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The Design of a PCR-Based Assay to Detect and Isolate the Serine Palmitolytransferase Gene From Environmental Bacteria

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THE DESIGN OF A PCR-BASED ASSAY TO DETECT AND ISOLATE THE SERINE PALMITOYLTRANSFERASE GENE FROM ENVIRONMENTAL BACTERIA

By

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ABSTRACT

LANA LEIGH TAYLOR: The Design of a PCR-Based Assay To Detect and Isolate the Serine Palmitoyltransferase Gene from Environmental Bacterial

(Under the Direction of Paul Boudreau)

Sphingolipids are a natural class of lipids that function as structural elements of cell membranes and signaling molecules for important cellular activities such as cell growth, differentiation, apoptosis, recognition, and adhesion. These lipids can be found universally in eukaryotic cells as well as some species of bacteria, such as those found in the human gut microbiome and in the environment in soils. Though sphingolipid production is rare in bacteria, both eukaryotic and prokaryotic sphingolipid biosynthesis begin with the condensation of serine and palmitoyl CoA into 3-ketodihydrosphingosine catalyzed by the enzyme serine palmitoyltransferase (SPT). In recent years, several studies have shown the connection between sphingolipid homeostasis and various diseases such as Alzheimer's disease and hereditary sensory neuropathy type I disease. Furthermore, these lipids play a significant role in the innate immune system as mediators of the inflammatory response and the CD1d pathway. These various biological functions and ready access to the compounds from bacterial producers have presented sphingolipids as possible targets for therapeutic development. However, our knowledge of bacterial sphingolipids and their therapeutic potential is limited. The purpose of this research is to develop an effective means of identifying environmental sphingolipid producers for further analysis of the structure of their sphingolipids and characterization of their bioactivity. Because the SPT gene is a commonality between all sphingolipid producers, our research has centered around creating a set

of polymerase chain reaction (PCR) primers that can accurately test for the presence of this gene amongst bacterial isolates from soil. Using previous literature and our own sequencing data, we have developed a set of primers that can detect the presence of the SPT gene in species of *Sphingomonas* bacteria and some species of *Novosphingobium*, both known sphingolipid producers. Additionally, our team has been working on using various methods of selective culturing to add to the efficiency of the screening process and optimize the procedure of finding environmental sphingolipid producers. Future work on this project will continue to analyze genetic sequences to create a complete set of primers that can isolate naturally occurring sphingolipid producers across several different species. Overall, we aim to create a screening technique that is efficient and useful in discovering more sphingolipid-producing bacteria from the environment with the hopes of isolating novel sphingolipids for development as medicines.

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LIST OF ABBREVIATIONS

SPT	Serine Palmitoyltransferase
PCR	Polymerase Chain Reaction
iNKT	Invariant Natural Killer T Cells
HSN1	Hereditary Sensory Neuropathy Type 1
AD	Alzheimer's Disease
B-3126	*Sphingomonas echnoids*
PBI8A	Novosphingobium Soil Isolate #1
PBI8H	Novosphingobium Soil Isolate #2
Milli Q	Nuclease-Free Water
kb	Kilobases

INTRODUCTION

Sphingolipids are a diverse class of lipids that are found in all eukaryotic cells and some prokaryotic cells.[1] The roles of sphingolipids in eukaryotic cells are numerous. They are most often noted as structural components of cell membranes, but they also function as intracellular and extracellular signaling molecules to mediate processes like cell cycle progression, apoptosis, and endocytosis.[1,2] Furthermore, eukaryotic organisms use these lipids, commonly ceramide or sphingosine-1-phosphate, to induce immune responses and recognize pathogens. Eukaryotic cells can modulate the reorganization of the cellular membrane using sphingolipid products, thus controlling the internalization of bacteria.[2] Production of sphingolipid enriched rafts also facilitates the fusion and degradation of extracellular pathogens.[3] Once the bacterium has been internalized, sphingolipids are also implicated in the release of cytokines, the inflammatory response, and the initiation of apoptosis of the infected cell by multiple different mechanisms.[2,4] Observations that these lipids play a significant role in the immune response have pointed to them as possible therapeutic targets in inflammation and pathology regulation.

Sphingolipid production is much less common in prokaryotes, with only a small subset of bacteria being known producers. Currently, the known sphingolipid-producing bacteria include the majority of the Bacteroides phylum, members of the Chlorobiota phylum, and a few species from the Alphaproteobacteria and Deltaproteobacterium.[5] Just as in eukaryotes, bacterial sphingolipids also function as an essential outer membrane component.[6] This is a distinguishing feature among sphingolipid-producing bacteria, as most bacteria have outer membranes composed only of glycolipids. Despite a small subset of species capable of making

sphingolipids, these producers can be found throughout the biosphere. Species such as *Acetobacter, Sphingomonas,* and *Novosphingobium* are commonly found in environmental soil or marine environments.[5] The *Bacteroides* are sphingolipid-producing bacteria which are key members of the human gut microbiome. In more recent years, it has been implicated that bacterially produced sphingolipids play a role in the relationship of the microbe and the host. For example, it was found that the presence of *Bacteroides* sphingolipids negatively correlated with inflammation in the intestinal tissue of inflammatory bowel disease patients.[6] The connection between sphingolipids and host cell reactions implicates sphingolipids as possible therapeutic targets. Additionally, the frequent appearance of bacterial sphingolipid producers in the environment easily enables their natural products to be gathered and isolated for further investigation of their bioactive properties.

The Structure and Biosynthesis of Sphingolipids:

The synthesis of all sphingolipids can be broken down into three parts. First is the creation of the sphingoid backbone by SPT, followed by the attachment of an amide-linked fatty acid, and finally, the generation of complex sphingolipids by the addition of head groups to the C1 terminal of the sphingoid base.[7,8] The first step of *de novo* sphingolipid synthesis is highly conserved across both prokaryotic and eukaryotic organisms. As seen below in **Figure 1,** it begins with serine palmitoyltransferase (SPT) condensing serine and palmitoyl coenzyme A into 3-ketodihydrosphingosine to create a sphingoid base, or backbone, for the lipid.[3] Because of the prevalence of SPT, this enzyme, or rather the genes that code for this enzyme, make for an identifying trait for all sphingolipid-producing organisms. However, there are some notable differences in SPTs across different organisms: in bacteria, the location of this enzyme is in the

cytoplasm for bacteria, whereas mammals, plants, and fungi will have the enzyme localized in the endoplasmic reticulum.[7] Bacterial SPT enzymes form a homodimeric complex, whereas fungal and mammalian SPTs are heterodimeric.[7] The eukaryotic forms of SPT have very little tolerance for substrates other than palmityl coenzyme A and L-serine compared to the prokaryotic SPT that exhibited tolerance to a range of CoA substrates.[7]

