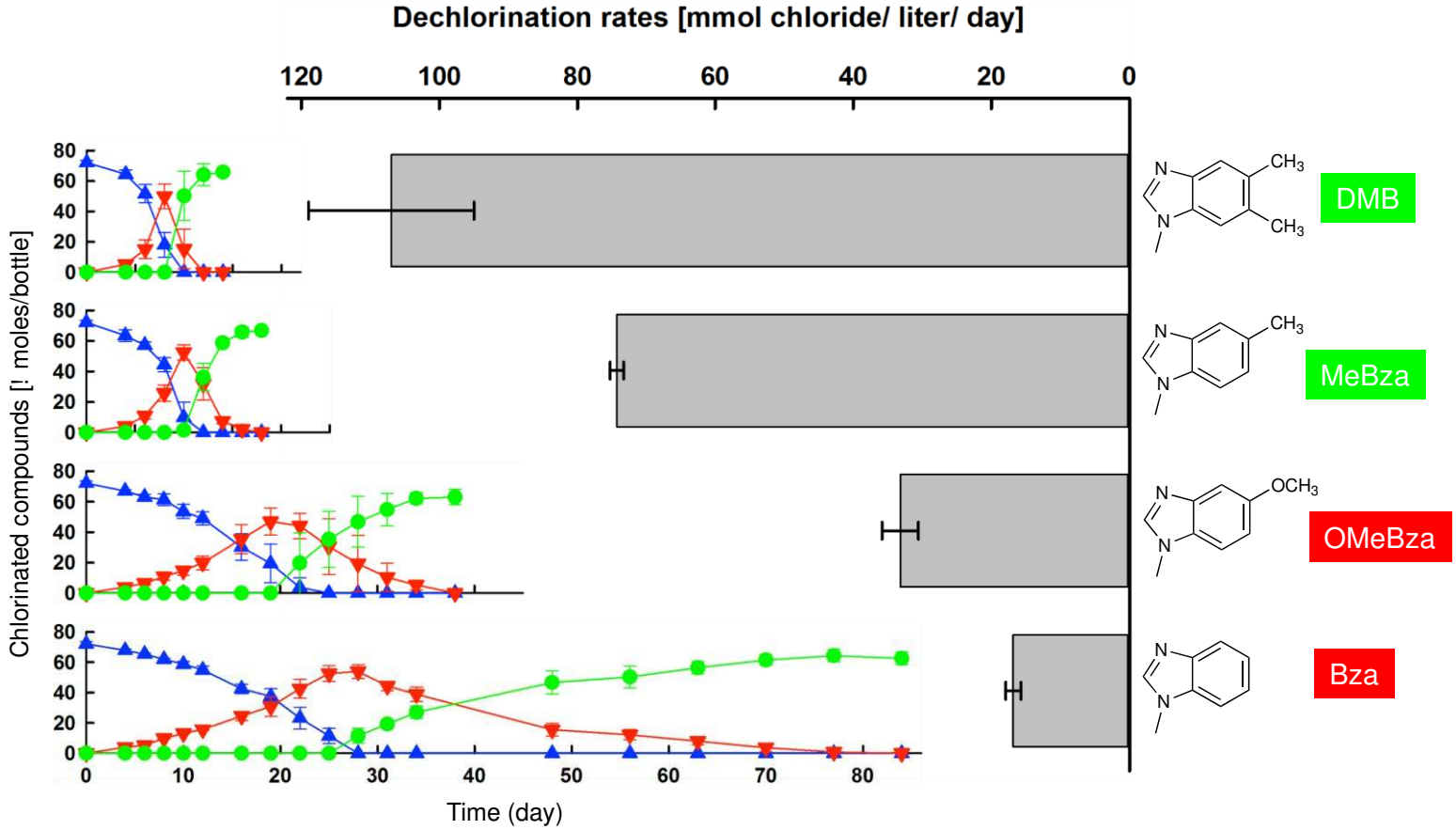

Yan *et al.*, 2013
-CH3 Methyl group
-OH Hydroxyl group
-CN Cyano group
5'-deoxyadenosyl
Lower base

Figure 2.

Figure 3. *Dhc* dechlorination depends on corrinoid structure.

When *Dhc* is supplemented with B12, which has dimethylbenzimidazole (DMB) as its lower base, the highest rates of dechlorination are achieved. However, when *Dhc* cultures are supplemented with corrinoids composed of other lower bases, such as methylbenzimidazole (MeBza), or benzimidazole (Bza), dechlorination occurse at much slower rates.



Yan *et al.* 2016

Figure 3.

Methane and Methanogens

Dhc is not the only microbe possessing a corrinoid-dependent metabolic pathway. **Corrinoids are required by a wide variety of metabolisms, leading us to hypothesize that other organisms that require corrinoid cofactors may also show bias toward one type of corrinoid over others.** One particularly important area of interest for this research is the corrinoid-dependent production of methane by methanogens.

Methanogens carry out methanogenesis, a process estimated to be responsible for more than 10% of greenhouse gas emissions in the United States alone. Methane is estimated to be 25 times more impactful on climate change than the most abundant greenhouse gas, carbon dioxide [14].

Most methane emissions are a result of human activity, stemming from industry, agriculture and waste treatment sources. More than 20% of total methane emissions can be attributed to rumen enteric fermentation, mostly as a result of cattle farming [14]. Microorganisms that produce methane within the rumen are called methanogens, which make up a diverse family of archaea. Methane production, or methanogenesis, is a complex metabolic pathway present only in methanogens. Methanogens can produce methane from several carbon compounds, such as carbon dioxide, methanol and acetate. Regardless of the substrates, all of the methanogenesis pathways intersect at one common step (boxed in red, Figure 4). This step is the methyl transfer step, which can be

divided into two sub-steps. First, the single carbon unit (methyl group) is transferred to a corrinoid cofactor. Then, the methyl group is transferred to coenzyme M, and subsequently reduced to methane [15]. Since the methyl transfer step is conserved across all methanogens, it is a target in the methanogenesis pathway for studies designed to manipulate methanogenesis.

Corrinoids

The corrinoid-dependent methyl transfer step of methanogenesis is not unique to methanogens. Corrinoid-dependent reactions appear in a variety of metabolic processes in all three domains, yet corrinoids are only produced by a small group of prokaryotes[16; 17]. Vitamin B_{12} is a corrinoid cofactor in pathways such as methionine synthesis in humans, reductive dechlorination in the bacteria *Dehaloccocoides mccartyi* [2; 18] and methanogenesis. An estimated 75% of bacteria have corrinoid dependent enzymes, but only half of these bacteria can produce corrinoids *de novo*[19; 20]. Previous work in the human gut microbiome discovered that many bacteria have multiple copies of corrinoid transporters, and although they appear to be redundant, they have very distinct preferences for different corrinoids[21]. Corrinoids cannot be synthesized by eukaryotes, and must be ingested as a dietary component. In many cases, bacteria living in our body synthesize the vitamins we need for our metabolic processes, creating an important symbiotic host-microbiome relationship [22; 23; 24].

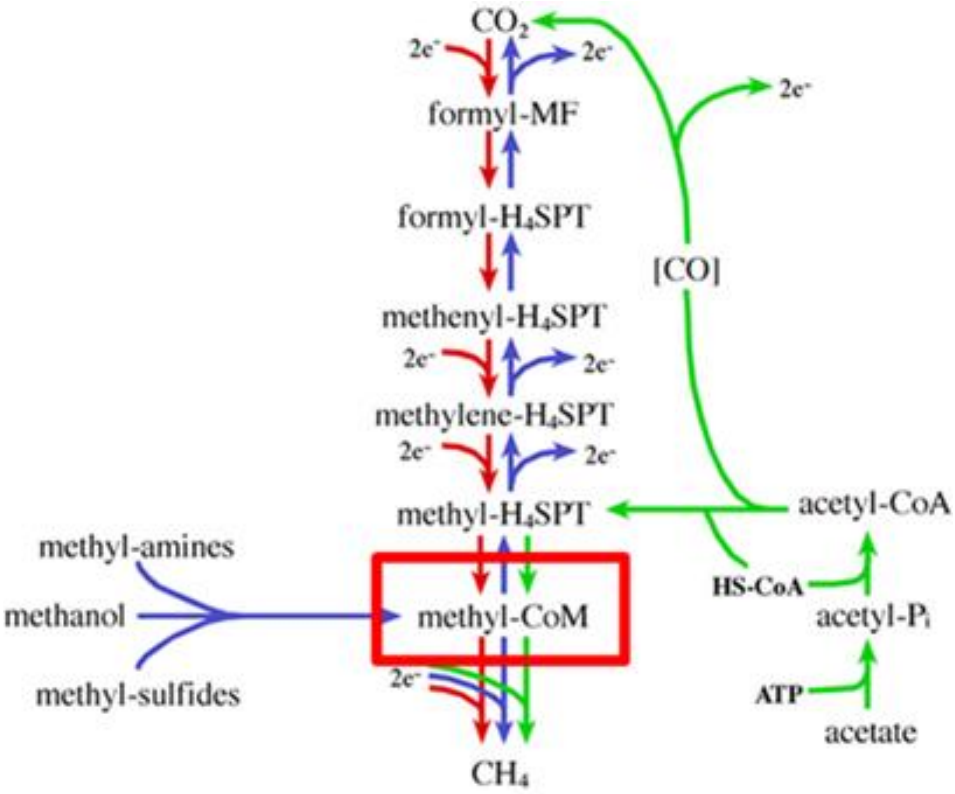


Figure 4. Methanogenesis Pathway.

Methanogenesis pathway with various substrates. Boxed in red is the methyl transfer step. This step of the pathway is shared by all methanogens, regardless of their species, or starting carbon material.

This symbiotic relationship also incites competition within the microbial community for selected corrinoids, as they can only be synthesized by a small subset of bacteria and archaea, but are a nutritional requirement for almost every organism on the planet [11; 21; 25]. Therefore, corrinoid cross-feeding is present in almost every system, and may be a result of the advantage of acquiring the metabolically expensive cofactors from the environment, as opposed to the lengthy ~30 step corrinoid biosynthetic pathway [26; 27].

