**Glutathione dependent $NADP^+$ linked enzymatic pathway as a possible pathway used by microorganisms in the detoxification of formaldehyde resins.**

By

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A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia

in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with Honours in Biology

April 2014, Halifax, Nova Scotia



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## Abstract

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Urea-formaldehyde polymers undergo hydrolysis to break into their monomeric components; one of which is formaldehyde. When resin containing wood chips are put into anaerobic bioreactors it is suspected that formaldehyde breaks down into carbon dioxide and methane gas, by microbes present in the system; this shares common characteristics with the MixAlco® fermentation process. The biochemical pathway with which this is achieved is unknown in this system, however throughout nature with respect to microbes, both eukaryotes and prokaryotes; their metabolic pathways are intensely studied and characterized. The purpose of this experiment was to determine if a glutathione (GSH) dependent $NADP^+$ linked pathway was used by microbes for this process. Custom primers for use with polymerase chain reactions (PCR) were created to detect the presence of a gene responsible for the synthesis of a key intermediate enzyme in this pathway; S-(hydroxymethyl) glutathione dehydrogenase (S-GSHDH). Agarose Gel electrophoresis determined a greater intensity of S-GSHDH gene PCR product in samples with urea formaldehyde resins as a primary carbon source. These findings correlate with the resource ratio competition models and support the hypothesis that this pathway may be one of the main pathways used in this system.

April 2014

## Acknowledgments

I would like to acknowledge all of the Saint Mary's University Biology Department, including their lab technicians for helping me throughout my research and making me feel at home. I would also like to thank Dr. Lihong Chai for her help with most of the molecular bench work conducted. I would like to thank Dr. David Dansereau for his help guiding me through endlessly seeming groups of electronic databases. A large thank you goes to Dr. Gavin Kernaghan for his help through this entire process. I would like to thank my caring and loving family. Without their support I would not have been able to achieve anything, and I thank you every day for that. Lastly I would like to thank my supervisor and mentor Dr. Zhongmin Dong. Your help throughout my entire undergraduate has been one of the most important and impactful things in my academic career and life, thank you.

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## Abbreviations

| | |
|---|---|
| Anaerobic Bioreactor | AB |
| Sample from just manure without MDF addition. Reaction had been running for a total of 9 days. | CM7 |
| Sample from just manure with the addition of MDF. Reaction had been running for a total of 9 days. | CM9 |
| Deoxyribonucleic Acid | DNA |
| Deoxyribonucleotide triphosphate | dNTP |
| Engineered Wood Products | EWP |
| Glutathione | GSH |
| Medium Density Fiberboard | MDF |
| Nicotinamide adenine dinucleotide | NAD |
| Nicotinamide adenine dinucleotide phosphate | $NADP^+$ |
| Polymerase chain reaction | PCR |
| Quantitative Polymerase Chain Reaction | qPCR |
| S-(hydroxymethyl) Glutathione | S-GSH |
| S-(hydroxymethyl) Glutathione Synthase | S-GSHS |
| S-(hydroxymethyl) Glutathione dehydrogenase | S-GSHDH |
| S-(hydroxymethyl) Glutathione dehydrogenase gene | S-GSHDH-g |
| Second Generation Primer | SGP |
| Super Optimal Broth + Glucose | SOC |
| UreaFormaldehyde | UF |
| Sample of CM9 after 31 days of reaction. Removal of possible inhibitors such as formic acid before tested. | WCMB |

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# Introduction

## 1.1 Engineered Wood Products

Urbanization of human civilization has been occurring ever since the development of agriculture. It is not a new phenomenon but in relative recent times the degree to which it is occurring has exponentially increased (Grimm, 2008). Approximately 80% of developed countries' populations are shown to be in urban environments, while the rest of the world's population is roughly 50% urbanized with a steady growth rate in recent years (Nations, 2006). This increase in urbanization has put new stresses and demands on certain industries, including the waste management industry. The most widely used systems of waste management are landfills; these sites hold numerous waste products and are quickly filling up each year with more waste (Grimm, 2008). One of the largest waste products that are being thrown into these landfills is engineered wood products (EWP); with nearly 201,600 tonnes of EWP's in Canadian landfills in 2010 (Canada, 2010).

EWP's are produced in an array of compositions for different functions. For 2014 the engineered wood industry is estimating a total of 25.7 billion square feet of plywood and oriented strand board will be created for North America (Press, 2013). These EWP's are used primarily as structural components for residential housing. The composition of these EWP's are a mixture of different wood types and adhesives. The adhesives used in a large number of these EWP are formaldehyde based resins (Laborie, 2002) (Dunky, 1998) (Bohm, et al., 2012).

Synthesis of many of these resins are based on the manifold reaction of their monomers, one of which is formaldehyde; other common monomers include phenol and urea (Tohmura, et al., 2001).These resins may contain more than just two monomers, such as in the example of melamine-urea-formaldehyde. Different monomer composition results in different resin structure and properties; including resistance to environmental stresses on the integrity of the resin (Tohmura, et al., 2001). Due to this, the choice of adhesive used is based upon the function and environment to which these wood products would be exposed. For example phenol-formaldehyde resins are commonly used in EWP's as a finishing laminate to protect the EWP from moisture (Hesse, 2000). Each of these formaldehyde resins **is** created for an intended purpose and place for their use; this does not mean that these are the only ones they will face. The lifetime of EWP's is not limited to only when in households or commercial use, when they are disposed of many of these products end up in municipal solid waste programs. The most common method used for treating municipal solid waste is landfills (Canada, 2010).

One of the most common EWP is known as medium density fiberboard (MDF). MDF is used in a wide array of commercial and residential furniture. It is a fibrous, homogenous EWP made from individual soft wood fiber strands (Philip Ye, et al., 2007).

### 1.2 Urea-Formaldehyde Resins

These individual wood fibers are combined with a resin, commonly urea-formaldehyde (UF), the bonding system is then cured under heat and pressure (Association, 2002). The end product is an economically cheap EWP that can serve multiple functions. UF resins are not intended to be in contact with water, due to this MDF is primarily found in interior furniture such as desks and chairs. EWP, including MDF, have been shown to

emit formaldehyde in humid climates in concentrations as high as .04 mg $m^{-3}$ (Risholm-Sundman, et al., 2007). These emissions are important factors when evaluating the potential effects of EWPs on human health and the environment (Risholm-Sundman, et al., 2007). Formaldehyde is primarily in its polymeric form in UF; this combined with a finishing seal in most MDF products produces relatively low amounts of formaldehyde emissions. However when these MDF products come in contact with water, relatively high emission rates are observed (Kelly, et al., 1999). This increase in emission of formaldehyde is due to the breaking of UF into its monomer components. These characteristics are due to the high susceptibility towards hydrolysis of the aminomethylene link within UF (Dunky, 1998).