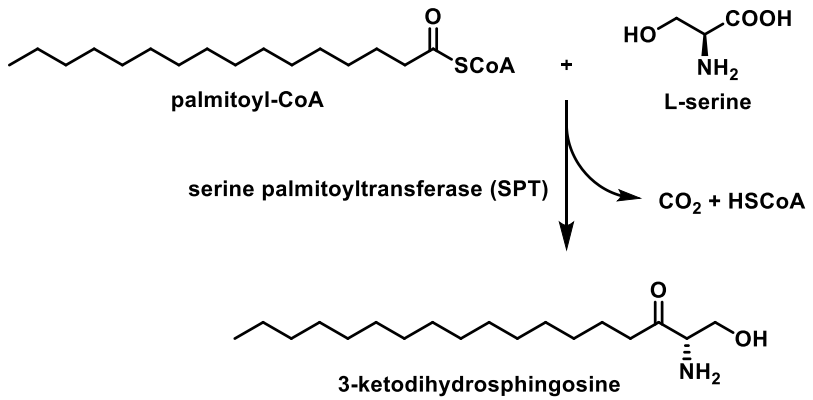


Figure 1: The first step of *de novo* sphingolipid production catalyzed by the enzyme serine palmitoyltransferase (SPT).

The basic structure of a sphingolipid is a long-chain sphingoid backbone amide linked to a fatty acid with a polar head group connected to the C-1 terminal via an ester bond.[3] Sphingolipid bases can vary in length and number of double bonds and hydroxyl groups in the fatty acid chains. The polar head groups range from the unelaborated alcohol of ceramide to highly complex glycoconjugates with many sugars. The different sphingolipid bases are signature to phylogenetically different organisms.[1] For example, a sphingosine or dihyrosphinsosine backbone is typical of mammalian sphingolipids, while phytosphingosine is seen in many plants and fungi.[8] The complex polar head groups are often markers for particular cell types and variable functions, such as bonding with other carbohydrate groups on neighboring

cells.[1] Because of the vast number of possible sphingoid backbone and headgroups, there are thousands of sphingolipids available among the environment. Therefore, in developing a method to identify sphingolipid producers we targeted the conserved SPT gene, rather than approaches, such as LCMS metabolomics, which would have to identify common structural features across this diverse class. After isolating these species, we will be able to analyze all the different sphingolipids amongst our isolated producers from the environment to understand their functions and therapeutic potential.

The Role of Sphingolipids in Diseases:

In 1994, α-galactosylceramide, a glycosphingolipid from the marine sponge *Agelas mauritianus*, was identified as an antigen for invariant natural killer T (iNKT) cells during a cancer antigen screen.[9] Several years later, it was discovered that α-galactosylceramide produced by the intestinal microbe *Bacteroides fragilis* acts as an agonistic ligand in iNKT cells resulting in a reduction of colonic iNKT cells and protection from induced colitis in adult mice.[5] iNKT cells are unique immune cells closely related to lipid-driven disorders because they are sensitive to lipid antigens presented by CD1 proteins. They can release copious amounts of cytokines, recruit neutrophils, and exhibit regulation of the inflammatory response upon activation.[9,10] Since these observations, sphingolipids have gained much attention in the pharmaceutical industry because of their correlation with metabolic disorders.[11] In both humans and mice, *Bacteroides* produced sphingolipids were significantly decreased in the stool of individuals with inflammatory bowel disease, and the reduced sphingolipid levels are associated with increased inflammation in the gut.[6] Sphingolipids have proven to be very important in maintaining homeostasis within humans leading to the possibility of using these lipids in medicine. Because

of the ability to induce an immune response, glycosphingolipids could be applied as regulatory compounds to combat inflammatory disorders such as inflammatory bowel disease or ulcerative colitis.

Within the last twenty years, sphingolipids as a therapeutic target have also been a trending topic amongst the pharmaceutical community because the disturbance of the sphingolipid homeostasis has been linked to several neurological diseases. One of the most relevant instances has been hereditary sensory neuropathy type 1 (HSN1), an autosomal dominant genetic disorder characterized by the degeneration of motor neurons in the limbs and extremities, leading to loss of pain and temperature sensation, atrophy, and weakness.[12] The causes of HSN1 have been liked to mutations in the SPTLC1 gene, which codes for one of the two subunits of the human SPT enzyme.[13] To date, there are at least nine mutations, with three of them being missense mutations, in the human SPTLC1 (LCBI) gene that are known to cause HSN1.[12–14] Because SPT is the first rate-limiting factor in all *de novo* synthesis of sphingolipids, the missense mutations cause an overall decrease in the SPT activity and *de novo* sphingolipid production. However, the mechanisms by which decreased SPT activity causes neuropathic symptoms are still unclear. Human and mice HSN1 studies revealed relatively stable levels of cellular sphingolipids in affected participants. This is likely due to the ability of cells to use the degradation of sphingomyelin to produce ceramides as compensation for decreased *de novo* synthesis.[12] Therefore, there is still much debate on how altered activity levels of the SPT are correlated with showing neuropathic symptoms. Sphingolipids are not only relevant to HNS1; they are implicated in the pathogenesis for other neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS), and Farber's diseases. As of 2021, AD was the most prevalent neurodegenerative disorder aside from

Parkinson's disease; however, the precise mechanism of development and maintenance for AD is still unknown.[15] Early reports suggest that genes controlling the synthesis of sphingolipids are upregulated, resulting in increased ceramide levels in the brain. This accumulation of ceramides could be a contributing factor to ER stress and neurodegeneration.[16] Furthermore, the sphingolipids found in neuronal plasma membranes are involved in the processing and aggregation of β-amyloid aggregates, which are known to be the main inducers of AD.[15] Because of the various roles sphingolipids play in the manifestation of these diseases, further investigation of sphingolipids as disease-regulating components could be very beneficial in combating these disorders and advancing medicine.