There are three ways an organism may obtain the corrinoids it needs. Corrinoids can be synthesized by the organism itself, but this method is limited to a small subset of the microbes. Another option is the selective import of corrinoid compounds into the cell, which allows microbes to take up corrinoids from its environment. The third option is remodeling of an existing corrinoid by the microorganism. Remodeling can occur in several ways; starting with the import of a whole corrinoid from the environment, or the uptake of corrinoid precursors. After importing a whole corrinoid, the organism can simply “remodel” the cofactor by removing the lower base and replacing it with a different one. The alternative option is to take up corrinoid precursors, then assemble them with the desired lower base. These lower bases may be synthesized by the organism or picked up from the environment.

Corrinoid Biosynthesis

The synthesis of vitamin B_{12} involves the synthesis of individual pieces of the cofactor, followed by their assembly, which is commonly seen in the

biosynthesis of vitamins. The main component of vitamin B_{12} is the corrin ring, which is synthesized from uroporphyrinogen III (UroIII), a precursor common to heme. After synthesis of the corrin ring, the central cobalt is added, followed by the addition of an aminopropanol side chain. Lastly, the lower ligand is added to the side chain to form a nucleotide loop that coordinates with the central cobalt center.

Corrinoid Uptake

Although selective transport of specific corrinoids in bacteria has not been explored, it may play a role in ensuring that bacteria take up the corrinoids they need for their metabolism. Corrinoid transporters are encoded in ~ 76% of bacterial genomes [20]. Previous work in the human gut microbiome revealed that many bacteria have multiple copies of corrinoid transporters, and although they appear to be redundant, they may have distinct preferences for different corrinoids [28]. Thus, it may advantageous to have multiple corrinoid transporter systems to allow selective uptake of preferred corrinoids based on the corrinoids present in the environment.

Corrinoid Remodeling

The alternative to selectively transporting corrinoids into the cell is corrinoid remodeling, or the removal of the lower ligand of an imported corrinoid and replacement with another lower ligand [29]. This strategy allows bacteria to take advantage of the already-assembled corrinoid structures available in the environment. This strategy is seen in *Dhc,* which requires corrinoids to perform

reductive dechlorination. *Dhc* can only use three types of corrinoid structures to successfully carry out its metabolic processes, so, even if the “preferred” corrinoid is not present in the environment, the bacteria are able to take up an existing corrinoid structure, and replace the lower ligand with one that *Dhc* can use [30; 31]. Corrinoid remodeling can also be performed by bacteria capable of producing corrinoids *de novo*[29]. This strategy allows the corrinoid producers to save the energy they would expend in the long, metabolically expensive process of corrinoid biosynthesis. Allen and Stabler have observed this energy-saving behavior in the human gut, where changes in fecal corrinoid profiles differed prior to, and post-ingestion of cobalamin supplements [32].

Role of Corrinoids in Methanogenesis

Previous work in bacteria has demonstrated that mediated processes, such as methyl transfers, are directly influenced by the structure of the corrinoid cofactor [1; 2]. Individual organisms show bias or preference for one type of cofactor over another, with the sole difference being the lower ligand attached to the corrin ring [4]. Most organisms have a “preferred” corrinoid structure, and when this structure is not available, are forced to use a non-ideal corrinoid instead [1; 2]. In some cases, a non-ideal corrinoid can slow down metabolic processes in the organism, while in other cases, the non-ideal corrinoid cannot be used at all. Availability of corrinoids and their precursors have serious implications on microbial metabolism, and the ability of a microbe to occupy its niche [5]

Due to the importance of corrinoid structure in microbial metabolism, we hypothesize that the structure of the corrinoid cofactor produced by methanogens could have a direct impact on the rate of methanogenesis, as well as total methane production by the microbe. Therefore, if we supplement methanogens with a lower base, rather than “waste” the energy to manufacture the corrinoid lower base themselves, they will take up the supplemented lower base, and incorporate it into the corrinoid structure. The newly assembled synthetic corrinoid may impact the process of methanogenesis. If we are able to slow down, or reduce total methane production, lower base supplementation may be a solution to lower greenhouse gas emissions. However, if we are able to increase the rate of methanogenesis, or produce more methane, lower base supplementation could be used to generate more methane in a controlled setting for biogas applications.

CHAPTER TWO DOES 5μM LOWER BASE SUPPLEMENTATION IMPACT METHANE PRODUCTION?

Materials and Methods

Chemicals

5,6-dimethylbenzimidaole (DMB), 5-chlorobenzimidazole, and 5,6-dibromobenzimidazole were purchased from Combi-blocks. Methanol was purchased from Sigma Aldrich.

Cultures

Pure *Methanosarcina barkeri* strain *fusaro* cultures were grown in 160 ml serum bottles with 50 ml of minimal salts medium with a vitamin B_{12}-free vitamin mix. Cultures were grown anoxically with a sodium bicarbonate buffer at pH 7.2 and 37 degrees Celsius. Minimal salt media is composed of (g/L): 1 g NaCl, 0.5 g $MgCl_2 \times H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2 \times 2H_2O$. Trace element solution (per liter): 10 ml HCl (25% solution w/w), 1.5 g $FeCl_2 \times 4 H_2O$, 0.19 g $CoCl_2 \times 6H2O$, 0.1 g $MnCl_2 \times 4 H2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4 \times 2 H_2O$, 24 mg $NiCl_2 \times 6 H_2O$, 2 mg $CuCl_2 \times 2 H_2O$. Vitamin solution contains (mg/L): 0.02 biotin, 0.02 folic acid, 0.1 pyroxidine hydrochloride, 0.05 riboflavin, 0.05 thiamine, 0.05 nicotinic acid, 0.05 pantothenic acid, 0.05 *p*-aminobenzoic acid, and 0.05 thioctic acid. Se/Wo solution includes (per L): 6 mg $Na_2SeO_3 \times 5 H_2O$, 8 mg $Na_2WO_4 \times H_2O$ and 0.5 g NaOH. Gas phase was composed of H_2 and CO_2 (90/10). 0.1% resazurin solution was added at 0.25

ml/L medium. 30 mM sodium bicarbonate, 2 mM L-cysteine monohydrate and 0.048 g/L Sodium sulfide x $9H_2O$ were added. Either 40 mM acetate or 5mM methanol was added as electron donor. Sterile-filtered lower bases were added to a final concentration of 5 µM (Figure 5). Treatments were set up in triplicates and included:

(1) Control (no lower base added)(5OHbza)

(2) 5,6-dibromobenzimidazole (5,6Brbza)

(3) 5-chlorobenzimidazole (5Clbza)

(4) 5,6-dimethylbenzimidazole (DMB)

Methane Measurements

Methane production was measured at regular intervals with an Agilent 7890 gas chromatograph equipped with FID (flame ionization detector) and DB-624 capillary column (60 m x 0.32 mm x 1.8 µm). 100 µl headspace samples were withdrawn using disposable syringes and manually injected into a split injector with a split ratio of 2:1. The GC inlet was maintained at 200 °C, and the GC oven temperature was kept at 60 °C for 2 min then increased to 200 °C at a rate of 25 °C min−1, and the FID detector was operated at 280 °C as described as described by [33].

Methanol Measurements

Consumption of methanol by methanogens to produce methane was measured via an autosampler attached to a gas chromatograph.

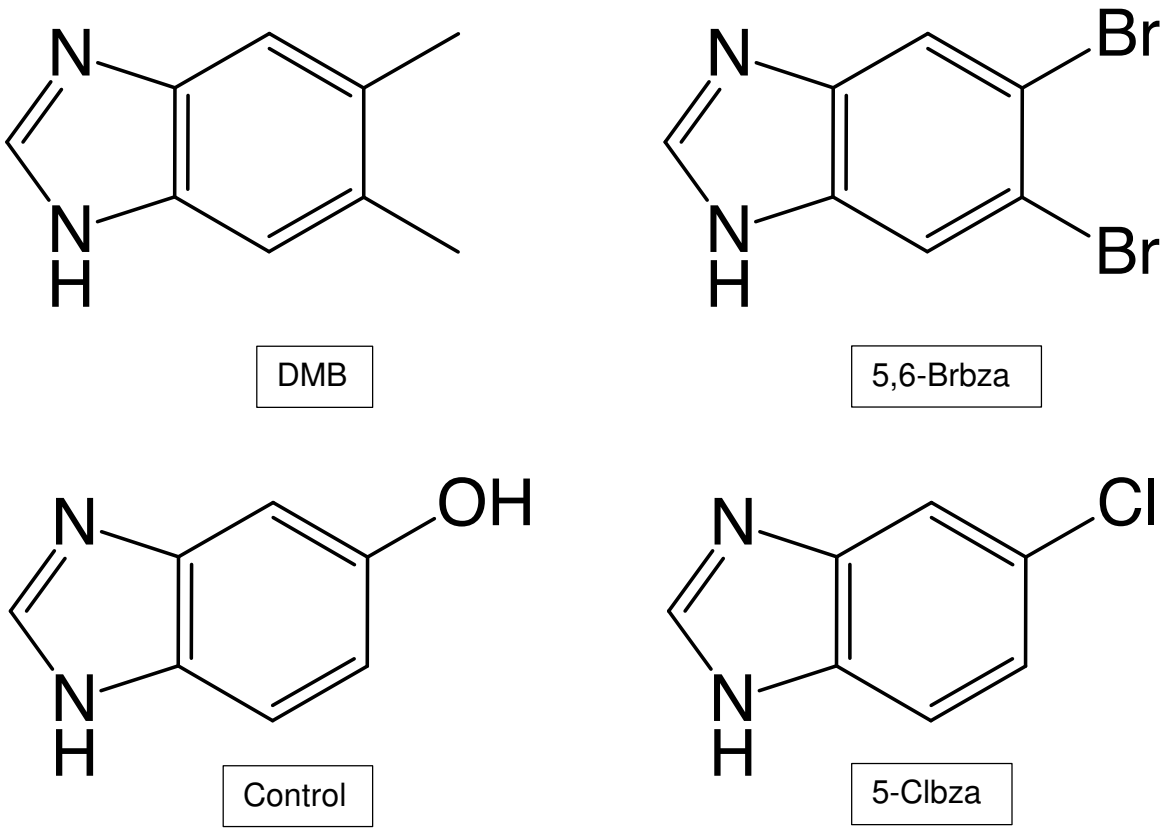


Figure 5. **Lower base structures.**

Lower base structures for each treatment. Each bottle contained 5 µM concentration of one lower base.