This can become a serious problem when these EWP are exposed to rain, primarily in landfills. This exposure activates the release of formaldehyde and urea within local environments. Formaldehyde concentrations ranging from 2.3-39.0 µg $m^{-3}$ have been found in landfills within the United States (Agency, 2007). Formaldehyde has been known to have a number of toxic effects on both microorganisms and larger organisms. In tests conducted on formaldehyde from EWP wastewater, inhibition of microbial activity at concentrations as low as 150 mg $litre^{-1}$ have been observed (Lu & Hegemann, 1998). The standard concentrations used for formaldehyde in the production of UF are 2-4 g $litre^{-1}$ (Idustrie, 1992). This concentration of formaldehyde present is more than 20 times the known minimum amount required to disturb microbial communities (Lu & Hegemann, 1998). Studies have also found formaldehyde to cause damage in plant tissue (Sunkar, et al., 2003). Plants and microbes are not the only organisms that are susceptible to formaldehyde. Some short term health effects from formaldehyde exposure for humans

include watery eyes, burning sensations in the eyes, nose, and throat, coughing, wheezing, nausea, and skin irritation (Institute, 2011). The International Agency for Research on Cancer has also listed formaldehyde as a human carcinogen (Organization, 2006).

### 1.3 MixAlco® Fermentation with Anaerobic Bioreactors

A possible solution towards EWP waste is through the use of fermentation within anaerobic bioreactors (AB) (Kaszycki, et al., 2001). Aldehyde fermentation is possible by a wide array of microorganisms including both fungi and bacteria (Adroer, et al., 1990). In spite of formaldehyde's toxic effects it can still be metabolised by methylotrophic microorganisms as a carbon source. Methylotrophic yeasts have shown unprecedented capacity to break down formaldehyde. (Kaszycki, et al., 2013). A system was created with the intended purposes of breaking down formaldehyde through the use of the MixAlco® fermentation process (Terrabon, 2008). This system used inoculum from cattle (*Bos primigenius*) manure. The manure was treated through a series of dilutions and macronutrients were added. Once the composition of the reactor was finalized the system was sealed and anaerobic conditions were maintained. The engineering and makeup of the bioreactor is beyond the scope of this paper. However, for additional information the reader is referred to Skouteris et. al, 2012 (Skouteris, et al., 2012). AB were constructed and maintained at Saint Mary's University by B.Sc. Biology honours candidate Damin Lee. The micro flora present in the inoculum (cattle manure) were unknown. Due to this, the enzymatic pathways used for formaldehyde breakdown were unknown as well.

The amount of free formaldehyde should be used by the microorganisms whenever it became readily available in solution. This results in common analytical techniques such as high performance liquid chromatography, not suitable for measuring formaldehyde breakdown rates. Testing for formaldehyde breakdown had to be done through secondary means of measurement. It was noted that formaldehyde in the MixAlco® fermentation process resulted in the production of alcohols such as ethanol and methanol, which can be further metabolised into gaseous components, such as carbon dioxide and methane. This resulted in a gas production curve which showed clear trends in all replicates, this can be seen in Figure 1.3.

Many of the underlying mechanisms involved in this system were unknown with respect to biochemical processes. The inoculum was predicted to have a high species richness; with any number of microbes detoxifying formaldehyde through multiple pathways (Figure 1.2). This uncertainty led to further investigation in regards to the gene expression and metabolism occurring within the AB.

## 1.4 Enzymatic pathways for the breakdown of formaldehyde including Glutathione dependant $NADP^+$ linked pathways

Biochemical pathways for formaldehyde metabolism are numerous. They include the use of dehydrogenases class enzymes, $NADP^+$, catalase, ribulose monophosphate, xylulose monophosphate, and other cofactors and enzymes (Yurimoto, et al., 2005) (Adroer, et al., 1990) (Xenia, et al., 2002).

A common enzymatic pathway for prokaryotes and eukaryotes are nicotinamide adenine dinucleotide (NAD) linked pathways (Marx, et al., 2004). It should be noted that many organisms have more than one type of pathway present in their system. Activation of protein synthesis is dependent on environmental conditions. Formaldehyde detoxification is initiated primarily through the use of cofactors, one of which being is glutathione (GSH). All of these reactants combine to form a cofactor-bound complex which then can undergo oxidation to convert formaldehyde into formate (Vorholt, 2002). Investigation into the presence and expression of a GSH dependant $NADP^+$ linked pathway within the AB was the focus of this study. This particular pathway may be present in the AB; the three crucial steps involved are as followed (Kaszycki, et al., 2013):

**1.5 Reaction for breakdown of formaldehyde through a GSH-dependant $NADP^+$ linked pathway** (Bioinformatics, 2012)

1. $\text{Formaldhyde} + \text{Glutathione} = (\mathbf{E})\ S - (hydroxymethyl)Glutathione\ Synthase = \text{S} - (\text{hydroxymethyl})\text{Glutathione}$
2. $\text{S} - (\text{hydroxymethyl})\text{glutathione} + \text{NAD(P)}+ = (\mathbf{E})S - (hydroxymethyl)\ glutathione\ dehydrogenase = \text{S} - \text{formylglutathione} + \text{NAD(P)H}$
3. $S - formlyglutathione + H2O = (E)S - Formlyglutathione\ Hydrolase = glutathione + formate$

## 1.6 Key Characteristics of the GSH-dependant $NADP^+$ linked pathway

The formation of the S-(hydroxymethyl) Glutathione (S-GSH) complex is formed spontaneously; it may also be catalyzed by S-(hydroxymethyl) Glutathione Synthase (S-GSHS) (E.C 4.4.1.22) (Bioinformatics, 2012). Several organisms including certain yeasts utilize NAD-linked pathways but not require the presence of glutathione (Marx, et al., 2004). The formation of S-formlyglutathione through a catalyzed reaction of S-GSH with $NAD(P)^+$ is the key characteristic of this biochemical pathway. The intermediate enzyme involved in this process, S-(hydroxymethyl) glutathione dehydrogenase (S-GSHDH) (E.C 1.1.1.284) has low expression levels in unstimulating conditions (Marletta, et al., 1988). The expression and presence of S-GSHDH is essential towards identification and determination of the usage of this pathway in formaldehyde detoxification.

## 1.7 Determination of presence of S-GSHDH

### 1.7.1 Gene Identification – Primer Design

Many different organisms contain the ability to express S-GSHDH, however their nucleotide sequences for the S-GSHDH gene (S-GSHDH-g) may not be identical (NCBI, 2014). Identification of gene presence can be done through analysis of gene sequences in electronic databases. S-GSHDH-g's will have highly conserved areas, especially within organisms of closely linked genus or families. These highly conserved regions within different S-GSHDH-g's may be used as blueprints for the designing of primers; for use with Polymerase Chain Reaction (PCR).

### 1.7.2 Gene Presence and Expression

The fermentation of formaldehyde within AB occurs over the span of approximately 31 days. If formaldehyde is the primary carbon source within AB then organisms, which can competitively break this down molecule down will thrive and proliferate. This competitive advantage will result in a higher amount of DNA present within the system. If these amplified DNA products contain a higher quantity of gene presence it will most likely be due to the S-GSHDH expressing microbes thriving. Band intensity analysis of PCR products over different reaction times can be used to identify DNA quantity.

### 1.7.3 Research Objectives

The purpose of this research was to determine if a GSH dependent $NADP^+$ linked pathway was one of the biochemical pathways used by microbes in the breakdown of formaldehyde in AB. A series of objectives were placed to help guide the research:

(1) – Determine if S-GSHDH-g is present in any of the microbes within the system.

(1a) – The use of custom primers from a wide array of sequences of S-GSHDH-g showing the presence of target gene, and therefore the potential for protein expression.

(1b) – Determine if target gene is being expressed in system.