Aside from being possible inflammatory therapeutics, sphingolipids have also presented themselves as potential antibacterial agents. Because sphingolipid production is uncommon among bacteria, they have been shown to inhibit growth and kill many Gram-positive and Gram-negative bacteria.[2] For example, *Pseudomonas aeruginosa* is a primary cause of morbidity in cystic fibrosis patients, but it is inhibited by sphingosine, a commonly produced sphingolipid in mammals.[2] Furthermore, sphingolipids play an active role in the respiratory tract as they are constituents of the mucus secreted by the alveolar epithelium in the lungs. They function as surfactants, maintain barrier integrity, and aid in protecting tissues from invasion by pathogens like *P. aeruginosa* and *Staphylococcus aureus*.[2] The antibacterial properties of sphingolipids have been shown to work on several types of bacterial infections, and therefore, have the potential to be a new tool used to fight them. This potential usage is also significant in today's healthcare because novel therapies are needed to combat antibiotic-resistant infections.

Polymerase Chain Reaction for Screening:

This project aims to develop a screening technique to isolate environmental sphingolipid producers from soil. However, thousands of different species may be present in just 1 gram of soil.[17] Therefore, we have chosen to use a polymerase chain reaction (PCR) to identify sphingolipid producers amongst the thousands of other microorganisms living in the environment. In 2010, a novel selective growth medium - PCR assay was developed as a means to isolate *Sphingomonas* species from environmental soils. Two sets of PCR primers were created that can accurately detect the SPT gene in over 70 different species of *Sphingomonas*.[18] Using this work as a basis for this research, I hypothesized that a set of similar primers that can amplify the SPT gene from a larger subset of sphingolipid producers can be made. Our hopes for this project are to make a PCR assay that can successfully detect the SPT gene in most environmental sphingolipid producers, such as species of *Sphingomonas*, *Zymomonas*, and *Novosphingobium.*

The PCR tests are used to detect the presence of the SPT gene, and the reaction will amplify that segment of DNA for sequencing and identification. Since all sphingolipid producers will contain the SPT gene, the sequence for the SPT gene is used as an identifying factor for our screening technique. There are several benefits to using PCR over other screening methods. PCR is a very prompt reaction that takes little time to complete if done correctly. As opposed to other, methods such as 16S rDNA sequencing, where we would need to sequence each PCR product to identify known sphingolipid producers, the SPT screen will quickly eliminate other bacteria where there is no amplification because there is no SPT gene. The screening technique is also very efficient because only a small amount of each primer, polymerase, and DNA sample are needed to get good results. This is especially beneficial for screening soil isolates because there

may be a smaller presence of sphingolipid-producing bacteria compared to other species, necessitating screening many colonies. Lastly, PCR is highly accurate because the primers can only adhere to and amplify very specific sequences of DNA. This research has centered around designing a PCR reaction that can rapidly identify sphingolipid producers across multiple species.

MATERIALS AND METHODS

Bacterial Strains and DNA Isolation:

Three known sphingolipid producers were used for the duration of the primer design and optimization. *Sphingomonas echinoids* (referred to as B-3126) culture was obtained from Agricultural Research Service (ARS) culture collection, and two strains of *Novosphingobium* (PBI8A and PBI8H) which were isolated from the soil of a tomato plant by Dr. Paul Boudreau. These strains were grown on a minimal defined medium, modified from *Acidovorax* Complex Medium (MACM) with 1x nystatin at room temperature. All three strains were kept for long-term storage at -70 ℃ in 1:1 media to 50% glycerol stock. The genomic DNA of each strain was isolated using the E.Z.N.A bacterial genomic DNA kit (Omega Bio-Tek). The protocol from the manufacturer was followed directly, except that the lysis steps of the bacteria were altered. The optional lysis step using glass beads was not performed, and the lysozyme was allowed to digest for 30 minutes at 37 ℃ instead of 10 minutes as the protocol suggests. Using this protocol, approximately 150 µL of genomic DNA diluted in elution buffer was recovered. Using the Invitrogen Qubit dsDNA high-sensitivity assay, the concentration of DNA for each DNA isolation was measured. B-3126 had a concentration of 260.0 ng/µL, PBI8A had 37.7 ng/µL, and PBI8H had 7.7 ng/µL. It was noted that PBI8H had a very low concentration of DNA, so the DNA isolation was repeated to get a new concentration of 39.7 ng/µL. All samples were stored in a -20 ℃ freezer for future use.

Development of the PCR Primers and Protocol:

The first sets of PCR primers were generated based on the sequences of *S. paucimobilis, S. mutlivorum,* and *S. spiritvorum* using the NCBI Primer-BLAST tool.[11] For each species, the accession number was used to download the nucleotide sequence for the SPT gene from GenBank. Using the tool, the targeted amplicon size was set to 1000 – 1200 bp, and the number of returned primers pairs was limited to 3. Geneious software was then used to compare the DNA sequence of the *S. paucimobilis* SPT to other species of *Sphingomonas* bacteria to create primers that worked across this whole clade. Comparing their sequences revealed a region of high homology across the interior portion of the SPT gene. Using these nucleotide sequences, two forward primers (LT 01 and LT 03) and seven reverse primers were generated (LT02, LT04, LT05, LT06, LT07, LT 08, and LT 09) and are shown in **Table 1.** Some of the reverse primers were designed with each possible stop codon, then mixed together to make reverse primer combinations to test with each of the forward primers. LT04, LT05, LT06 were combined together to create a reverse primer mixture REV2, and LT05, LT06, and LT 07 were also combined together to create a reverse primer mixture REV3. Various combinations of these primers were used in a PCR reaction to determine their ability to amplify the SPT gene from PBI8A, PBI8H, and B-3126.

After the failure of these initial sets of primers, three more forward primers, and two reverse primers were developed. Using Geneious software, known sequences of the SPT gene from NCBI blast data were pulled and aligned. The interior regions of the gene were highly conserved, and so three new forward primers (LT10, LT11, and LT12) and one reverse primer (LT 13) were created (see **Table 1**). Each of the forward primers was used in with one of the

reverse primers in a PCR reaction to determine their ability to isolate and amplify the SPT Gene from PBI8A, PBI8H, and B-3126.

To determine the proper annealing temperature of each of the primer sets, the NEB Tm calculator tool was used; in the settings, the polymerase was set as Q5, the protocol/kit as Q5 2x Master Mix, and the primer concentration to 100 nM. Each primer pair was entered into the calculator to get an estimated annealing temperature while noting any issues the tool raised. A PCR reaction was carried out using a temperature gradient to test these calculations. In a single PCR tube, 1.20 µL of one forward primer, 1.20 µL of reverse primer, 1.20 µL of genomic DNA (from B-3126, PBI8A, or PBI8H), 26.4 µL of nuclease-free water (Milli Q), 30.0 µL of Q5 2x Master Mix (New England Biolabs) all were added into one PCR tube and split into five samples of 12.0 µL each. The thermocycler was programmed to do the annealing step at various temperatures around the calculated annealing temperature from ca. 55 - 65 °C (specifically 54.4 °C, 55.2 °C, 59.0 °C, 63.9 °C, and 65.6 °C). The reaction started with an initial denaturation at 98 °C for 30 seconds, followed by 35 cycles of 10 seconds at 98 °C, 20 seconds at the annealing temperatures, and, lastly, 35 seconds at 72 °C, after these cycles there is a final extension step at 72 °Cf for 2.0 minutes and a then hold at 4 °C.