Acetate Measurements

Acetate was analyzed using an Agilent 1200 series HPLC system including an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a UV detector at 210 nm. The column temperature was set to 30 °C, and the eluent (4 mM H_2SO_4) flowed at a rate of 0.6 mL min^{-1}. Samples for HPLC analysis were acidified to a final concentration of 4 mM H_2SO_4 prior to HPLC analysis.

Corrinoid Extraction and Purification

Cells were harvested to extract corrinoids and determine which corrinoids were produced by methanogens when supplemented with lower bases. The procedure was carried out as described by [34]. Cultures were centrifuged (Thermo Scientific sorcall Rc 6+) at 10,000 RPM for 20 minutes at 4°C. Supernatants were removed, and cell pellets were re-suspended in 5 ml deionized water. pH was then adjusted to 5-6 with 3% glacial acetic acid and 10 mM KCN, and boiled for 20 min in a water bath. Following the water bath, cultures were centrifuged again for 20 minutes at 10,000 RPM. Supernatants were collected, and cell pellet was extracted again. Supernatant fluids were combined and mixed with 0.01 volumes of acetic acid, then loaded into Sep-Pak C18 cartridges. Cartridges were first equilibrated with 2 ml of pure methanol and 60ml deionized water. Supernatant was pushed through column, and corrinoids were collected by washing the column with 3ml pure methanol, and collecting the pink-colored fluid to be vacuum dried. After vacuum drying was complete, samples were resuspended in 0.3 ml sterile deionized water in preparation for HPLC analysis.

HPLC Analysis

Analysis of corrinoids was performed with an Agilent 1200 series HPLC system equipped with an Eclipse XDB-C18 column (5 mm, 4.6 × 250 mm) and a diode array detector set at a detection wavelength of 361 nm. Samples were injected and separated at a flow rate of 1 mL per min at 30°C using 0.1% (v/v) formic acid (≥88%, w/v) in water (eluent A) and 0.1% (v/v) formic acid in methanol (eluent B) as mobile phases. The column was equilibrated with 82% eluent A / 18% eluent B, and a linear change to 75% A / 25% B was applied after sample injection over a time period of 12 minutes. Eluent composition then decreased immediately to 25% A / 75% B over 3 minutes, followed by a 5 minute hold. The column was then equilibrated to initial conditions.

qPCR Assay

To assess the number of methanogens in each treatment, quantitative PCR (qPCR) was performed. DNA was filtered by running 3ml of culture through a .22 μm Durapore membrane filter (Millipore, Tulagreene, Ireland). DNA was then extracted from filters using the QIAGEN PowerSoil DNA Isolation Kit (QIAGEN, Hilden, Germany). After DNA extraction, DNA was checked for purity and concentration with nandrop and qubit, then diluted 1:100 for qPCR analysis. All samples were run in triplicate, including standard curves and negative controls. Each well included 100 μM forward primer, 100 μM reverse primer, SYBR green master mix (Life Technologies), 2 μl DNA, and the remaining volume nuclease-free water. 96-well plates were then covered with an adhesive

cover, and centrifuged for 30 seconds at 2000 RPM (Sorvall Legend XTR, ThermoScientific). Forward primer Methgen835F-MI2 (5'-GGGRAGTACGKYCGCAAG-3') and reverse primer Methgen918R-MI2 (5'-GAVTCCAATTRARCCGCA-3') were obtained from Integrated DNA Technologies. Data obtained from qPCR (Life Technologies ViiA7, ThermoFisher) was analyzed for relative cell numbers by previously established methods [35].

Results – Methanol Experiments

Methane Production and Methanol Consumption

In *M. barkeri* cultures supplemented with 5 mM methanol and 5 μM lower base, we saw no alteration in methane production, or consumption of methanol by cultures amended with DMB, 5Clbza or 5,6Brbza. Relative rate and total methane production remained the same, regardless of treatment group. (Figure 6). Based on these results, it appears that 5 μM lower base supplementation has no impact on the methanol pathway of methanogenesis in *M. barkeri* cultures.

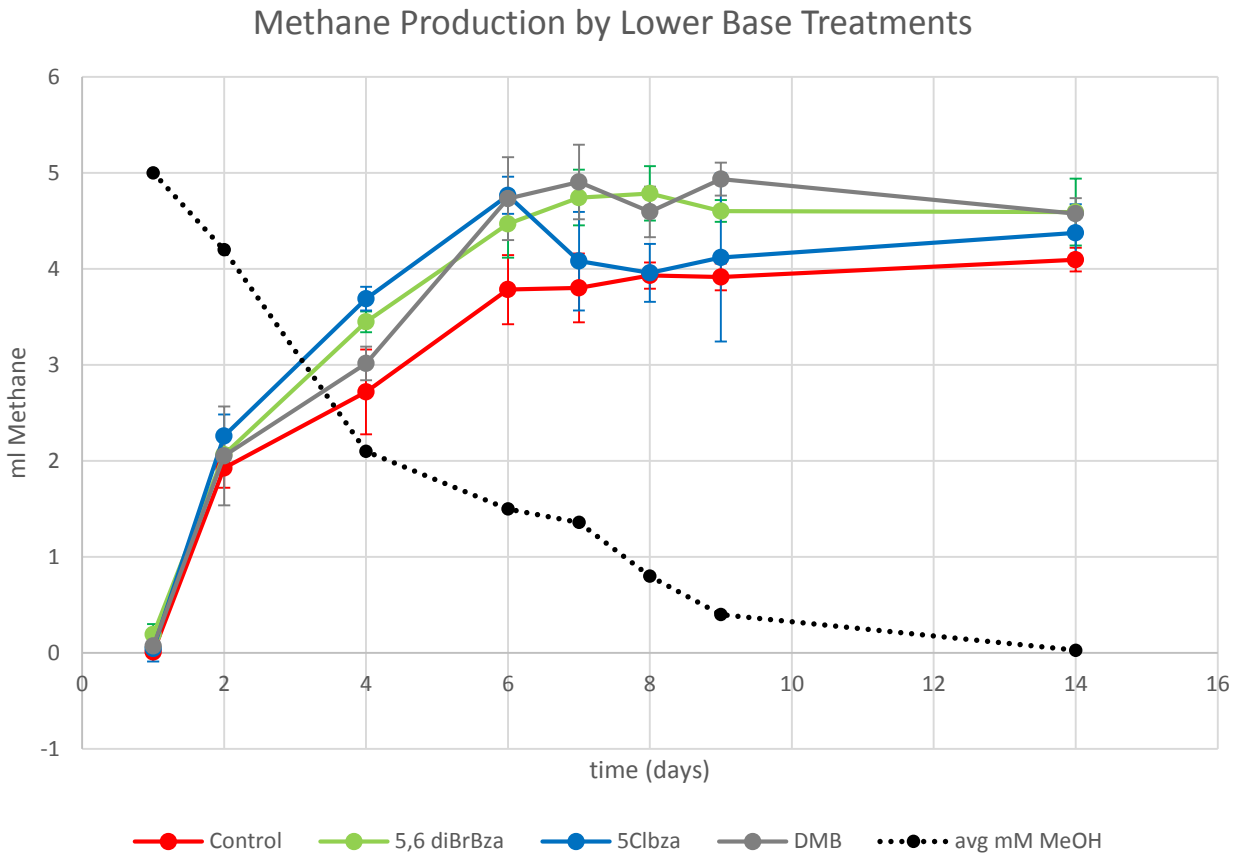


Figure 6. Methane production and methanol consumption by 5 μM lower base treatments.

There was no detectable difference in methane production or methanol consumption in culture supplemented with lower bases compared to the control.

HPLC/Corrinoid Extraction Results

In order to determine whether the 5 μM lower base supplemented to *M. barkeri* cultures caused the production of alternative corrinoid structures, corrinoid extraction and HPLC was performed (Figure 7). In the control cultures (A), we observed the production of a single corrinoid at the retention time ~26.5 minutes. In cultures amended with DMB (B), we see the production of a single corrinoid at the same retention time as the control chromatogram. Without further analysis, it is not possible to determine whether or not this corrinoid is the native corrinoid, however, this is the most likely outcome. In cultures amended with 5,6Brbza (C), we observed a peak that eluted at the same time as our control culture, therefore, it is most likely the native corrinoid that was produced in these cultures. Finally, in cultures amended with 5Clbza (D), we observed the production of two distinct corrinoid peaks, once cross-checked with UV Vis spectra. One peak eluted at the same time as the native corrinoid, and the other peak eluted later in the chromatogram. Without further testing, it is impossible to determine whether or not this peak is a corrinoid with the lower base 5Clbza, however, if we did expect this corrinoid to be produced, we would expect it to elute after the native corrinoid on the chromatogram, so this is certainly a possible explanation.