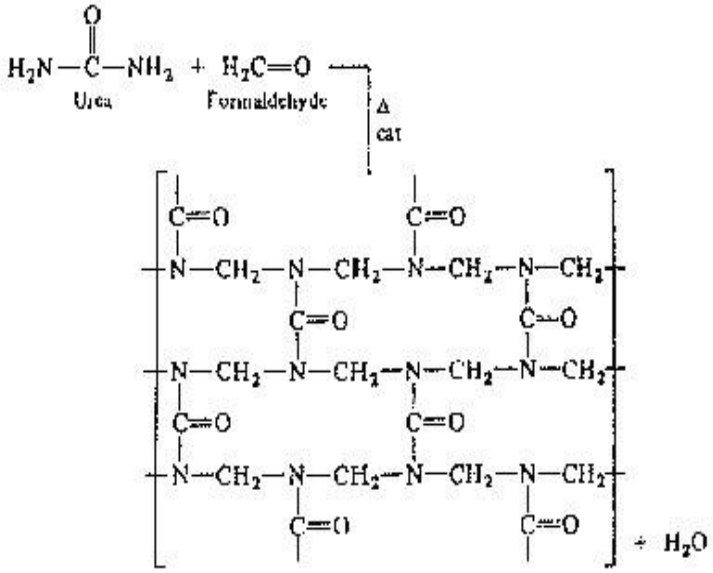
(2) – Determine if gene expression and gene quantity change over time

(2a) – Is gene quantity and expression in the system increasing or decreasing over time.

(2b) –Does gene quantity and expression change dependent on inoculum nature.

**Figure 1.1 – Urea-Formaldehyde Polymer formation through monomer components.**

**Source:** (Engineers, 2006)




Urea formaldehyde polymer

**Figure 1.2 – Multiple biochemical pathways used in to breakdown of formaldehyde.**

**Source:** (Lidstrom, 2006)

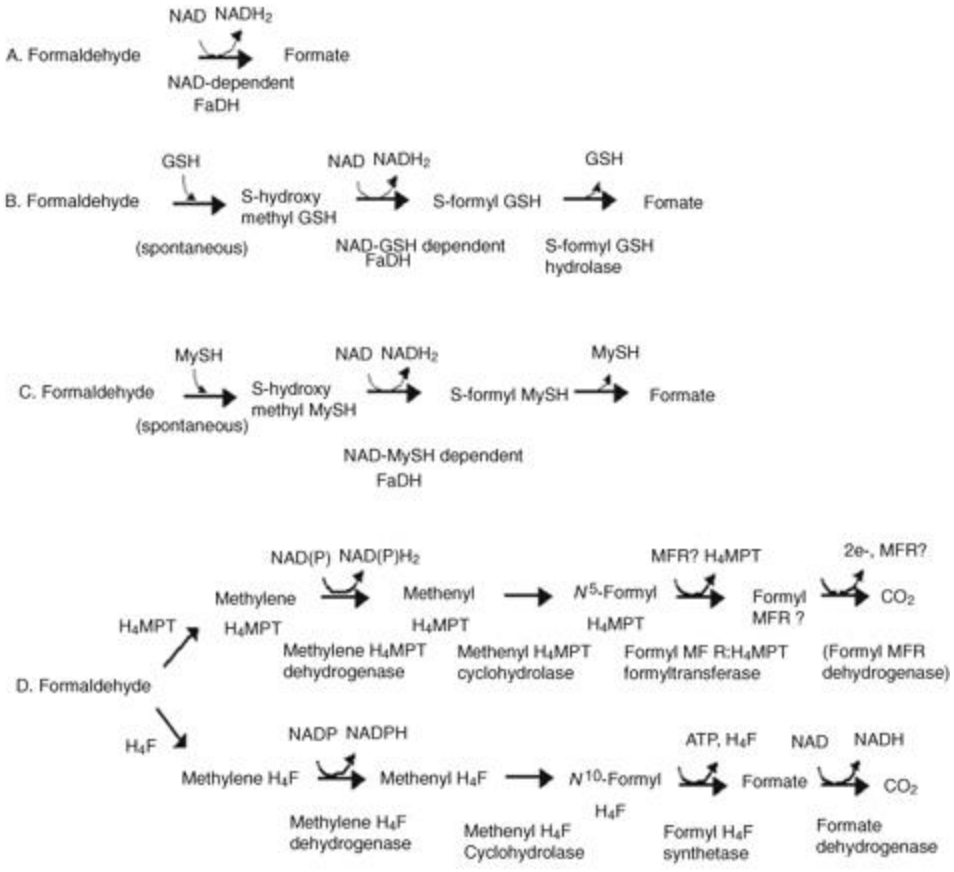
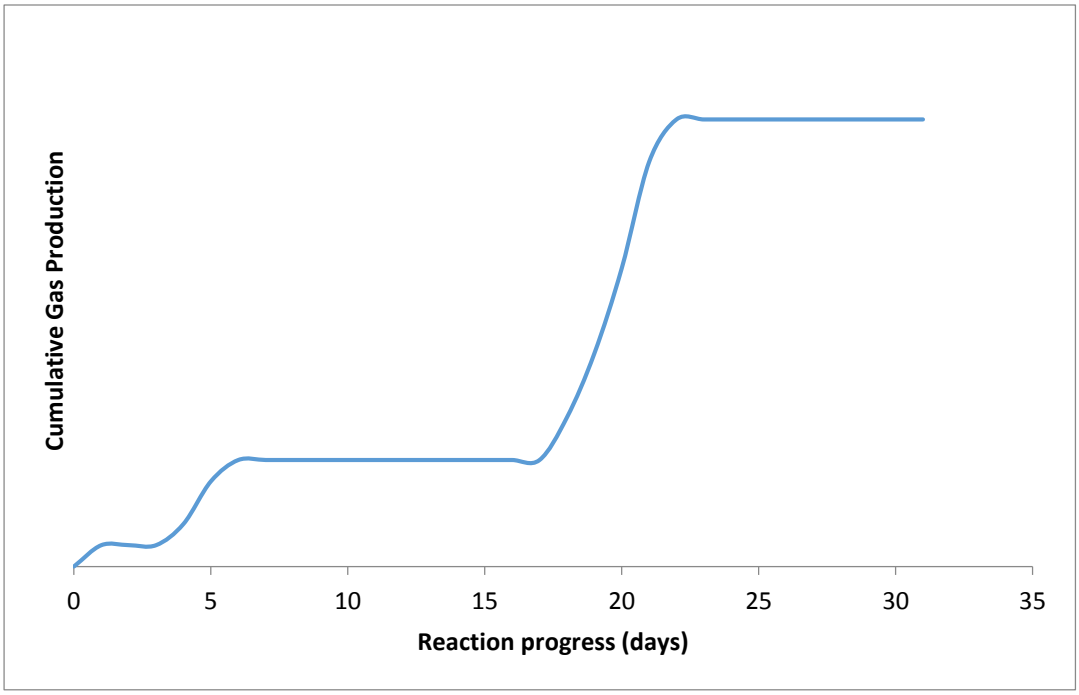