Table 1: Generated PCR Primers and their sequences. This table also includes the Primer Number, Primer Name, Direction, and Sequence of each primers used through the duration of these experiments.

Primer Number	Primer Name	Direction	Sequence
LT01	Sph FWD1	Forward	ATGWSSAAYTTCGARGGSCTSATCGC
LT02	Sph REV	Reverse	AGSRMATSYCHCCSYRBSDVRGNCC
LT03	Sph REV2	Forward	ATGACCGAAGCCGCYGCYCAGCCC
LT04	Sph REV2A	Reverse	TCAGCCGATCACGCCGACGGCCTGGCCCGC
LT05	Sph REV2B	Reverse	CTAGCCGATCACGCCGACGGCCTGGCCCGC
LT06	Sph REV2C	Reverse	TTAGCCGATCACGCCGACGGCCTGGCCCGC
LT07	Sph REV3A	Reverse	TTAGCCGATCACGCCGACGGCCTGGCCCGC
LT08	Sph REV3B	Reverse	TCAGCCGATSACKCCSACSGCCYGRCCSGC
LT09	Sph REV3C	Reverse	TTAGCCGATSACKCCSACSGCCYGRCCSGC
LT10	Sph Interior FWD1	Forward	CGATCGTGATGGA
LT11	Sph Interior FWD2	Forward	CGATCGTGATGGA
LT12	Sph Interior FWD3	Forward	CTCGACGCCGACAGCCA
LT13	Sph Interior REV1	Reverse	GAGCAGCGCAGCAG

A 1% agarose gel was made by adding 1.0 g of agarose to 100 mL of 1X TAE buffer. The solution was heated in the microwave until all the agarose was dissolved, being careful not to boil the solution. Once cool enough to handle, 5.0 µL of ethidium bromide (Sigma Aldrich 10

mg/mL in H_2O Ethidium Bromide Solution) was added, and the gel was poured into a mold and allowed to solidify. 2.5 µL of 6x purple loading dye with no sodium dodecyl sulfate (SDS) was added to each sample before loading. The 5 PCR products were run on the 1% agarose gel for 30 minutes at 1000 volts (V) against New England Biolab's 1 kb Plus DNA Ladder. Once the PCR reaction was finished, the agarose gel was viewed under ultraviolet light to determining the optimal annealing temperature of each set of primers. Comparing the presence of off target amplicons, as assessed by the presence of multiple bands, and yield, as assessed by brightness of the desired band, were the metrics used.

PCR Protocols:

Three separate PCR protocols were used in order to determine if the generated primers were capable of isolating and amplifying the SPT gene from each of our Bacterial strains. The Q5 SPT PCR protocol was used to decide which set of primers were best suited to amplify the SPT gene. Each primer pair was used in a PCR reaction with each of the bacterial strains B-3126, PBI8A, and PBIH8. The reaction mixture contained 1.20 µL of DNA, 1.20 µL of forward and reverse primer, 30.0 µL of Q5 2x Master Mix, and 26.4 µL of Milli Q water. The mixture was mixed thoroughly by pipette and split into five smaller samples of 12.0 µL each. The thermocycler was programmed for a 30-second initial denaturation at 94 ℃ followed by 35 cycles of denaturation at 94 ℃ for 10 seconds, annealing at the optimal temperature for 20 seconds, and the extension at 72 ℃ for 35 seconds. Lastly, there is a final extension at 72 ℃ for 2 minutes and then the reaction is held at 4 ℃.

The M13 PCR protocol was used to verify that the gene of interest was isolated by each primer set and successfully inserted into the pCR®4 Blunt-TOPO® plasmid and taken up by

TOP10 Chemically Competent *E. coli* cells. A new PCR reaction is performed using 1.0 µL of M13 Forward, 1.0 µ µL of M13 reverse, 34.0 µL of Milli Q, and 40.0 µL of OneTaq 2x Master Mix (New England BioLabs), all components were mixed and further divided into 9.5 µL aliquots in separate PCR tubes. Into every tube, 0.5 µL of a cell dilution in sterile phosphate-buffered saline (PBS) was added. The thermocycler is programmed to do an initial denaturation at 94 ℃ for 8 minutes, followed by 30 cycles of 94 ℃ for 30 seconds, 45 ℃ for 20 seconds, and then 68 ℃ for 2 minutes, then a final extension at 68 ℃ for 5 minutes, and a final hold at 4 ℃.

The Q5 SPT Colonies PCR protocol was used to detect the SPT gene in environmental bacteria. A PCR reaction using these isolated strains of bacteria as a screening mechanism for the SPT gene. For the reaction, 1.5 µL of LT11, 1.5µL of LT13, 51 µL of Milli Q, and 60 µL of Q5 2x Master mix are all mixed and split into 12 x 9.5 µL samples. 0.5 µL from the cell dilutions is added to the one-sample and repeated for all ten cell cultures in each sample. A positive control using B-3126 genomic DNA and a negative control using purified water are made using the other two samples. The thermocycler was programmed to run an initial denaturation for 8 minutes at 98 ℃, followed by 35 cycles of 10 seconds at 98 °C, 20 seconds at 55 °C, and 35 seconds at 72 degrees, then a final extension at 72 ℃ for 2 minutes, and a final hold at 4 ℃.

Testing the Ability of the Primers to Amplify the SPT Gene:

To determine which set of primers were best suited to amplify the SPT gene, each primer pair was run at the optimal annealing temperature as determined above using the Q5 SPT PCR protocol with each of the bacterial strains B-3126, PBI8A, and PBIH8. The PCR products were then dyed and run on a 1% agarose gel with ethidium bromide for 20 minutes at 100V. The gel was then viewed under UV light to determine which primer pair was able to amplify the SPT

gene segment from each of the bacteria. The primer pair that had a product closest to 1 kb was used for the duration of the experiment, and the other products were discarded.