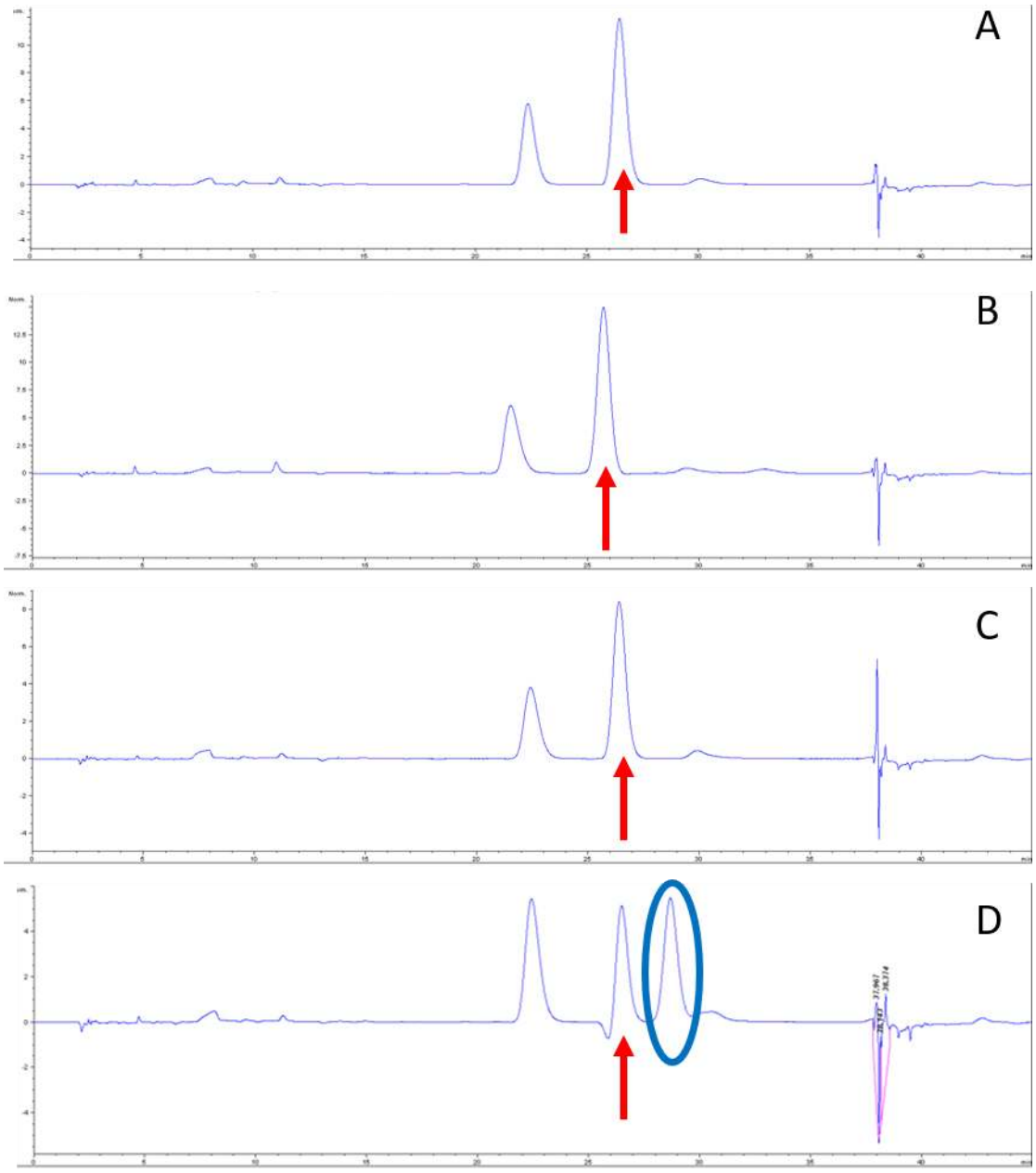


Figure 7. Corrinoids produced by cultures amended with 5μM lower base and 5 mM methanol.

(A) Control, (B) DMB, (C) 5,6Brbza, (D) 5Clbza.

Acetate Results

Methane Production

In *M. barkeri* cultures supplemented with 40 mM acetate and 5 μM lower base, we saw no difference in methane production in comparison with control cultures. Despite the supplemented lower base, there was no difference in rate or amount of methane produced between treatment (Figure 8). These results are consistent with our findings from the methanol pathway experiment.

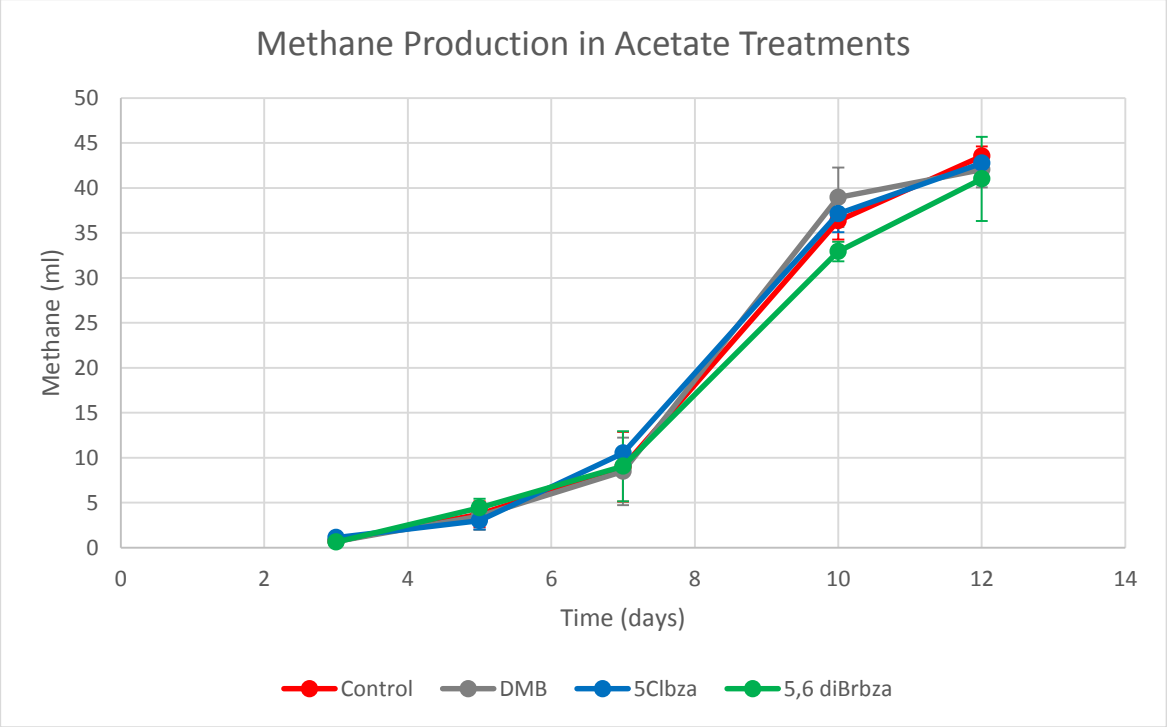


Figure 8. Production of methane in cultures grown with acetate. Despite some cultures containing 5 μM lower base, there was no difference in methane production between the treatments.

HPLC/Corrinoid Extraction

To determine whether the 5 μM lower base supplemented to *M. barkeri* cultures grown in 40mM acetate caused the production of alternative corrinoid structures, corrinoid extraction and HPLC was performed (Figure 9). In control cultures (A), we observed the production of a single corrinoid at the retention time ~26.5 minutes. In cultures amended with DMB (B), we see the production of only one corrinoid at the same retention time as the control chromatogram. Without further analysis, it is impossible to determine whether this corrinoid is the native corrinoid, however, this is the most likely the case. In cultures amended with 5,6Brbza (C), we observed a peak that eluted at the same time as our control culture, therefore, similar to DMB (B) cultures, it is most likely the native corrinoid that was produced in these cultures. Finally, in cultures amended with 5Clbza (D), we observed the production of two individual corrinoid peaks. One peak eluted at the same time as the native corrinoid, and the other peak eluted later in the chromatogram. Without further analysis, it cannot be determined whether or not this peak is a corrinoid with the lower base 5Clbza, however, if we did expect this corrinoid to be produced, we would expect it to elute after the native corrinoid on the chromatogram, so this is certainly a possible explanation.

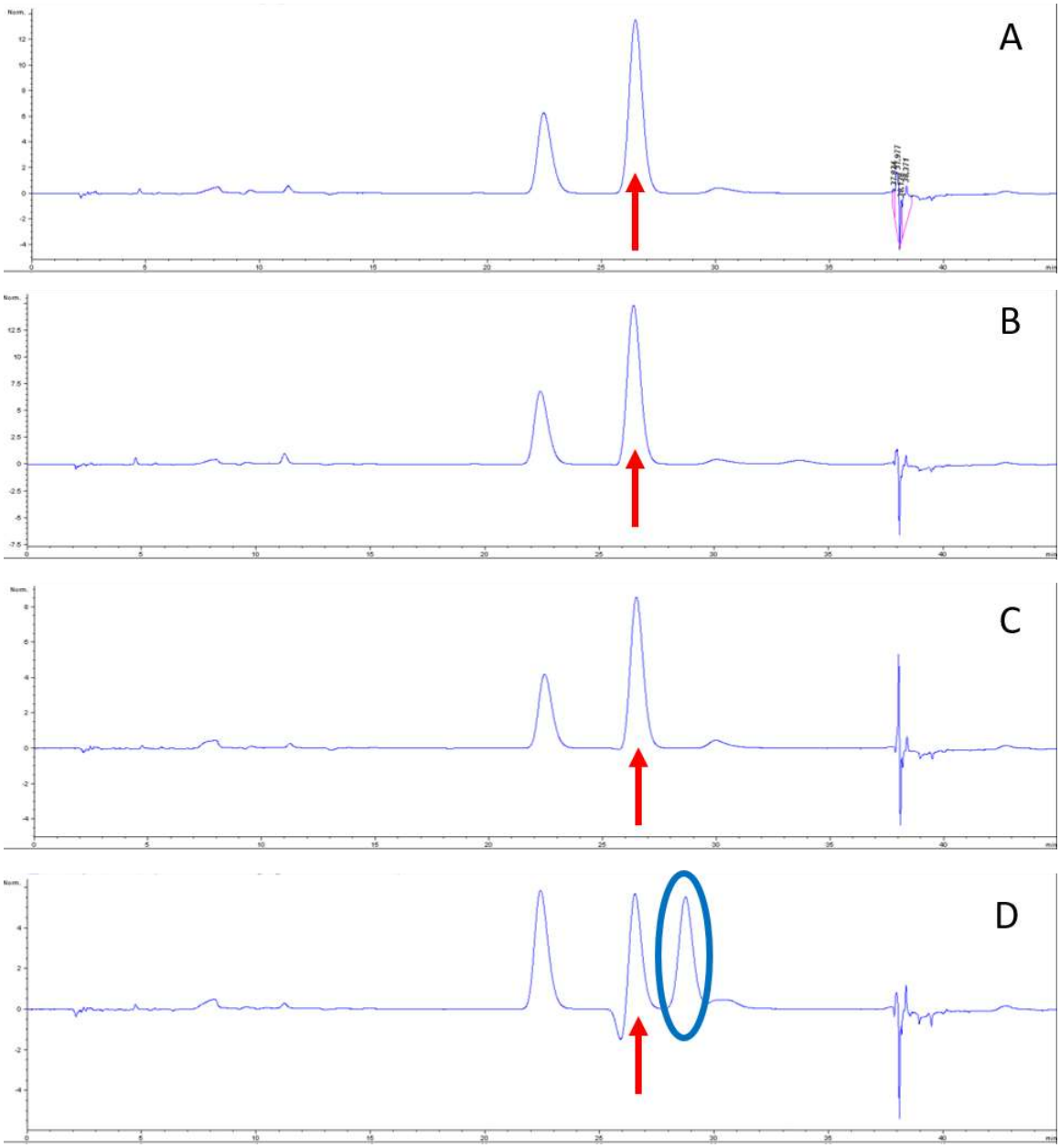


Figure 9. Corrinoids produced by cultures amended with 5µM lower base and 40 mM acetate.