A. Formaldehyde
NAD NADH2
Formate
NAD-dependent FaDH
B. Formaldehyde
GSH
S-hydroxy methyl GSH
(spontaneous)
NAD NADH2
S-formyl GSH
NAD-GSH dependent FaDH
GSH
Fornate
S-formyl GSH hydrolase
C. Formaldehyde
MySH
S-hydroxy methyl MySH
(spontaneous)
NAD NADH2
S-formyl MySH
NAD-MySH dependent FaDH
MySH
Formate
D. Formaldehyde
H4MPT
Methylene H4MPT
NAD(P) NAD(P)H2
Methylene H4MPT dehydrogenase
Methenyl H4MPT
Methenyl H4MPT cyclohydrolase
N5-Formyl H4MPT
MFR? H4MPT
Formyl MF R:H4MPT formyltransferase
Formyl MFR ?
2e-, MFR?
CO2
(Formyl MFR dehydrogenase)
H4F
Methylene H4F
NADP NADPH
Methylene H4F dehydrogenase
Methenyl H4F
Methenyl H4F Cyclohydrolase
N10-Formyl H4F
ATP, H4F
Formyl H4F synthetase
Formate
NAD NADH
CO2
Formate dehydrogenase

**Figure 1.3 – Gas production curve for the breakdown of urea-formaldehyde polymers within MDF.**


Cumulative Gas Production
0
5
10
15
20
25
30
35
Reaction progress (days)

# Materials and Methods

## 2.1 Samples and Environmental Conditions

Samples were collected from manure obtained from a cattle farm located in Noel Shore Maitland, Hants County, Nova Scotia, Canada. Manure was first diluted in a 1:1 ratio with tap water then sifted through two sieves; with 1mm and 2mm in diameter pore sizes. Four hundred milliliters of this diluted material was then further processed along with 400mL of distilled water and 10g of MDF with 14% urea-formaldehyde resin by volume was added into 1L bioreactors. The bioreactors were sealed airtight and put into a 37°C water bath as per industry standard (Skouteris, et al., 2012). Anaerobic conditions were maintained within the vessel throughout the entire period of formaldehyde biodegradation. Gas production and inoculate community analysis were investigated but this is beyond the scope of this thesis. Samples were collected from the manure immediately after it was processed but before it was added into a bioreactor and the addition of MDF. Samples were also collected from bioreactors which had already been in the AB for 9 days with MDF wood chips added (CM9), and just inoculum without MDF added (CM7). A sterile syringe attached to a clear piece of plastic tubing which was attached to a 4-way gas valve was used to extract physical samples. A third sample was made from inoculum from CM9 after 31 days of gas production. This sample underwent "washing" as described in section 2.2. (WCMB) A full index of samples is shown in Table 2.2

## 2.2 DNA extraction and sample physical properties

Samples were collected in 2mL aliquots and placed into 2mL microcentrifuge tubes. (Fisher cat. 05-408-138) Samples were then centrifuged at 13,000rpm for 10 minutes the supernatant discarded to remove excess liquid. A PowerSoil DNA Kit (cat. 12888-50) by MO BIO laboratories was used to extract DNA from each sample. The centrifuge protocol for DNA isolation was used (cat. 12888-50, protocol 12888). DNA concentrations and purity was determined by a nanodrop (Nanodrop 2000 Thermo Scientific). 1µL of each sample was used for concentration testing, 260/280 ratios above 1.8 were deemed acceptable.

In order to rid samples of possible inhibitors (e.g. formic acid, DNAases, proteases etc.) a protocol for "washing" the manure was created. Prior to DNA extraction each sample was placed in a 1.5ml centrifuge tube and centrifuged for 10 minutes at 10,000g. The supernatant was discarded and 250µL deionized water was added to the tube. This process was repeated three more times. 250µL of deionized water were added to the tubes and vortexed for 5 minutes. DNA was extracted from the samples using the protocol provided above.

Triplicates of purified DNA samples were created. One of each replicate was stored at -20°C for short term storage; the other two were stored at -80°C.

## 2.3 Sequence Analysis and Primer Design

The genomic sequences of the microorganisms involved were unknown. Primers were created for an array of different organisms, including bacteria, fungi, plants and echinoderms. PCR was used to test which primers amplified S-GSHDH-g successfully.

The amino acid sequence for S-GSHDH was available through a number of protein bank indexes; in this case the Nation Center for Biotechnology Information (NCBI) protein index was used (NCBI, 2013). A nucleotide sequence search through a protein query was used with the S-GSHDH amino acid sequence. A Basic Local Alignment Search Tool (BLAST) search on the NCBI website was used with the resulting nucleotide sequence (Medicine, 2014). Over 100 matches with genes which were observed to be the S-GSHDH-g were found; the 25 results with the closest matching alignment percentage were chosen.

The nucleotide sequences were aligned using the Clustal Omega provided by the European Bioinformatics Institute (Institute, 2014). A phylogram was created using the closest matching nucleotide sequences with a total of five distinguishable groups. Primer3, an open sourced code for primer design and analysis, was used to create primers for four of the groups (Groups 1, 2, 4, & 5). The annealing temperatures for the primers ranged between 57-62 °C. The product lengths were approximately 150bp for each pair of primers. Group three 3 on the phylogram tree did not give 100% sequence matches within the group, and for this clade, degenerative primers were created. Primers were created through aligning the nucleotide sequences and manually choosing 17-20 base pairs with the desired characteristics of optimal primers. Primers were ordered from Eurofins MWG Operon.

**2.4 PCR, Gel Electrophoresis, and Staining**

PCR was used to determine gene presence via the custom synthesized primers sets. Twenty five µL reaction volumes were used with template volumes determined through testing positive primer controls, BSF8/20 and BSR 534, with template volumes of .5µL, 1.0µL, 1.5µL, and 2.0µL. Template volume of 1.0µL was eventually determined to be the most appropriate volume. Applied Biosystems® 2720 Thermal Cycler (cat. 4359659) was

used for the heating and cooling of PCR tubes. Refer to Table 1 for the exact PCR recipe. Positive controls using *Escherichia coli* DNA were set using universal primers BSF 8/20 and BSR 534. Taq polymerase and dNTP's were obtained through New England BioLabs (cat. M0273S & NO447S). The annealing temperature for all samples except those classified within group 3 primers were 55°C. For primers 31, 32, & 34 annealing temperatures were 47°C. A was used. Primer group 3 had different reaction conditions than the other primers All PCR runs were set to 35 cycles, with non-primer group 3 annealing temperatures set to 57°C and an extension time of 45s. Primer group 3 has an annealing temperature of 47°C and an extension of 45s. For a complete reference as to the characteristics of each primer including annealing temperature refer to Table 3.3

The products expected were 117-150bp. For this a 100bp genome marker was used (Thermo Fisher cat. SMU0241) was used to determine product size. Gel electrophoresis was done with 45ml of 1.5% agarose gel. DNA to loading dye ratios were 5:1, resulting in 5µl to 1µl volumes, respectively. Gel electrophoresis voltage was set to 110V, with a current of 113-120mA. Gels were run for 35 minutes to allow enough differentiation of bands, but not too long as to allow potential product fall off.

Originally staining had been done with an ethidium bromide solution. However due to the toxic nature of this compound, an initiative was taken to develop a safer method of staining. ACTGene DGel by Hydragreen$^{TM}$ DNA Stain (Cat. ACT-IDMG04) was used as a pre-run stain into the agarose gel. A High performance Ultraviolet Transilluminator (HPUT) (cat. 95-0423-02) from UVP combined with the new safer fluorescent stain allowed for safer, and cleaner analysis of gels.