Next, a knife or razor blade was used to slice the DNA band at 1 kb out of the agarose gel. The DNA was cleaned and concentrated from the gel using the Zymoclean Gel DNA recovery kit. All steps were followed according to the manual except for a modification in the elution step. Instead of DNA elution buffer, 12 µL of nuclease-free water was added directly to the column matrix and allowed to incubate for 1 minute at room temperature. The column was put into a 1.5 mL microcentrifuge tube and spun for 30-60 seconds to elute the DNA. This step was repeated again using 8.0 µL of water, yielding 20 µL of eluted DNA.

To verify that the primers isolated the gene of interest from the genomic DNA, the PCR product was inserted into the pCR®4 Blunt-TOPO® plasmid and taken up by TOP10 Chemically Competent *E. coli* cells using the Invitrogen Zero Blunt® TOPO® PCR Cloning Kit for Sequencing. Per the provided protocol, 1.0 µL of salt solution, 1.0 µL of vector, and 4.0 µL of PCR product were carefully added into a PCR tube and incubated for 5 minutes at room temperature. All 6 µL were then transferred to a new microcentrifuge tube containing the *E. coli* and allowed to sit on ice for 15 minutes. Next, the cells were put onto a 42 ℃ heat block and heat-shocked for precisely 30 seconds to enable the uptake of the plasmid. The cells were then rested on ice briefly before being incubated for 1 hour at 37 ℃ with 250 µL of sterile S.O.C media. The newly grown *E. coli* cells were plated on lysogeny broth (LB) media with kanamycin for antibiotic selection and incubated at 37 ℃ overnight. Once colonies began to form, a colony was picked from the plate and put into 100 µL of sterile phosphate-buffered saline (PBS). These picked colonies were tested using the M13 PCR protocol as described above. Once completed, the PCR product is run on a 1% agarose gel for 20 minutes at 100 V to check for a product at the

desired length. Several more *E. coli* colonies are picked from the LB and kanamycin plate to be sent for sequencing of the plasmid via Sanger sequencing with the M13 primers within the TOPO vector using a commercial vendor (Genewiz). The resulting nucleotide sequences are analyzed using Geneious software by comparing our isolated sequences with SPT gene sequences of other bacteria from the literature.

Re-Optimizing the Primers:

To allow the PCR reaction to screen for a wider range of sphingolipid producers, LT11 and LT13 are combined with another set of primers created by Dr. Paul Boudreau. This combined set of primers is tested using PCR on PBI8H only. In one PCR tube, 5.0 µL of PAVL forward, 2.5 µL of PAVL reverse, 17.5 µL of Milli Q, and 25 µL of Q5 2x Master Mix are added to make 47.5 µL total. Into a new PCR tube, 9.5 µL of the mixture is added, and 0.5 µL of PBI8A is added as a positive control. The remaining 38 µL of the mixture is split equally into two PCR tubes, adding 1.0 µL of PBI8H genomic DNA to each sample. They are run in the thermocycler for an initial 30 seconds at 98 ℃, followed by 35 cycles of 10 seconds at 98 ℃, 20 seconds at 55 ℃, and 35 seconds at 72 ℃.

Gel electrophoresis was used to see if there is DNA amplification at the desired length of 1 kb. The samples were run using the same protocol as described above, using 1% agarose gel with ethidium bromide. The results were viewed under UV illumination. The band visible at 1 kb was cut out of the gel using a very sharp razor blade, and the DNA was extracted and cleaned using the Zymoclean Gel DNA recovery kit. All steps in the kit were followed according to the manufacturer's instructions until except the DNA elution step. Instead of using DNA elution buffer, 12.0 µL of MilliQ is added directly to the cotton at the bottom of the filter and allowed to

incubate at room temperature for 1 minute. The column is then placed into a clean microcentrifuge tube, spun for 30 seconds at 21.0 g, and the flow-through is kept. This adapted step is repeated, except with 8.0 µL of Milli Q. The result is 20 µL of eluted DNA.

To determine if these new sets of primers were capable of isolating the SPT gene from PBI8H, the PCR product is inserted into a vector plasmid and up taken by chemically competent TOP10 *E. coli* cells. These steps were carried out using the Invitrogen PCR cloning kit from Thermo Fisher Scientific, and all directions were followed as described in the manufacturer's protocol. The *E. coli* cells containing the vector plasmid are cultured on LB with kanamycin agar plates at 37 ℃ for 24 hours. Once cultured, several colonies are picked from the LB with kanamycin plate, and each is diluted into 100 µL of sterile PBS. PCR is run on the *E. Coli* cells containing the vector plasmid using the M13 PCR Protocol.

The finished products were run on gel electrophoresis using 1% agarose gel with ethidium bromide for 15-20 minutes at 100 V. The gel was viewed under UV light to analyze any amplified regions of DNA. For any colony with a band at 1 kb, the remaining PBS dilution was re-cultured in 2.0 mL of liquid LB with kanamycin in a 5 mL test tube and put on a shaker at room temperature. Once growth appeared, these cultures were sent to have the vector plasmid sequenced to determine if the new sets of primers could amplify and isolate the SPT gene from the original PBI8H strain.

Screening for Environmental Sphingolipid Producers:

To test the ability of LT 11 and LT 13 to identify environmental sphingolipid producers, the primers were used in a PCR reaction against bacteria that were isolated from the soil at various locations at the University of Mississippi. A small amount of soil was taken from the

flower beds in front of Thad Cochran Research Center, the Grove, the Circle, and the grass in front of Vaught-Hemingway Stadium. The samples were placed into a sterile test tube, sealed, and returned to the laboratory within 24 hours for processing. To isolate the bacteria from the soil samples, about 1.0 gram of soil was scooped out of the original sample and put into 1000 µL of sterile PBS media in a 1.5 mL microcentrifuge tube. The tubes were then vortexed for 10-15 minutes to suspend the soil in the media. Under sterile conditions, 10 µL of each cell suspension was pipetted onto a MACM with nystatin agar plate. A glass spreader was used to push the liquid around until it is absorbed into the medium. The following steps were repeated except pulling 50 µL of liquid for each cell suspension. The glass spreader was sterilized between each use to maintain sterility and prevent cross-contamination. All the plates were wrapped in parafilm and left to grow for at least 24 hours at room temperature.

Once colonies began to form on the plates, any colony that had similar morphology to the *Novosphingobium* and *Sphingomonas* colonies were picked from the plate using a sterile loop and restruck on a new MACM with nystatin plate. After new colonies formed on the plates, one from each was picked using a sterile loop and put into 100 µL of sterile PBS media. The presence of the SPT gene in each of these soil isolates was tested using the Q5 SPT Colonies PCR protocol. Once finished, the samples are run on gel electrophoresis on a 1% agarose gel with ethidium bromide for 15 minutes at 100 V. The gel is analyzed under UV light to determine if any amplification of the SPT gene had occurred in the reaction.