(A) Control, (B) DMB, (C) 5,6Brbza, (D) 5Clbza.

CO_2 Results

Methane Production

Much like previous results with 5 µM lower base supplementation, cultures grown on a combination of CO_2 and H_2 demonstrated no detectable difference in methane production compared to control cultures (Figure 10).

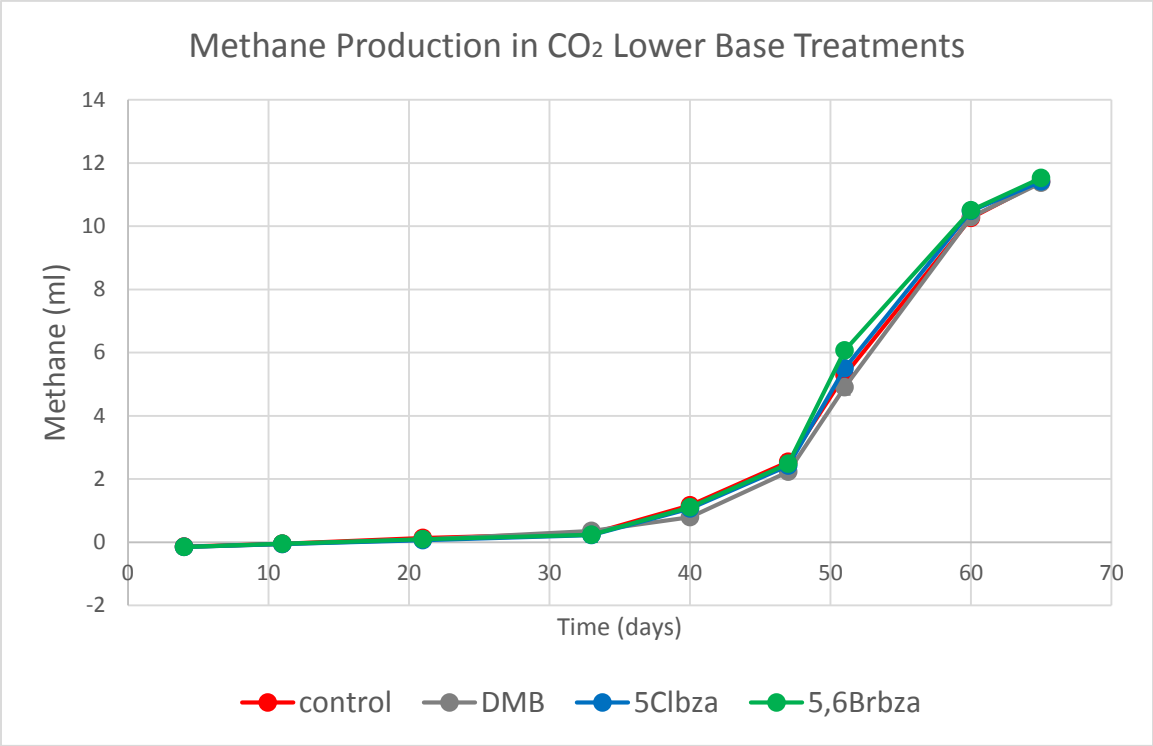


Figure 10. Methane production of cultures grown with CO_2 and 5µM lower base.

HPLC/Corrinoid Extraction

Once cultures had reached stationary phase of growth, they were sacrificed to extract corrinoids. Corrinoids were extracted and measured via HPLC to determine whether the native corrinoid was produced by the treatment cultures (Figure 11). In control cultures (A), we observed the production of a single corrinoid at the retention time ~26.5 minutes. In cultures amended with DMB (B), we see the production of a single corrinoid at the same retention time as the control chromatogram at about 26.5 minutes. Without further analysis, we could not determine whether this corrinoid is the native corrinoid, however, this is the most likely the case. In cultures amended with 5,6Brbza (C), we observed a peak that eluted at the same time as our control culture (~26.5 minutes), therefore, similar to DMB (B) cultures, it is most likely the native corrinoid that was produced in these cultures. Finally, in cultures amended with 5Clbza (D), we observed the production of two separate corrinoid peaks. One peak eluted at the same time as the native corrinoid, and the other peak eluted later in the chromatogram. Without further analysis, it cannot be determined whether or not this peak is a corrinoid with the lower base 5Clbza, however, if we did expect this corrinoid to be produced, we would expect it to elute after the native corrinoid on the chromatogram, so this is certainly a possible explanation.

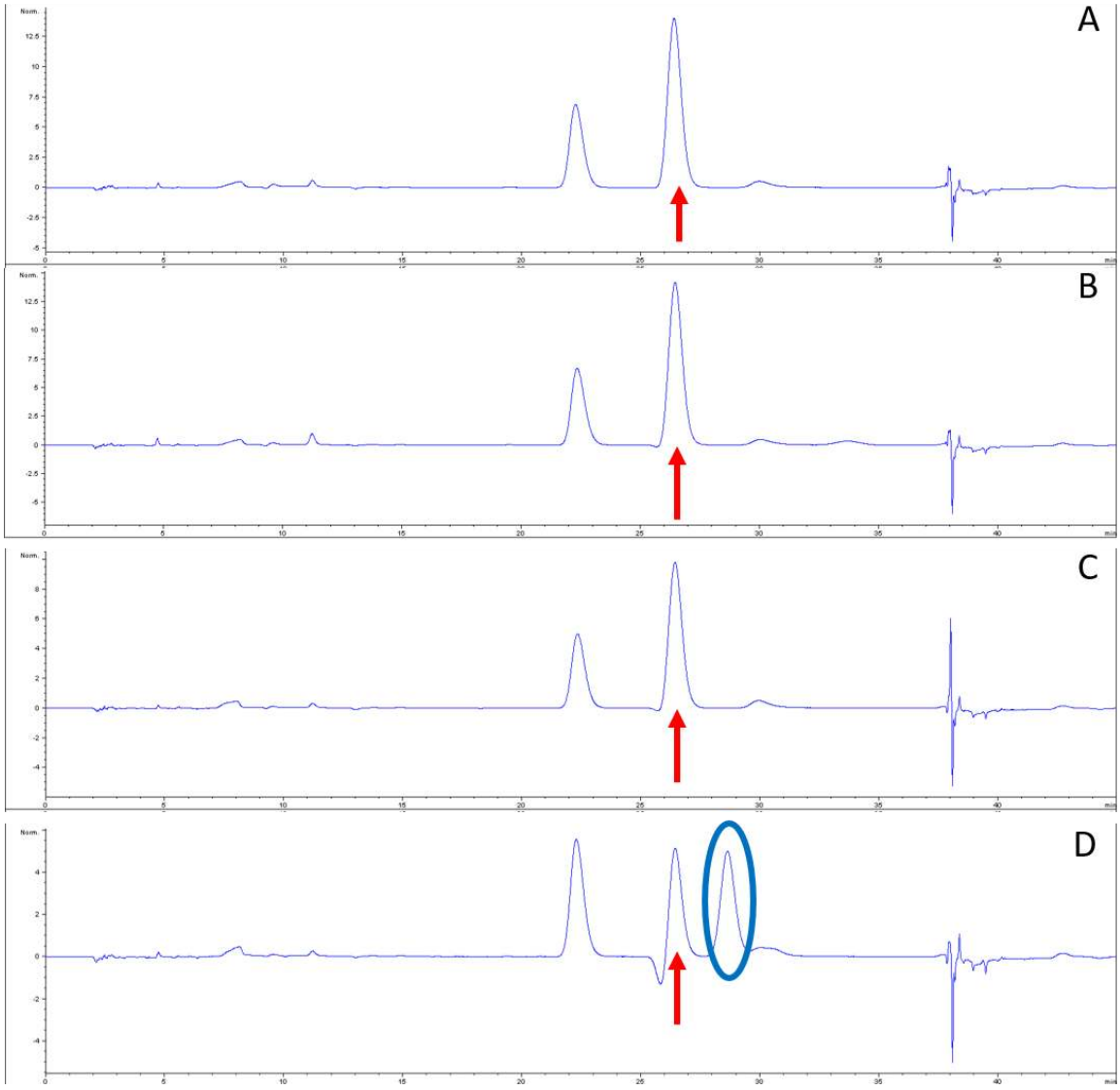


Figure 11. Corrinoids produced in cultures amended with CO_2, H_2 and 5 µM lower base.

(A) Control, (B) DMB, (C) 5,6Brbza, (D) 5Clbza.

qPCR Results

Once all cultures were grown up, 3 ml of culture was filtered for DNA extraction for qPCR to determine relative quantities of cells within each culture. qPCR was utilized to determine whether any differences in methane production could be attributed to greater quantities of cells in one particular culture (Figure 12). In the methanol experiments, we observed relatively identical quantities of cells in all four treatments. In the acetate experiment, once again, we saw relatively similar numbers of methanogens within each individual treatment. Finally, in the CO_2 and H_2 experiment, we observed very similar quantities of methanogens within each treatment, although there was a slight drop-off in the quantities of cells in the 5,6Brbza cultures. For the most part, the number of cells within each culture, across all three pathways and all four treatments was relatively similar, although there appears to be slightly more in the methanol and acetate experiments compared to the CO_2 experiment. This is not entirely surprising, as the CO_2 cultures appeared less dense than the cultures of the methanol and acetate experiments.