## 2.5 Cloning and Sequencing

Products from working sets of primers were cloned. The products were first purified using the QIAquick PCR purification kit (cat. 28106).The ProMega pGEM-T Easy Vector System I (cat. A1360) was used for ligation of the PCR products as per manufacturer instructions.

Super Optimal Broth media with added glucose (SOC) and Lysogeny Broth (LB) medium were prepared previous to ligation and heat shock. SOC and LB media recipes were readily available online (Microbiology, 2013).

NEB 5-alpha competent E. coli cells (NEB cat. C2988J) were used to host the cloned product. The cloned cells were incubated at 37°C for two hours on SOC media.

Samples were then tested for blue/white screening through α-complementation of the β-galactosidase reporter marker gene. Concentrations of 45µg/mL of X-gal and IPTG in a mixed solution were spread onto the prepared LB plates. Plates were left to settle for 30 minutes. 50µL of 100mg/mL ampicillin was added to each plate and then left to settle for an addition 30 minutes. Cells were spread onto these plates in 3 different volumes: 50µL, 150µL, and 300µL. Twelve plates (and two controls) were screened for positive cloning results. Samples were incubated overnight at 37°C.

Positive white colonies were isolated and selected by a sterile toothpick and put into an LB liquid media with final concentration of 100mg/mL of Ampicillin. Samples were then left overnight in a shaker at 250rpm and 37°C.

Plasmids extraction was performed with a QIAprep spin Mini prep kit (cat. 27106). An EcoR1 enzyme was used to cut plasmids to identify positive samples. If samples had

been successfully cloned, a band of cloned product would appear around the 100-300bp mark (Inc., 2014).

All sequencing was sent to Korean to be performed by Macrogen (Macrogen Inc., Seoul, Korea). Sequences were returned electronically where they were analyzed by aligning sequences and BLASTing any regions between primers.

**2.6 Primer Efficiency and Second Generation Primers**

Primer efficiency was used to determine if quantitative PCR (qPCR) and reverse transcriptase PCR could be used as an effective method to determine gene quantity and expression. Sequences were sent out in batches of 16-32 samples per primer group. Samples were screened for matching primers and inserts within plasmids, counting the number of positive insertions over the total samples submitted. If these insertions had a less than 50.0% success rate, second generation primers (SGP) had to be created.

SGP were designed based on successful primer sequences and positive match for the S-GSHDH-g. Inserts were aligned and highly conserved regions were selected for SGP synthesis.

**Table 2.1 – Recipe for standard PCR protocol.**

**Template DNA concentration was found with nanodrop. Concentration varied within samples. All other reagents were made from dilution of stock solutions into working solutions.**

| **Reagent** | 25µL RXN Volume (µL) | Concentration |
|---|---|---|
| 10x Standard Taq Buffer | 2.5 | 1.0x |
| 10uM Forward primer | 0.5 | 0.2 µM |
| 10uM Reverse Primer | 0.5 | 0.2 µM |
| 2.5mM dNTP | 2.0 | 200 µM |
| Template DNA | 1.0 | X |
| Taq DNA polymerase | 0.125 | 0.125 units/50µL |
| dH20 | 18.375 | - |

**Table 2.2 – Key and description of template DNA**

| Sample | Description |
|---|---|
| CM7A CM7B | Treated cow manure from Group 7 of the anaerobic bioreactors (Manure) [Aug 2013] |
| CM9A CM9B | Treated cow manure from Group 9 of the anaerobic bioreactors (Manure + MDF) [Aug 2013] |
| WCMB | Treated and "washed" cow manure from Group 9 of the anaerobic bioreactors. (Manure + MDF) [Sept 2013] |

# Results

## 3.1 S-GSHDH-g electronic database sequences

Please refer to Table 3.3 for ENA/NCBI protein accension number. These proteins are the amino acid sequences of the top 25 matching sequences for the S-GSHDH-g.

## 3.2 S-GSHDH-g primer group design

Alignment of the S-GSHDH-g sequences resulted in a insufficient amount of conserved regions within the entire 25 sequences for one universal primer design. A percent identity matrix was used to look for highly similar sequences within the larger sequence set. These values can be found within Table 3.2.

Within the larger sequence set, four smaller groups were found, each group with 100% match of S-GSHDH-g within the groups. Four groups of primer sets were created with 20 of the sequences. The last five sequences did not have 100% gene sequence match with any other sequences, but had enough highly conserved regions that degenerative primers could be made. A total of three sets of degenerative primers was created. For a complete list of primers, including their nucleotide sequences and other characteristics please refer to Table 3.4

The resulting five groups of primers can be found in Figure 3.1.

## 3.3 PCR and Electrophoresis

### 3.3.1 First generation primers

A total of eight first generation primer sets were used to test for gene presence. Of the 8 first generation primers tested, two showed positive results on samples. These two were primers G3_SGDHDP and G2. The target lengths were 117bp and 150bp, respectively.

### 3.3.2 Sample condition for band presence and intensity

Primers G3_SGDHDP and G2 showed bands for target product length with varied band intensities dependent on template DNA used. The three template DNA samples used were CM7A, CM9B and WCMB.

CM7A showed faint bands for primer G2. There was no band present for primer G3_SGDHDP. For sample CM9 both primers showed bands. Band intensities were greater than those of in CM7. For primer G2, sample C9B showed two bands. Both bands were less than 200bp.

PCR conducted on sample WCMB showed the greatest PCR product band intensity. These band intensities were greater for both primers used.

Refer to Figure 3.2 to see agarose gel electrophoresis sample and band intensities.

### 3.4 Cloning and sequencing

#### 3.4.1 First generation cloning and sequencing

Cloning and sequencing was done twice for 1st and 2nd generation primer development. EcoRI endonucleus enzymes cutting detected 56/64 of the cloned samples with target inserts.

Sequenced samples showed low percentage of successful insertion of intended target gene. G3_SGDHDP had a 25.0% success rate in total sequences. G2 had a 12.5% success rate. Both primers had too low of a successful insertion percentage to be used for qPCR.

New primers were designed with G3_SGDHDP as the template primer.

#### 3.4.2 Second generation cloning and sequencing

Second generation primer sequences are shown in Table 3.4. A total of 16 samples was cloned and sequenced for second generation primers. 0.0% returned with successful insertions. Many sequences had both primers present (forward and reverse) but insert sequences were not representative of the intended gene.