Tree Making

In the Geneious 2022.1.1 software, gene sequences for known bacterial SPT genes and putative SPT genes from closely related organisms, identified by BLAST searches for highly

homologous genes, were aligned to the Sanger sequences of the SPT genes isolated from B-3126 and PBI8A via MUSCLE alignment with a maximum of 8 iterations. The alignment was trimmed to the length of our interior sequences and the Geneious Tree Builder was used to make a phylogenetic tree of this alignment with the Jukes-Cantor genetic distance model using the Neighbor-Joining tree building method with a putative SPT gene sequence from *Bacteroides fragilis* Q1F2 as an outgroup with bootstrapping of 50,000 replicates and a support threshold for the tree of 50%.

RESULTUS AND DISCUSSION

The Initial Primer Sets:

The first primer sequences generated were done using the NCBI primer BLAST tool based on previously known sequences of the SPT gene from *S. paucimobilis, S. mutlivorum,* and *S. spiritvorum.*[11] Two forward (LT01 and LT03) and six reverse primer sequences (LT04, LT05, LT06, LT07, LT08, and LT09) were created and tested. For each forward and reverse primer set, the optimal annealing temperatures were determined experimentally. This was done to ensure that proper binding of the primers and amplification of the DNA could occur for every reaction. The forward primer LT01 was run on a PCR reaction either with REV2 or REV3 which are a combination of reverse primers. A PCR reaction using these primers on genomic DNA from each of our strains of bacteria was run with sample 1 at 54.4 ℃, sample 2 at 55.2 ℃, sample 3 at 59.0 ℃, and sample 4 at 63.9 ℃ and sample 5 at 65.6 ℃. Our gene of interest, the SPT gene, is predicted to be 1000 to 1200 base pairs long, meaning that a PCR product showing to be 1 to 1.2 kilobases (kb) long is desirable. When viewed on gel electrophoresis, the temperature that showed as the clearest and brightest band at the correct length was chosen as the optimal annealing temperature for that particular primer set. This reaction was repeated again for The forward primer LT03 with REV2. The optimal annealing temperature was determined for each one of the sets, as seen in **Table 2.**

Running LT01 and REV2 on PCR resulted in PBI8A having two bands: one at 1.2 kb and one slightly higher at 2 kb. PBI8H had no product, and B-3126 had a product around 3 kb whic

was discarded. Since 2 and 3 kb are slightly larger than desired, it could be inferred that the amplified region of DNA was not the SPT gene. However, the product at 1.2 kb could likely be the gene of interest. Therefore, the 1.2 kb PCR product was inserted into E. coli cells via plasmid vector so that the isolated DNA could be sequenced to be certain.

Forward primer LT03 and with REV 2 initially showed a PCR product around 1.2 kb for B-3126 and PBI8A. It also showed three bands for PBI8H, around 1 kb (low), one at 1.2 kb (middle), and one slightly above at 2 kb (high). To determine if these PCR products were the SPT gene, all of these products were spliced out of the gel, including each band from the PBI8H strain, and inserted into *E. coli* cells via plasmid vector. Before sending these off for sequencing, another PCR reaction was performed to verify that the transformed *E. coli* cells had the PCR products from the LT03 and REV2 reactions. The results of the PCR reaction were not as expected. Only one colony containing PBI8H (high) produced a product at the desired length. This meant that only one colony out of the 11 tested had the amplified region of DNA from the PBI8H strain. Therefore, those cells were saved and sent for sequencing to determine if the isolated DNA was our target gene.

LT01 and REV3 primers resulted in a PCR product at 1.2 kb for B-3126 genomic DNA. To determine if these primers had amplified the correct region of DNA, the PCR product was put into a vector plasmid and inserted into *E. coli* cells. PCR has performed again on selected cultures to confirm that the PCR product had been correctly inserted into the cells. Two cultures came back with a band at 1.2 kb after gel electrophoresis, so the remaining cells from these cultures were regrown and sent off for sequencing to determine if the isolated PCR product was our target gene.

The DNA sequences isolated from the LT01 and REV 2 PCR reaction, the LT03 and REV2 PCR reaction, and the LT01 and REV3 PCR reaction were analyzed using Geneious software. Already known sequences of the SPT were compared to our sequences to determine if the SPT gene had been isolated. There were few homologous regions in all of our DNA sequences compared with the literature strains. There were also premature stop codons in the sequencing data for many of our sequences when translated into protein. For these reasons, it was concluded that these sets of primers were not successful at isolating and amplifying the SPT gene from any of our bacteria. Since these initial primer combinations yielded a product around 1.2 kb after running their PCR products on gel electrophoresis; it likely meant that these primers are binding and amplifying a different region of DNA in PBI8A, PBI8A, and B-3126. The inability of these primers to amplify the gene of interest indicated that the sequences of the primers were not analogous with the sequence of the SPT gene and that different sets of primers were needed.

Successful Primer Set:

Using Geneious, aligning all the literature sequences of the SPT gene together revealed that there was a region of high homology on the interior portion of the gene. Therefore, three new forward primers (LT10, LT11, and LT12) and one new reverse primer (LT13) were generated using these regions. Using the consensus sequence, the regions with little to no ambiguity were targeted for the forward primer design. (see **Figure 2**) Similarly, the reverse primer LT 13 was designed and generated based on the consensus sequence with the least ambiguity. (see **Figure 3**) Because these new primers targeted the front and back interior region of the SPT gene, a slightly shorter PCR product around 1 kb would be produced.

Figure 2: Gene Sequence Alignment for Primer LT 11 Design. The alignment shows the highly homologous region on the front, interior portion of the SPT gene which was targeted by the Forward Primer LT 11.