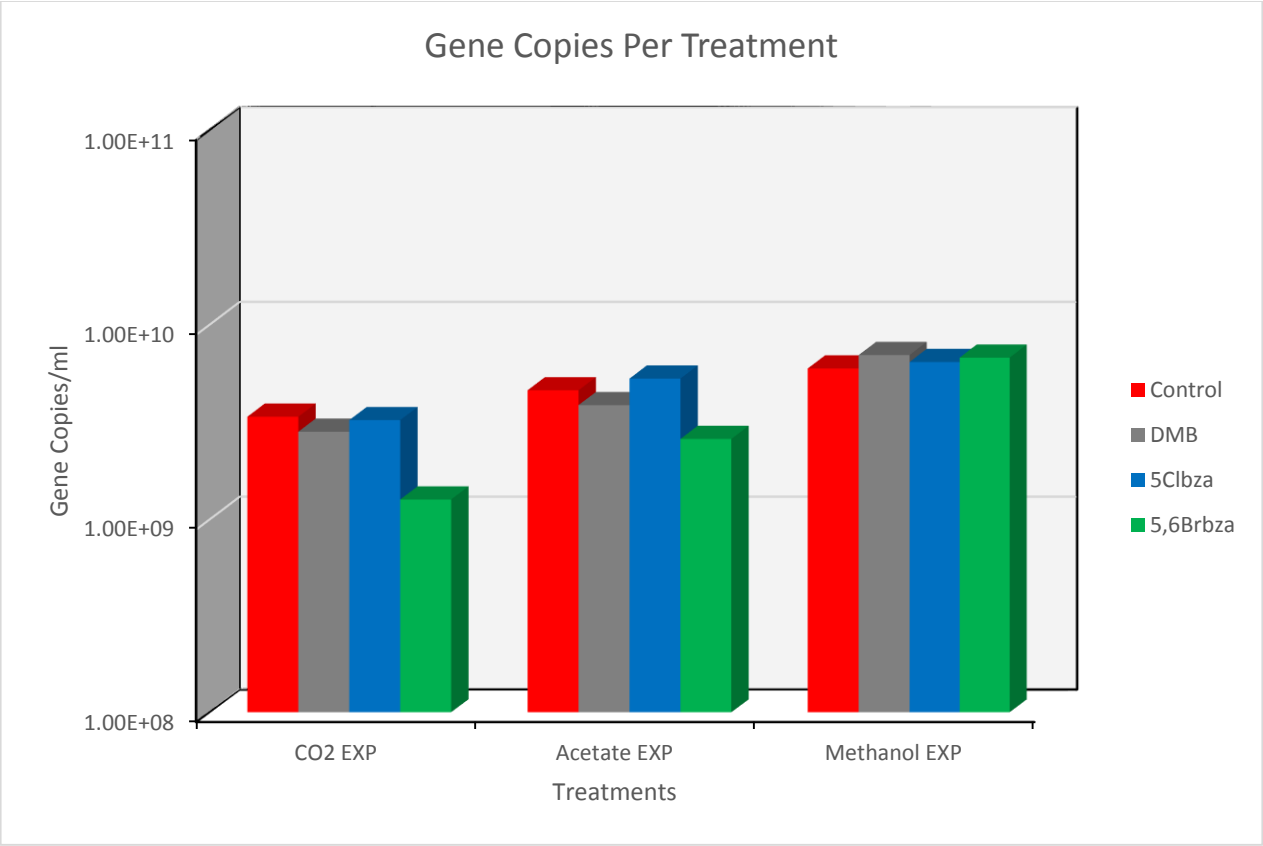


Figure 12. Gene copies per experimental treatment.

Discussion

Despite the investigation of three different potential pathways of methanogenesis, the results across all three experiments remained the same. Overall, when cultures were amended with 5 µM lower base, there appeared to be no difference in methane production, or abundance of methanogens growing within each individual treatment. However, previous research in bacteria has demonstrated that we can observe changes in microbial activity with supplemented lower bases when they are supplied at 200 µM concentration [3].Therefore, it is entirely possible that the quantity of lower base supplemented (5 µM) was much too low to see any sort of effect on the growth or metabolism of the cultures.

To determine whether an elevated concentration of lower base could impact our cultures, we continued the experiments at a concentration of 50 µM lower base.

CHAPTER THREE DOES 50μM LOWER BASE SUPPLEMENTATION AFFECT METHANOGENESIS?

Materials and Methods

Chemicals

5,6-dimethylbenzimidaole (DMB), 5-chlorobenzimidazole, and 5,6-dibromobenzimidazole were purchased from Combi-blocks. Methanol was purchased from Sigma Aldrich.

Cultures

Pure *Methanosarcina barkeri* strain *fusaro* cultures were grown in 160 ml serum bottles with 50 ml of minimal salts medium with a vitamin B_{12}-free vitamin mix. Cultures were grown anoxically with a sodium bicarbonate buffer at pH 7.2 and 37 degrees Celsius. Minimal salt media is composed of (g/L): 1 g NaCl, 0.5 g $MgCl_2 \times H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2 \times 2H_2O$. Trace element solution (per liter): 10 ml HCl (25% solution w/w), 1.5 g $FeCl_2 \times 4 H_2O$, 0.19 g $CoCl_2 \times 6H2O$, 0.1 g $MnCl_2 \times 4 H2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4 \times 2 H_2O$, 24 mg $NiCl_2 \times 6 H_2O$, 2 mg $CuCl_2 \times 2 H_2O$. Vitamin solution contains (mg/L): 0.02 biotin, 0.02 folic acid, 0.1 pyroxidine hydrochloride, 0.05 riboflavin, 0.05 thiamine, 0.05 nicotinic acid, 0.05 pantothenic acid, 0.05 *p*-aminobenzoic acid, and 0.05 thioctic acid. Se/Wo solution includes (per L): 6 mg $Na_2SeO_3 \times 5 H_2O$, 8 mg $Na_2WO_4 \times H_2O$ and 0.5 g NaOH. Gas phase was composed of H_2 and CO_2 (90/10). 0.1% resazurin solution was added at 0.25

ml/L medium. 30 mM sodium bicarbonate, 2 mM L-cysteine monohydrate and 0.048 g/L Sodium sulfide x $9H_2O$ were added. Either 40 mM acetate or 5mM methanol was added as electron donor. Sterile-filtered lower bases were added to a final concentration of 50 µM. Treatments were set up in triplicates and included:

(1) Control (no lower base added)(5OHbza)

(2) 5,6-dibromobenzimidazole (5,6Brbza)

(3) 5-chlorobenzimidazole (5Clbza)

(4) 5,6-dimethylbenzimidazole (DMB)

Methane Measurements

Methane production was measured at regular intervals with an Agilent 7890 gas chromatograph equipped with FID (flame ionization detector) and DB-624 capillary column (60 m x 0.32 mm x 1.8 µm). 100 µl headspace samples were withdrawn using disposable syringes and manually injected into a split injector with a split ratio of 2:1. The GC inlet was maintained at 200 °C, and the GC oven temperature was kept at 60 °C for 2 min then increased to 200 °C at a rate of 25 °C min−1, and the FID detector was operated at 280 °C as described as described by [33].

Cells were harvested to extract corrinoids and determine which corrinoids were produced by methanogens when supplemented with lower bases. The procedure was carried out as described by [34]. Cultures were centrifuged (Thermo Scientific sorcall Rc 6+) at 10,000RPM for 20 minutes at 4 degrees C.

Supernatants were removed, and cell pellets were re-suspended in 5 ml deionized water. pH was then adjusted to 5-6 with 3% glacial acetic acid and 10 mM KCN, and boiled for 20 min in a water bath. Following the water bath, cultures were centrifuged again for 20 minutes at 10,000 RPM. Supernatants were collected, and cell pellet was extracted again. Supernatant fluids were combined and mixed with 0.01 volumes of acetic acid, then loaded into Sep-Pak C18 cartridges. Cartridges were first equilibrated with 2 ml of pure methanol and 60ml deionized water. Supernatant was pushed through column, and corrinoids were collected by washing the column with 3ml pure methanol, and collecting the pink-colored fluid to be vacuum dried. After vacuum drying was complete, samples were resuspended in 0.5 ml sterile deionized water in preparation for HPLC analysis.

HPLC Analysis

Analysis of corrinoids was performed with an Agilent 1200 series HPLC system equipped with an Eclipse XDB-C18 column (5 mm, 4.6 × 250 mm) and a diode array detector set at a detection wavelength of 361 nm. Samples were injected and separated at a flow rate of 1 mL per min at 30°C using 0.1% (v/v) formic acid (≥88%, w/v) in water (eluent A) and 0.1% (v/v) formic acid in methanol (eluent B) as mobile phases. The column was equilibrated with 82% eluent A / 18% eluent B, and a linear change to 75% A / 25% B was applied after sample injection over a time period of 12 minutes. Eluent composition then

decreased immediately to 25% A / 75% B over 3 minutes, followed by a 5 minute hold. The column was then equilibrated to initial conditions.

Methanol Results

Methane Production

In M. *barkeri* cultures supplemented with 50 μM lower base, we saw a difference in the rate and total quantity of methane production between treatments. The cultures amended with 50 μM synthetic lower bases (5-chlorobenzimidazole and 5,6-Dibromobenzimidazole) saw slower rates of initial methane production, as well as lower total volume of methane produced (Figure 15). Control treatments were not amended with any lower base. Therefore, the control cultures were required to synthesize their native lower base, 5OHbza. 50 μM lower base supplementation of DMB, a naturally occurring lower base, had little to no impact on rate or total quantity of methane produced compared to the control. 5Clbza supplementation led to a slower initial rate of methane production, but eventually the cultures were able to recover and produce roughly equivalent amounts of methane compared to the control. However, the 5,6Brbza treatment appears to have severely impeded methanogenesis, as very little methane was produced over the course of the entire experiment.