**Table 3.1 – Percent identity matrix of 25 aligned sequences for S-GSHDH-g.**

Percentage identity matches of nucleotide sequences for S-GSHDH-g. Sequences show range of 100% identity match within specific groups. ClustalW was used to electronically create percent identity matrix with inputed sequence in FASTA format file.

| |
|---|
| 1: Klebsiella_pneumoniae_strain_NK245_plasmid_pK245 100.00 100.00 100.00 100.00 100.00 40.77 39.89 39.89 39.89 40.04 39.38 39.16 38.99 40.49 42.70 38.38 39.71 39.71 39.71 39.71 41.42 41.37 41.37 41.37 41.32 |
| 2: S-_hydroxymethyl_glutathione_dehydrogenase__P072206B_0005_ 100.00 100.00 100.00 100.00 100.00 40.53 39.95 39.95 39.95 39.72 39.14 38.91 38.97 40.65 42.62 38.10 39.49 39.49 39.49 39.49 40.83 40.77 40.77 40.77 40.75 |
| 3: Escherichia_coli_plasmid_pRAx 100.00 100.00 100.00 100.00 100.00 40.53 39.95 39.95 39.95 39.72 39.14 38.91 38.89 40.65 42.62 38.10 39.49 39.49 39.49 39.49 40.83 40.77 40.77 40.77 40.75 |
| 4: Aeromonas_hydrophila_plasmid_pRA1__complete_sequence. 100.00 100.00 100.00 100.00 100.00 40.53 39.95 39.95 39.95 39.72 39.14 38.91 38.89 40.65 42.62 38.10 39.49 39.49 39.49 39.49 40.83 40.77 40.77 40.77 40.75 |
| 5: Yersinia_pestis_biovar_Orientalis_str._IP275_plasmid_pIP1202_compl_sequence. 100.00 100.00 100.00 100.00 100.00 40.53 39.95 39.95 39.95 39.72 39.14 38.91 38.89 40.65 42.62 38.10 39.49 39.49 39.49 39.49 40.83 40.77 40.77 40.77 40.75 |
| 6: PREDICTED__Ceratitis_capitata_LOC101460851 40.77 40.53 40.53 40.53 40.53 100.00 82.13 82.13 82.04 69.36 68.91 69.18 67.20 71.53 77.48 72.79 73.78 73.78 73.78 73.78 75.05 75.05 75.05 75.05 75.05 |
| 7: Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._515920-2 39.89 39.95 39.95 39.95 39.95 82.13 100.00 100.00 99.91 69.09 70.80 72.15 71.05 72.25 71.26 74.05 74.23 74.23 74.23 74.23 73.33 73.33 73.33 73.33 73.33 |
| 8: Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._4952974 39.89 39.95 39.95 39.95 39.95 82.13 100.00 100.00 99.91 69.09 70.80 72.15 71.05 72.25 71.26 74.05 74.23 74.23 74.23 74.23 73.33 73.33 73.33 73.33 73.33 |
| 9: Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._414877 39.89 39.95 39.95 39.95 39.95 82.04 99.91 99.91 100.00 69.00 70.80 72.15 71.05 72.34 71.26 74.14 74.23 74.23 74.23 74.23 73.24 73.24 73.24 73.24 73.24 |
| 10: Gluconobacter_frateurii_adhC_gene__strain__NBRC_103465. 40.04 39.72 39.72 39.72 39.72 69.36 69.09 69.09 69.00 100.00 75.20 74.57 73.58 73.87 73.51 74.05 72.88 72.88 72.88 72.88 73.51 73.51 73.51 73.51 73.51 |
| 11: Providencia_stuartii_MRSN_2154__complete_genome. 39.38 39.14 39.14 39.14 39.14 68.91 70.80 70.80 70.80 75.20 100.00 78.26 76.28 77.03 71.80 75.50 73.87 73.87 73.87 73.87 74.77 74.77 74.77 74.77 74.77 |
| 12: PREDICTED__Strongylocentrotus_purpuratuss__LOC756103_ 39.16 38.91 38.91 38.91 38.91 69.18 72.15 72.15 72.15 74.57 78.26 100.00 79.87 77.39 71.80 77.57 76.31 76.31 76.31 76.31 74.95 74.95 74.95 74.95 74.95 |
| 13: Acinetobacter_baumannii_glut-dependent_formaldehyde_dehydrogenase_gd-faldh 38.99 38.97 38.89 38.89 38.89 67.20 71.05 71.05 71.05 73.58 76.28 79.87 100.00 74.68 69.73 75.05 71.98 71.98 71.98 71.98 71.32 72.70 72.70 72.70 69.54 |
| 14: Xenorhabdus_bovienii_SS-2004_chromosome__complete_genome. 40.49 40.65 40.65 40.65 40.65 71.53 72.25 72.25 72.34 73.87 77.03 77.39 74.68 100.00 74.95 76.22 76.22 76.22 76.22 76.22 77.30 77.30 77.30 77.30 77.30 |
| 15: Pseudomonas_entomophila_str._L48_chromosome_complete_sequence. 42.70 42.62 42.62 42.62 42.62 77.48 71.26 71.26 71.26 73.51 71.80 71.80 69.73 74.95 100.00 78.92 77.75 77.75 77.75 77.75 79.28 79.28 79.28 79.28 79.28 |
| 16: E.coli_plasmid_DNA_for_formaldehyde-dehydrogenase 38.38 38.10 38.10 38.10 38.10 72.79 74.05 74.05 74.14 74.05 75.50 77.57 75.05 76.22 78.92 100.00 80.72 80.72 80.72 80.72 76.58 76.58 76.58 76.58 76.58 |

| |
|---|
| 17: Escherichia_coli_strain_TW14359 39.71 39.49 39.49 39.49 39.49 73.78 74.23 74.23 74.23 72.88 73.87 76.31 71.98 76.22 77.75 80.72 100.00 100.00 100.00 100.00 80.36 80.36 80.36 80.36 80.36 |
| 18: Escherichia_coli_strain_TB182A 39.71 39.49 39.49 39.49 39.49 73.78 74.23 74.23 74.23 72.88 73.87 76.31 71.98 76.22 77.75 80.72 100.00 100.00 100.00 100.00 80.36 80.36 80.36 80.36 80.36 |
| 19: Escherichia_coli_strain_87-14 39.71 39.49 39.49 39.49 39.49 73.78 74.23 74.23 74.23 72.88 73.87 76.31 71.98 76.22 77.75 80.72 100.00 100.00 100.00 100.00 80.36 80.36 80.36 80.36 80.36 |
| 20: Escherichia_coli_strain_86-24_alcohol_dehydrogenase_class_III 39.71 39.49 39.49 39.49 39.49 73.78 74.23 74.23 74.23 72.88 73.87 76.31 71.98 76.22 77.75 80.72 100.00 100.00 100.00 100.00 80.36 80.36 80.36 80.36 80.36 |
| 21: Photobacterium_damselae_subsp.piscicida_plasmid_pP9014 41.42 40.83 40.83 40.83 40.83 75.05 73.33 73.33 73.24 73.51 74.77 74.95 71.32 77.30 79.28 76.58 80.36 80.36 80.36 80.36 100.00 100.00 100.00 100.00 100.00 |
| 22: Escherichia_coli_plasmid_pE66An 41.37 40.77 40.77 40.77 40.77 75.05 73.33 73.33 73.24 73.51 74.77 74.95 72.70 77.30 79.28 76.58 80.36 80.36 80.36 80.36 100.00 100.00 100.00 100.00 100.00 |
| 23: Photobacterium_damselae_subsp._piscicida_plasmid_pP9014_DNA_complete_seq 41.37 40.77 40.77 40.77 40.77 75.05 73.33 73.33 73.24 73.51 74.77 74.95 72.70 77.30 79.28 76.58 80.36 80.36 80.36 80.36 100.00 100.00 100.00 100.00 100.00 |
| 24: _Photobacterium_damselae_subsp._piscicida_plasmid_pP99018_DNA__complete_sequence. 41.37 40.77 40.77 40.77 40.77 75.05 73.33 73.33 73.24 73.51 74.77 74.95 72.70 77.30 79.28 76.58 80.36 80.36 80.36 80.36 100.00 100.00 100.00 100.00 100.00 |
| 25: Enterobacter_cloacae_plasmid_pEC-IMP__complete_sequence**1__1210bp_ 41.32 40.75 40.75 40.75 40.75 75.05 73.33 73.33 73.24 73.51 74.77 74.95 69.54 77.30 79.28 76.58 80.36 80.36 80.36 80.36 100.00 100.00 100.00 100.00 100.00 |