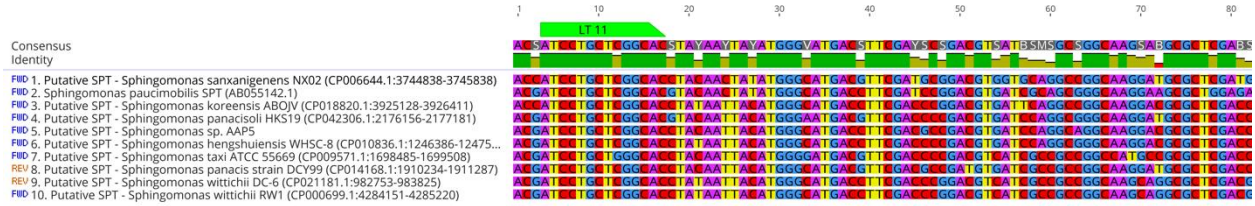
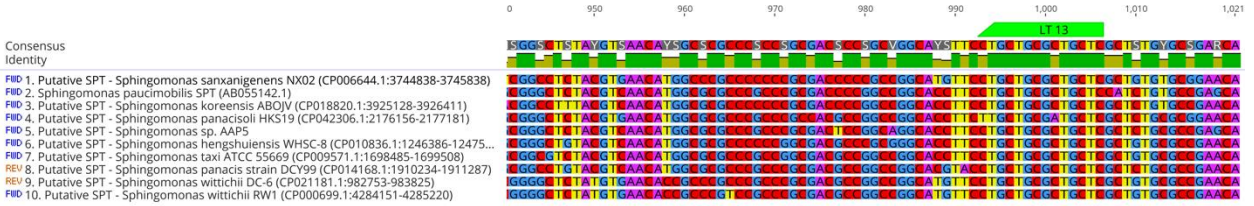


Figure 3: Gene Sequence Alignment for Primer LT13. The alignment shows the highly homologous region on the back, interior portion of the SPT which was targeted by Reverse Primer LT.



The first thing that was done was to generate and test for the optimal annealing temperatures for each forward primer with the reverse primer. For LT 12 and LT 13, the optimal annealing temperature was predicted to be 59 ℃ by the NEB Tm calculator. A PCR reaction using these primers on B-3126 genomic DNA was run on a temperature gradient in order to confirm the optimal temperature. After running the reaction of gel electrophoresis, no band was present indicating that these primers failed at all temperatures. For LT 11 and LT13, the NEB Tm calculator also predicted the optimal annealing temperature to be 59.0 ℃. A PCR reaction using LT11 and LT13 on B-3126 genomic DNA was carried out to verify this temperature on a temperature gradient from 55 ℃ to 65 ℃. The results of the reaction showed a singular clear band at 1 kb for the temperature at 54.5 ℃. (see **Table 2**) This slightly shorter length was

optimal since our primers were complementary to the interior portion of the SPT gene. Therefore, it could still be concluded that this PCR product may contain the gene of interest.

To confirm that the PCR reaction using LT11 and LT13 was successful at amplifying the SPT gene from B-3126, the PCR product was extracted from the gel, cleaned, and put into a plasmid vector for uptake by TOP10 *E. coli* cells. The plasmid-bearing cells were then sent off for sequencing so that the identity of the sequence could be confirmed. The results were as suspected; the isolated sequence was highly homologous with the other known sequences, meaning that the interior portion of the SPT gene had been isolated from B-3126. Similar PCR reactions were run on PBI8A and PBI8H using 55 ℃ for the annealing temperature. Water was used as a negative control whereas B-3126 was used as a positive control for this experiment. After running the samples on gel electrophoresis, PBI8A had a singular, distinct band at 1 kb just as B-3126 did. However, PBI8H did not have any clear amplification of the SPT gene on the gel electrophoresis.

The PCR product from PBI8A was extracted from the agarose gel and put into a plasmid vector for uptake by *E. coli* cells. The plasmid was again sequenced to confirm that it was the SPT gene that was isolated from the PBI8A genomic DNA. The sequence data when aligned with other SPT gene sequences also showed significant similarity and high homology. This analysis confirmed that the interior portion of the SPT gene was successfully isolated from PBI8A using LT11 and LT13 primers.

Table 2: The Results of the Generated Primer Sets. All of the tested PCR primer sets with their tested optimal annealing temperatures, approximate length of DNA that was amplified using the primers, which bacteria they worked on, and whether sequencing data confirmed the amplification of the SPT gene.

Primer Set	Optimal Annealing Temperature (°C)	Approximate Length of Band Observed (kb)	Bacterial Species	Sanger Sequencing Confirmation of the SPT Gene (Yes/No)
LT 01 & REV2 (LT04, LT05, LT06)	55.8	1.2, 2	PBI8A	No
LT01 & REV3 (LT07, LT08, LT09)	55.8	1.2	B-3126	No
LT03 & REV 2 (LT04, LT05, LT06)	63.8	1, 1.2, 2	PBI8A, PBI8H, B-3126	No
LT12 & LT13	N/A	N/A	none	No
LT11 & LT13	55.0	1	PBI8A, B-3126	Yes

Screening for Environmental Sphingolipid producers:

The purpose of developing primers for the SPT gene is to use them to identify sphingolipid producing bacteria from the environment. Therefore, LT11 and LT13 were used to screen isolated strains of bacteria from the soil from the various locations on the University of Mississippi campus. This was done in order to test the application of the primers, as well as to develop a systematic and efficient means to screen environmental bacteria.

To start the screening process, cells from the soil were suspended in sterile PBS and then cultured on MACM with nystatin on agar plates. Nystatin was used for the selection of bacteria from the soil by inhibiting the growth of fungi. After the first cultures were allowed to grow,

several different morphologies started to appear on the plates due to the soil containing an abundance of different bacteria. Any colonies that had a yellow or white-yellow color to them were picked off of the plate and restruck to get a pure isolated strain. This particular morphology was selected because it is distinctive of the *Sphingomonas* species and because our original bacterial strains also had this morphology.[18]