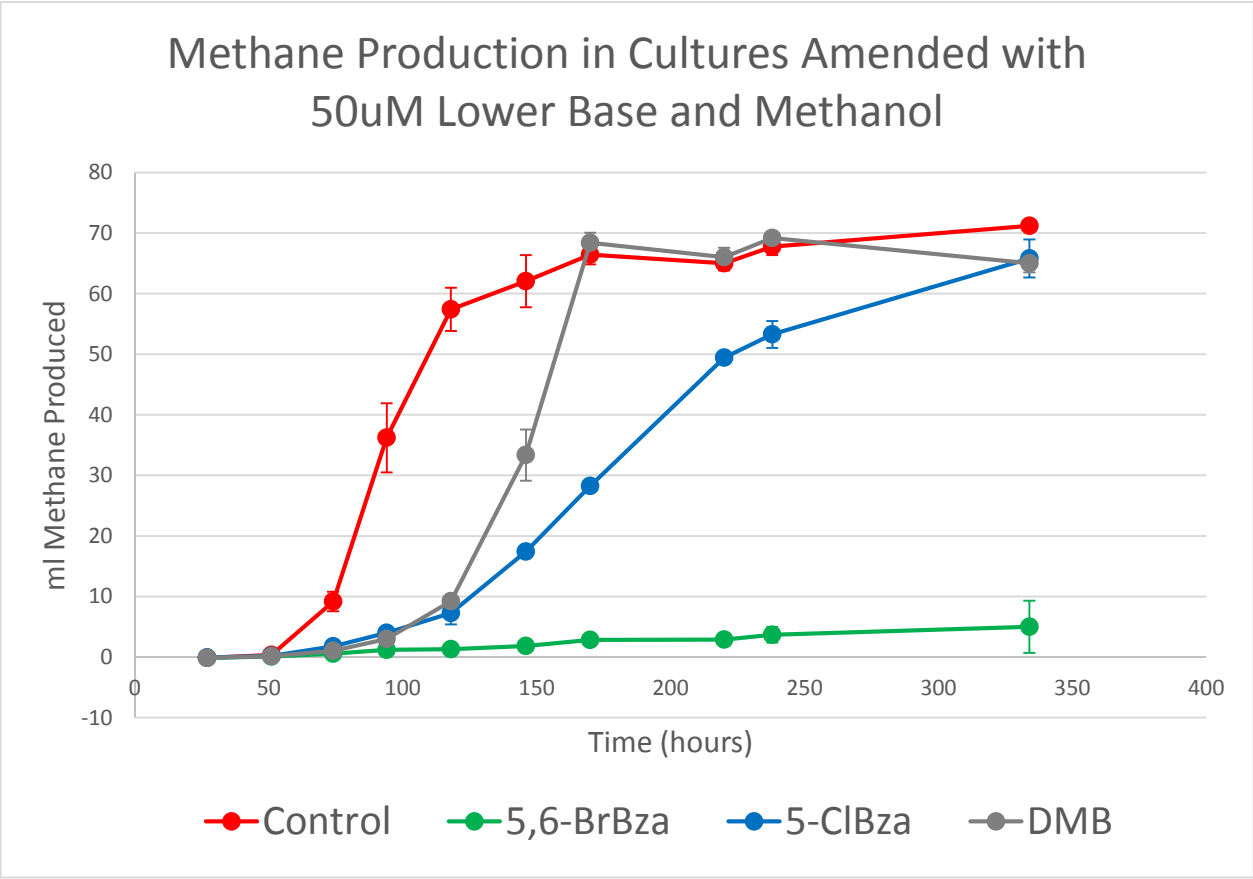


Figure 13. Methane Production in Methanol and 50 μM Lower Base.

Corrinoid Analysis

HPLC was used to determine whether *M. barkeri* cultures amended with 50 μM lower bases produced their native corrinoid, a different corrinoid, or a combination of the two. In the case of our control cultures, not amended with any lower base, we observed a corrinoid peak at a retention time ~21.5 minutes, with a corresponding UV-Vis peak at 361 nm, suggesting the presence of a corrinoid (Figure 16). When the control (A) HPLC chromatogram is compared with the two cultures amended with 50 μM synthetic lower bases (5,6-dibromobenzimidazole (B) and 5-chlorobenzimidazole (C)), they also contain a peak, with UV-Vis peaks suggesting the presence of a corrinoid eluting at the same time as the native corrinoid would be expected to elute. In 5-Chlorobenzimidazole (C) cultures, we observed two potential corrinoid peaks. One has the same retention time as the native corrinoid, while the other, larger peak suggests the presence of a corrinoid other than the native. This data suggests that *M. barkeri* cultures were able to take up 5-chlorobenzimidazole, as well as activate the lower base and incorporate it into a corrinoid. The apparent mixture of corrinoids presents an interesting question: Does the appearance of some native corrinoid explain the ability of the culture to still produce some methane, although not nearly as much as the control cultures?

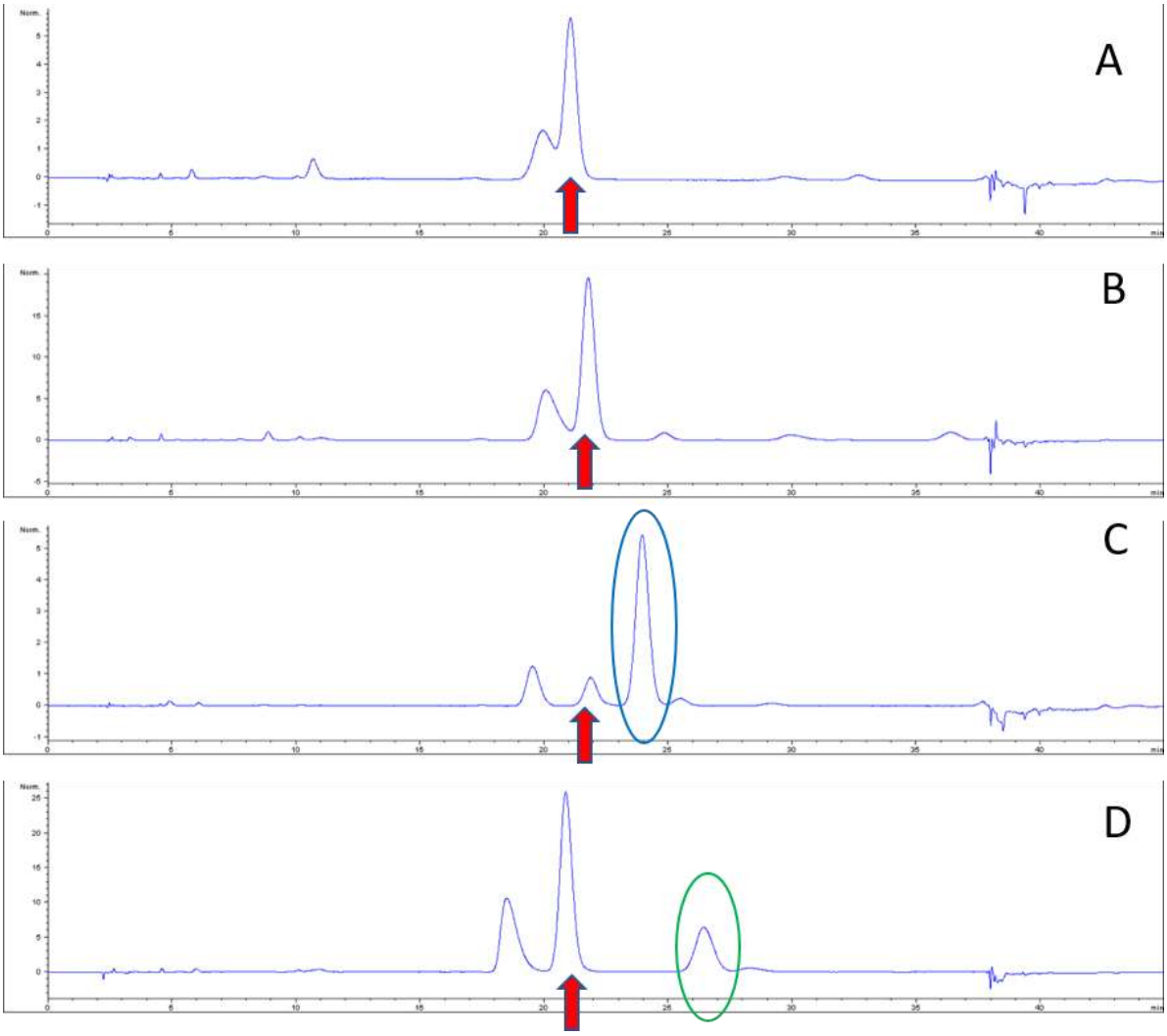


Figure 14. (A) control, (B) 5,6-diBrbza, (C) 5-Clbza, (D) DMB.

In the 5,6-dibromobenzimidazole cultures, we observed a similar phenomenon. A corrinoid elutes at the same time as the native corrinoid would be expected. There is also the presence of a much smaller potential corrinoid peak later on in the chromatogram. This evidence suggests the production of both a native corrinoid, and a new corrinoid from the supplemented lower base. This, once again, begs the question: is the presence of the native corrinoid responsible for the small amount of methane that the culture was able to produce?

In the case of 5,6-dimethylbenzimidazole, a naturally occurring (but not produced by methanogens) corrinoid, we observed measurable difference in methane production from the control. Upon HPLC analysis of corrinoids in the cultures, we saw a mixed production of corrinoids. There is a peak in the HPLC chromatogram that suggests the presence/production of the native corrinoid, as well as the presence of a B12 Corrinoid.

Acetate Results

Methane Production

Cultures grown with 40 mM acetate and various lower base supplements were monitored for methane production over time to determine whether lower base supplementation may impact methanogenesis (Figure 15).