**Table 3.2 – Protein ENA/NCBI Accession numbers.**

Accession numbers for AA sequence and name of protein. Sequences are a result of translation of the 25 nucleotide sequences used to create first generation primers.

| Protein ENA/NCBI Accession Number | Protein ENA/NCBI Accession Number | Protein ENA/NCBI Accession Number | Protein ENA/NCBI Accession Number | Protein ENA/NCBI Accession Number |
|---|---|---|---|---|
| AF130307 | YP_001102243.1 | BAM31234.1 | AFJ27445.1 | CCN79918.1 |
| ADF47466.1. | AEK68083.1 | AFH95945.1 | AFJ27445.1 | CCN79918.1 |
| ACN66820.1. | AEK68083.1 | CBJ81804.1 | CAA52057.1 | BAH83624.1 |
| ABG56790.1 | WP_000842142.1 | XP_001193488.1 | AFJ27445.1 | BAH83624.1 |
| YP_002995631.1 | XP_001193488.1 | AFJ27445.1 | AFJ27445.1 | ACO54013.1 |

**Figure 3.1 – Phylogram and grouping of organisms which S-GSHDH-g sequences were used to create first generation primers.**

25 organisms which were used in the production of first generation primers. Sequences were aligned and a phylogram was created based on sequence similarity. A total of 5 groups were created.

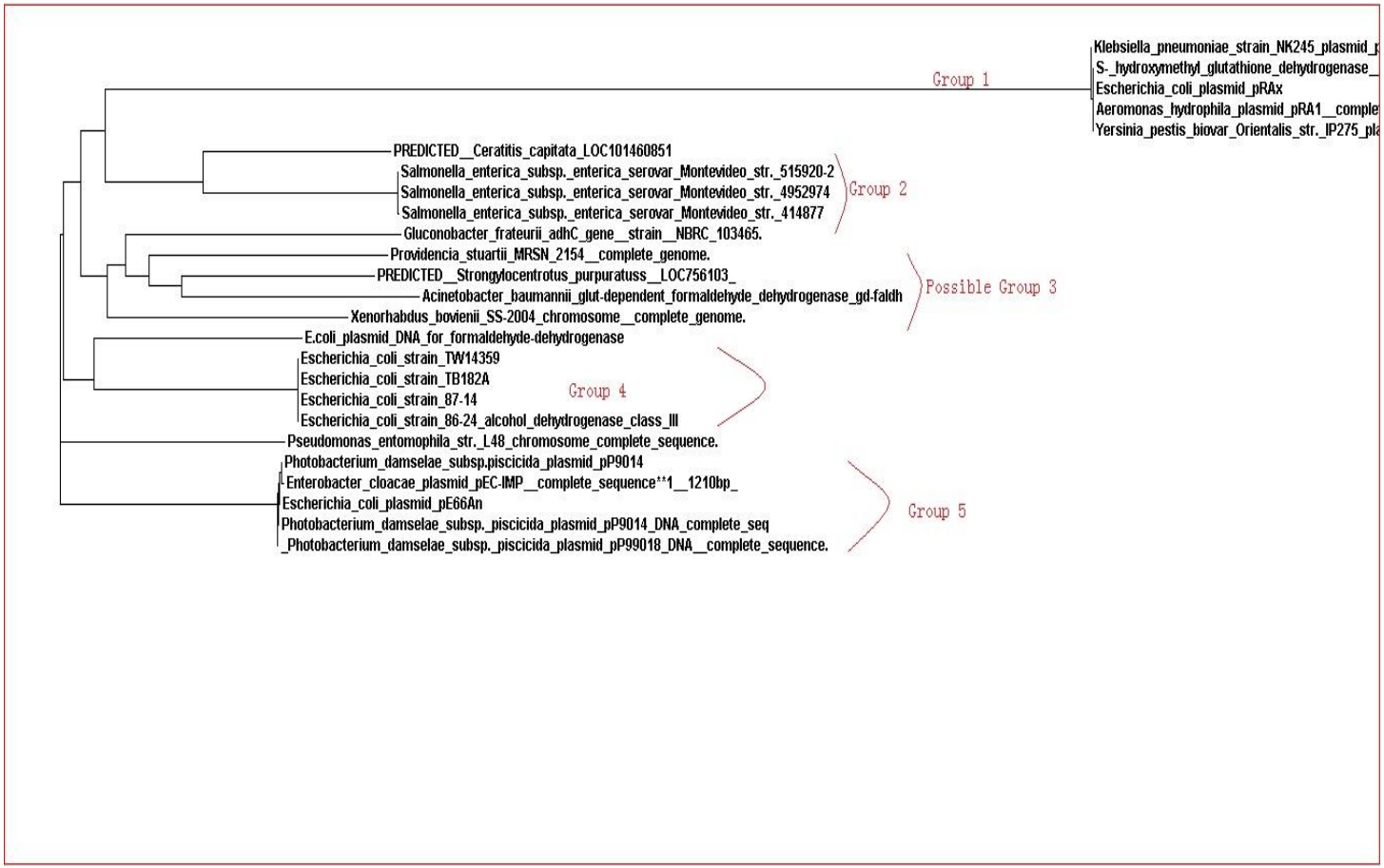

Group 1
Klebsiella_pneumoniae_strain_NK245_plasmid_p
S-_hydroxymethyl_glutathione_dehydrogenase_
Escherichia_coli_plasmid_pRAx
Aeromonas_hydrophila_plasmid_pRA1__comple
Yersinia_pestis_biovar_Orientalis_str._IP275_pla
PREDICTED__Ceratitis_capitata_LOC101460851
Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._515920-2
Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._4952974
Salmonella_enterica_subsp._enterica_serovar_Montevideo_str._414877
Group 2
Gluconobacter_frateurii_adhC_gene__strain__NBRC_103465.
Providencia_stuartii_MRSN_2154__complete_genome.
PREDICTED__Strongylocentrotus_purpuratuss__LOC756103_
Acinetobacter_baumannii_glut-dependent_formaldehyde_dehydrogenase_gd-faldh
Possible Group 3
Xenorhabdus_bovienii_SS-2004_chromosome__complete_genome.
E.coli_plasmid_DNA_for_formaldehyde-dehydrogenase
Escherichia_coli_strain_TW14359
Escherichia_coli_strain_TB182A
Escherichia_coli_strain_87-14
Escherichia_coli_strain_86-24_alcohol_dehydrogenase_class_III
Group 4
Pseudomonas_entomophila_str._L48_chromosome_complete_sequence.
Photobacterium_damselae_subsp.piscicida_plasmid_pP9014
Enterobacter_cloacae_plasmid_pEC-IMP__complete_sequence**1__1210bp_
Escherichia_coli_plasmid_pE66An
Photobacterium_damselae_subsp._piscicida_plasmid_pP9014_DNA_complete_seq
_Photobacterium_damselae_subsp._piscicida_plasmid_pP99018_DNA__complete_sequence.
Group 5

**Figure 3.2 – Agarose Gel Electrophoresis of S-GSHDH-g.**

Bands A, B, C, and D were amplified via primer G3_SGDHDP. Bands E, F, G, and H were amplified via primer G2. Template DNA from bands A & E was from sample CM7A. Template DNA from bands B, C, F, and G was from sample CM9B. DNA bands for D and H were from WCMB. Ladder markers were 100-1200bp in 100bp increments.