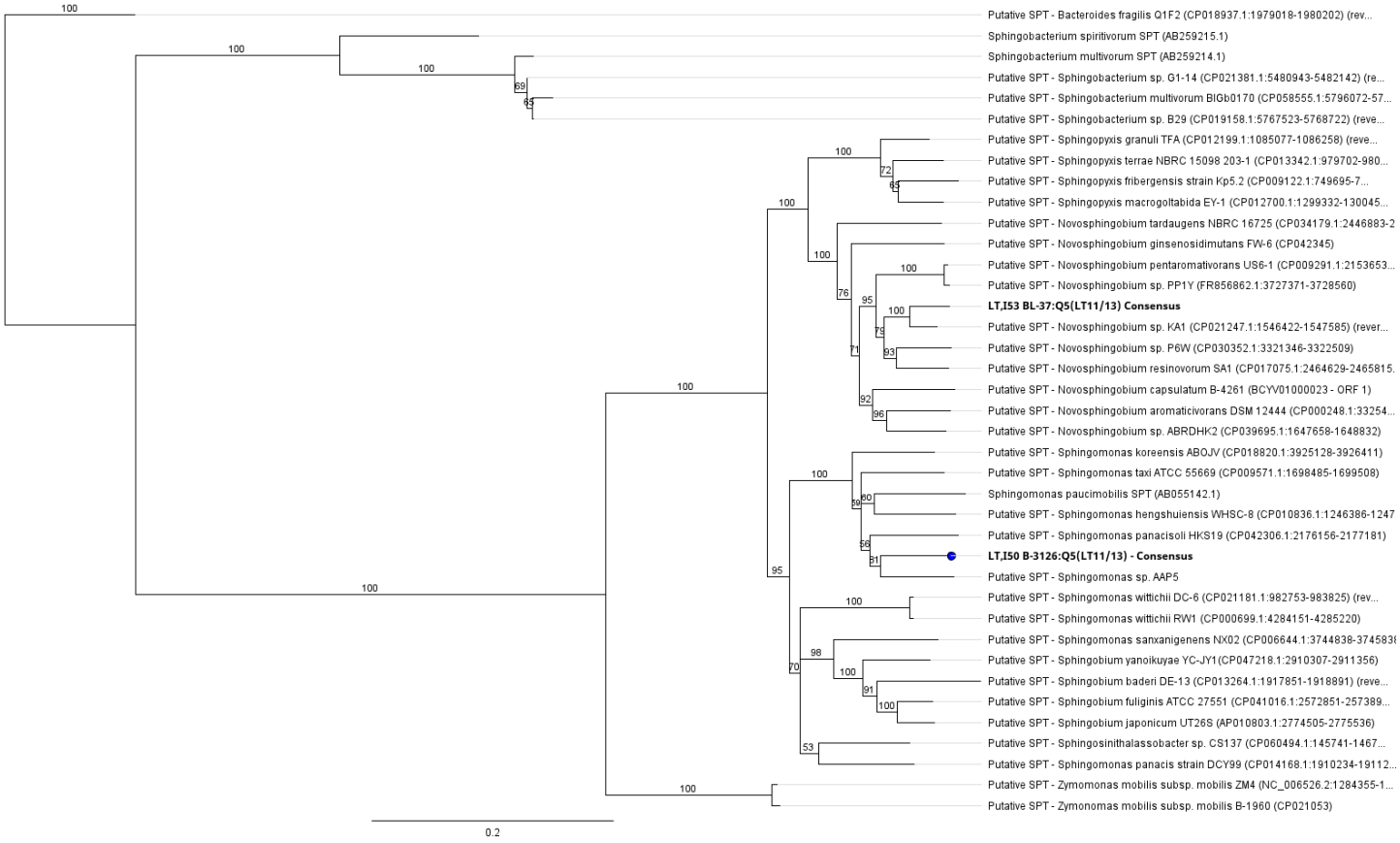
Once a pure strain with no other morphological differences appeared on the plates, the PCR protocol was carried out by using LT11 and LT13 to screen the unknown strains for the SPT gene. Two PCR screens were run on 23 total soil isolates. When viewed under gel electrophoresis, there was no DNA amplification that occurred at the optimal length of 1 kb for any of the soil isolates. There are a few possible reasons that our screening protocol did not detect positive hits from the soil isolates. The first reason is that sphingolipid-producing bacteria are relatively uncommon, so it could have been that the bacteria showing the selected morphology were simply not sphingolipid producers. Another possible explanation would be that our primers, LT11, and LT13, may not be effective in identifying all sphingolipid producers across various species. Upon examination of their SPT gene sequences in relation of other species, it reveals that they are more closely related to those of the *Sphingomonas* and *Novosphingobium* Genera. (see **Figure 4**) However, LT11 and LT13 were not successful in amplifying the SPT gene out of PBI8H which also belonged to the genus *Novosphingobium*. Therefore, it is likely that LT11 and LT13 are only capable of isolating the SPT gene from certain species of *Sphingomonas* and *Novosphingobium* as seen from the positive results with B-3126 and PBI8A.

The results of the PCR screening concluded that there needed to be adjustments made to the screening protocol. In order to streamline the culturing and PCR process, the use of liquid

cultures could be implemented to produce faster outputs and results. The tedious process of isolating a pure colony on an agar plate from the soil showed to be very time-consuming and slowed the screening process significantly. The use of liquid cultures in future screening experiments would likely aid in speeding up the process and yield a higher number of cultures tested. Liquid cultures are less labor-intensive because they would eliminate the need to create a cell dilution for every colony tested since the liquid cell cultures can be used directly in the PCR reaction. Furthermore, they may yield better results as more bacteria could be tested in a shorter amount of time, increasing the odds that there will be a positive result for an environmental sphingolipid producer.

The next step taken for the screening protocol was to find a primer set that will also isolate the SPT gene from PBI8H. Two new sets of forward and reverse primers were designed by Dr. Boudreau and combined with LT11 and LT13. These primers were tested in the same methods that the previous primers were. The results have yet to be determined.

Figure 4: A Phylogenetic Tree of the Sequenced SPT genes. The phylogenetic tree shows the relationship of PBI8A (LT,I53 BL-37:Q5 (LT11/13) Consensus) and B-3126 (B-3126 BL-37:Q5 (LT11/13) Consensus) to other known sphingolipid producers based on their SPT gene sequences.



CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of this project was to develop an effective method to identify environmental sphingolipid producers to analyze the structures of their sphingolipids and characterize their function and activity. The majority of this thesis centers around using PCR as a screening tool by creating a set of primers capable of amplifying the SPT gene. The findings of this thesis conclude that creating a screening protocol of such will likely be a combined effort. Because multiple species of environmental bacteria can produce sphingolipids, several primer sets will likely be needed to encapsulate all of the varying SPT gene sequences. In the course of this thesis, one forward and reverse primer set were designed and were successfully used to amplify the SPT gene from a species of *Novobsphingobium* and a species of *Sphingomonas* bacteria. It has been concluded that these primers alone cannot amplify the SPT gene from all bacterial sphingolipid producers. However, these primers represent a starting point in which a more universal screening technique can be developed.

The goal of this project remains to create a means of identifying environmental sphingolipid producers using a PCR assay with hopes of analyzing their lipids as possible therapeutic agents. The most pressing need for this project would be the continued search for effective PCR primers that can be used in conjunction with LT11 and LT13 primers. At the moment, Ph.D. student Tahir Ali has successfully designed primers for several other sphingolipid-producing bacteria. Creating a library of effective primers would allow for a more significant number of sphingolipid producers to be identified from soil isolations and would

further advance this project toward its end goal. Additionally, future work includes optimizing and streamlining the screening protocol to ensure the best results when testing soil isolates. The use of liquid cultures could be explored to increase the number of isolates screened. Testing new primer combinations and PCR methods may also be applicable as this project evolves to encapsulate a broader range of bacterial sphingolipid producers.

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