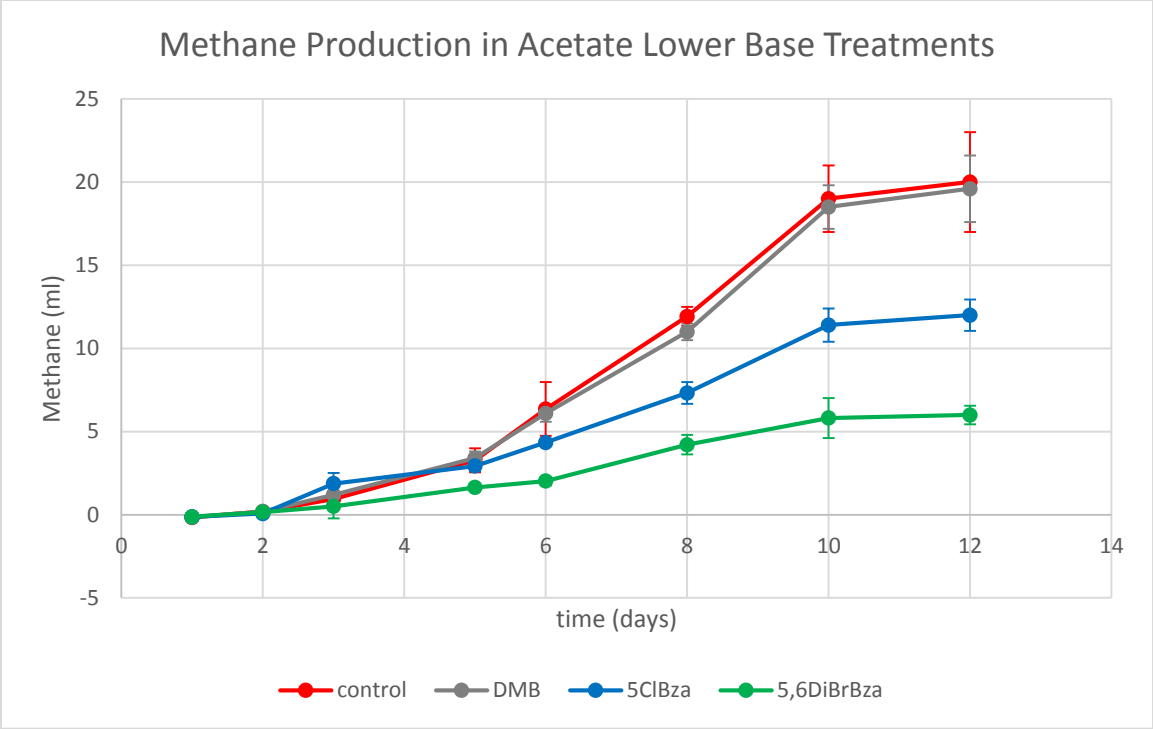


Figure 15. Methane production in cultures amended with 40mM acetate and 50 μM lower base.

Cultures amended with DMB showed methane production nearly identical to control treatments. However, in the treatments supplied synthetic lower bases (5,6Brbza and 5Clbza), there are marked differences in methane production throughout the course of the experiment. In the 5Clbza treatments, initial methane production over the first few days keeps pace with the control treatments. After day 5, Clbza treatments rate of methane production slows in comparison to control cultures, and does not catch up throughout the remainder of the experiment, eventually producing only about half as much methane in comparison to control treatments. We observed a similar phenomenon in cultures amended with 5,6Brbza. 5,6Brbza treatments kept pace with control treatments from day 0-3, but after day 4, there were obvious differences in methane production that continued through the course of the experiment. When the experiment was finished, 5,6Brbza treatments only produced only about a third of the overall methane that was produced by control treatments.

Corrinoid Analysis

To determine whether or not *M. barkeri* treatment cultures were able to activate and incorporate the supplemented lower bases into a corrinoid produced by the organism, corrinoids were extracted from cultures and separated with HPLC (Figure 16).

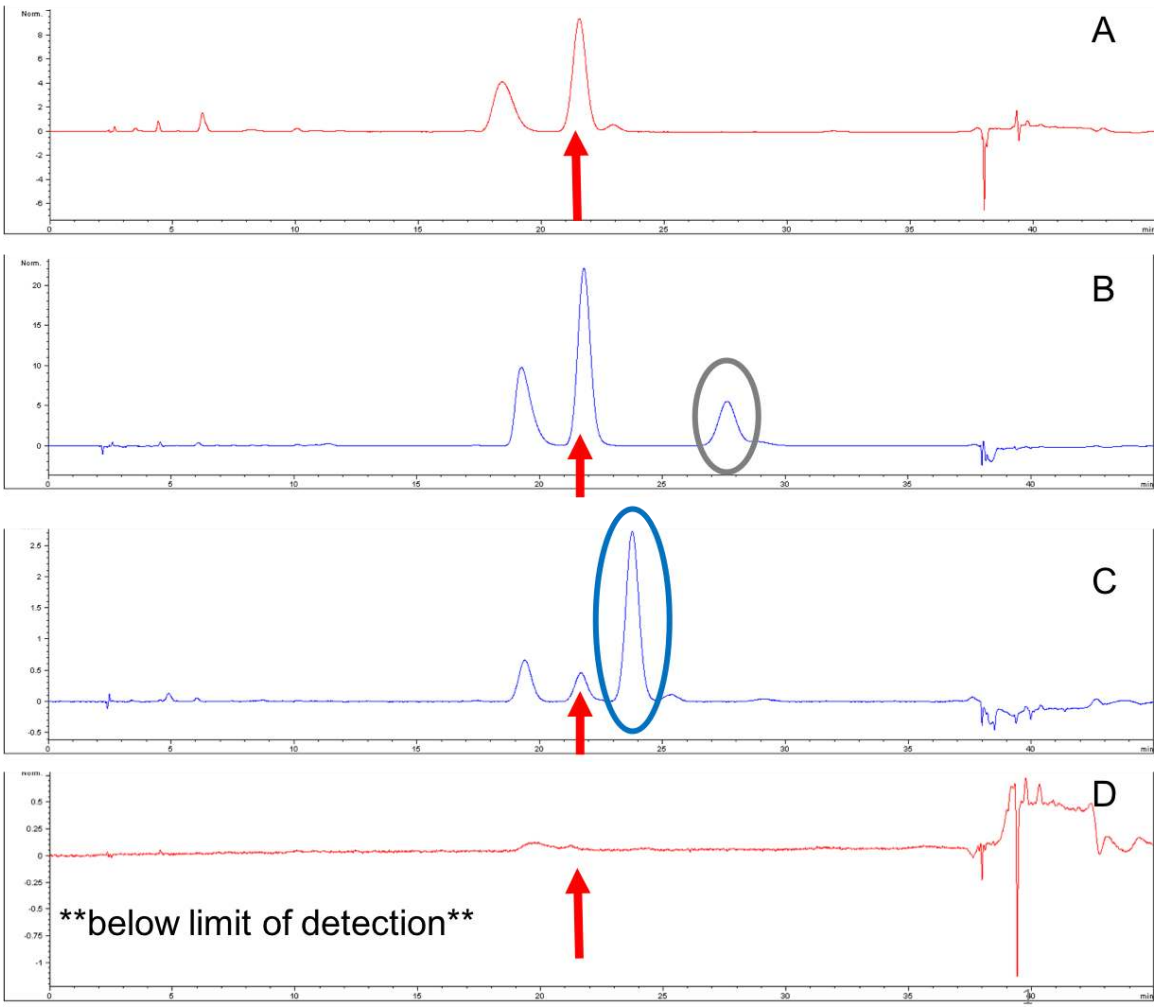


Figure 16. HPLC Chromatograms of (A) Control, (B) DMB, (C) 5Clbza and (D) 5,6Brbza treatments.

In chromatogram (A), we observed the elution of the native corrinoid at ~21.5 minutes (red arrow), and a UV Vis peak of 361nm, indicating that the peak we observed corresponds to a corrinoid. In DMB treated cultures (B), the chromatogram presented two different peaks with UV Vis peaks of 361nm. This indicated the presence of two different corrinoids in the sample. Based on the elution time of the first peak (21.5 minutes), it is likely the native corrinoid (factor III). The other peak (circled in grey) eluted at ~27.5 minutes, and is likely B12 corrinoid, based on standards previously run. DMB is the lower base of B12 corrinoid, which would indicate that *M. barkeri* culture supplemented with DMB are able to activate DMB and produce B12. In 5Clbza treatments (C), we observed chromatograms similar to DMB treatments. There was a corrinoid peak in the chromatogram at 21.5 minutes, suggesting the cultures still produced their native corrinoid. However, there was a second corrinoid peak later in the chromatogram at ~24 minutes. In the 5,6Brbza treatments (D), there were no observable corrinoids, most likely due to the low culture volume and poor growth of these cultures. There was a tiny peak at the time point we would expect the native corrinoid to elute, however, the volume was too small to be cross-checked with UV Vis.

Discussion

The 50 uM lower base experiments have presented the most interesting results of the project thus far for several reasons:

1) A noticeable difference in methane production in cultures treated with certain lower bases
2) The production of foreign corrinoids confirmed with HPLC in cultures treated with certain lower bases.

Treatments of *M. barkeri* cultures with another naturally-occurring lower base, DMB, proved ineffective at altering methane production. However, it is interesting to note that *M. barkeri* cultures appear able to activate and utilize this lower base to continue normal metabolic activity, ie. Methanogenesis. Perhaps more interesting, is the cultures amended with the synthetic lower bases 5Clbza and 5,6Brbza. Cultures amended with these lower bases demonstrated greatly reduced methanogenesis activity. In the case of 5Clbza treatments, we observed about half the amount of methane production, as well as the assembly of two different corrinoids. Most interestingly, these cultures appeared to produce a greater quantity foreign of corrinoids than the native corrinoid, factor III. Unfortunately, 5,6Brbza treatments inhibited methanogenesis to the point that very little methane production or culture growth was observed, leading to inconclusive HPLC results. Due to the low culture volume, we are unable to determine the quantities and types of corrinoids produced by the 5,6Brbza treatments.

CHAPTER FOUR CONCLUSION

The high global warming potential of methane, combined with human activity directly attributed to increased methane production warrants further research on methanogens, the microbes that produce methane. Due to the great diversity of methanogenic archaea, it is critical that research in methanogenic activity be applicable across all species of methanogens. A dependence on a corrinoid cofactor to perform the methyl transfer step required before the final reduction of the compound to methane is conserved across all methanogen species, making it an attractive target for researchers interested in manipulating methanogenic activity.

Previous work in bacteria demonstrated that corrinoid mediated processes, such as methyl transfers, are directly influenced by the structure of the corrinoid cofactor[1; 2; 3]. Therefore, corrinoid cofactors represent a promising target for those who chose to investigate methanogenic activity as well. Our research has provided the foundation for further investigation into methanogenic preference of corrinoid structure. It has been demonstrated that supplementing at least 50 µM of lower base to a pure culture may impact methanogenic activity, although much remains to be determined about how this mechanism may occur.

At this point, we can proceed with more detailed experiments regarding the isolation and characterization of the other corrinoids produced in pure *M. barkeri* cultures grown with other lower bases. Upon confirmation that these lower bases are in fact incorporated into another corrinoid being utilized by the

methanogen, it would be appropriate to expand these experiments into mixed cultures. One culture of interest may be rumen fluid, as the agricultural industry would be a potential target for this type of remediation. Ultimately, further studies on this topic may lead to potential applications for reducing methanogenic activity, and therefore, reducing the production of greenhouse gasses directly related to human activity.

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VITA

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