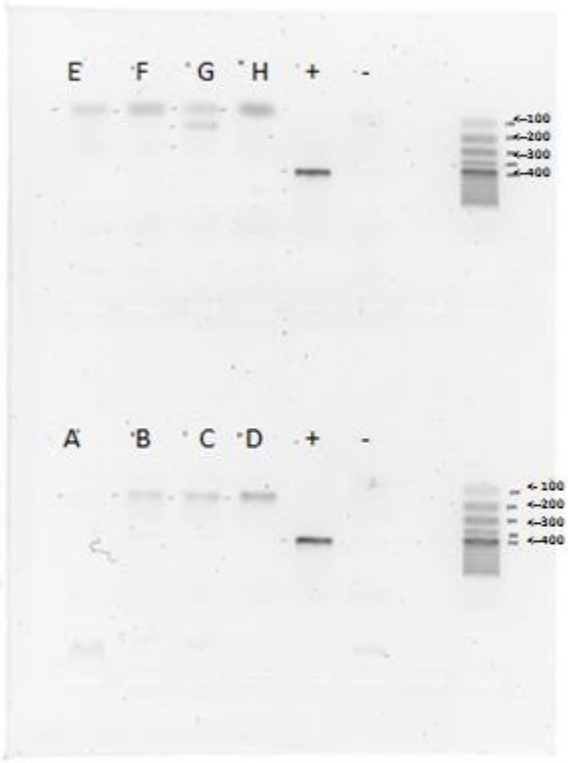

E
F
G
H
+
-
<-100
<-200
<-300
<-400
A
B
C
D
+
-
<-100
<-200
<-300
<-400

**Table 3.3 – Primer sequence and characteristics of both first generation and second generation primers.**

Primers were synthesized in pairs. A total of 18 primers were created over the course of this experiment. 8 primers were degenerate. 2 primers were SGP.

| PRIMER NAME | PRIMER DIRECTION – PRIMER LENGTH – ANNEALING TEMP – CG% - SEQUENCE – REVERSE COMPLIMENT (5'-3') |
|---|---|
| G1F1 | LEFT PRIMER 20 58.71 55.00 GTGTACAGCGGGATCACATG (CATGTGATCCCGCTGTACAC) |
| G1R1r | RIGHT PRIMER 20 58.94 50.00 TGTAGGCATCGGTATGGCAT (ATGCCATACCGATGCCTACA) |
| G2F1 | LEFT PRIMER 20 58.99 55.00 GTTGAGCTTACTGATGGCGG (CCGCCATCAGTAAGCTCAAC) |
| G2R1r | RIGHT PRIMER 20 59.03 50.00 GGCAGGAAATCAAAACCCGT (ACGGGTTTTGATTTCCTGCC) |
| G3_SGDHDP_F1[1] | LEFT PRIMER 19 - - AAATTGTTGAARTTGATGT |
| G3_SGDHDP_R1[1] | RIGHT PRIMER 20 - - ACDACVCCHGCVCCTTCATG |
| G3_SGDHDP_F2[1] | LEFT PRIMER 17 - - GGTGATCAYGTDATYCC |
| G3_SGDHDP_R2[1] | RIGHT PRIMER 17 - - TATTCRCTRAANGTDGA |
| G3_SGDHDP_F3[1] | LEFT PRIMER 17 - - GAAATGACWGRMTGGGG |
| G3_SGDHDP_R3[1] | RIGHT PRIMER 20 - - CCTTTVACHCCYCCAAAHGC |
| G3_SGDHDP_F4[1] | LEFT PRIMER 16 - - CHACNTTYAGYGAATA |
| G3_SGDHDP_R4[1] | RIGHT PRIMER 17 - - ATACCVCCKARVCCAAA |
| 2G1F[2] | LEFT PRIMER 20 60.33 45.00 TGGTAAATCGGCTCACCATT (AATGGTGAGCCGATTTACCA) |
| 2G1Rr[2] | RIGHT PRIMER 20 60.01 50.00 CCGGGTGATCATGTTATTCC (GGAATAACATGATCACCCGG) |
| G4F1 | LEFT PRIMER 20 59.05 50.00 AAACCATGAACACGTCTGCC (GGCAGACGTGTTCATGGTTT) |
| G4R1r | RIGHT PRIMER 20 58.86 55.00 CGAAAGCGGGACGGATTATC (GATAATCCGTCCCGCTTTCG) |
| G5F1 | LEFT PRIMER 20 58.75 50.00 CATCCAAGTTTGAGCTGGCA (TGCCAGCTCAAACTTGGATG) |
| G5R1r | RIGHT PRIMER 20 58.99 50.00 ATGTCAATGTGATGCGGTCG (CGACCGCATCACATTGACAT) |

[1] – First generation degenerative primers.

[2] –Second generation degenerative primers, synthesized based on successful PCR amplification of 1st generation primers.[1]

# Discussion

## 4.1 Primer Efficiency

As stated in the objectives, a quantitative analysis S-GSHDH-g modulation over time would have been optimal. A common technique for this is qPCR, which requires the creation of standard curves. These standard curves require primers which have high efficiency in template DNA replication. However when primers were designed and PCR products were sequenced, only low efficiency in successful target gene insert was observed. This stifled the experiment and resulted in the need for SGP. Insert efficiency for SGP was also very low. This limited the use of quantitative methods for gene tracking.

## 4.2 Presence of S-GSHDH-g

The presence of S-GSHDH-g was confirmed within the AB. Two of the primer groups worked for CM9 samples and only one for CM7. There is S-GSHDH-g present in the system. The intensity of the gel electrophoresis bands increased for samples with MDF present, this can be seen in Figure 3.2.

This difference in band intensity can be used as a semi quantitative form of analysis. However, because PCR templates amplify exponentially relative differences within the band intensities correlate toward the relative differences in template DNA (Bio-Rad, 2014). These band intensities will eventually plateau at a maximum intensity due to the depletion of PCR reagents. However, with the intensity further increased in WCMB this is unlikely.

If formaldehyde is the primary carbon source within AB then organisms, which can efficiently metabolize this molecule will thrive and proliferate. This competitive advantage will result in a higher amount of S-GSHDH-g containing DNA present within the system.

If these amplified DNA products contain a higher quantity of gene presence it is likely due to the S-GSHDH expressing microbes thriving. Microbes in the presence of MDF may be acclimatizing to the nutrients available in the system (Tilman, 1977). If formaldehyde is their major carbon source this can explain for the increase in genomic DNA containing S-GSHDH-g. This is known as the resource ratio competition model (Hibbing, et al., 2010). The microorganisms which are successful at using formaldehyde will thrive during the detoxification process. S-GSHDH-g increase when formaldehyde is the major food source supports the trends described in the resource ratio competition model.

Although it cannot be concluded that GSH dependent NAD linked pathways are the only processes used in this system, there is evidence to suggest that it is one of the pathways used. The use of quantitative analysis of gene expression is the next step in strengthening this hypothesis. Much of the work done for this thesis is preliminary work, the first of its kind of this brand new model. Important foundation has been laid for further studies and advancements into this complex system and all the interactions occurring.

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