The Role of Lipoprotein Biosynthesis in the Pathogenicity of *Streptococcus bovis*

Albert M. Blackburn ’23 and Michael J. Wolyniak

Department of Biology, Hampden-Sydney College, Hampden-Sydney, VA 23943

Abstract

Enzyme prolipoprotein diacylglyceryl transferase (LGT) was extracted from *Streptococcus bovis* for genetic disruption to decrease its virulence and pathogenicity. Although this research is yet to be completed, the gene was successfully cut along three PAM sites via Cas9 protocols. The bacteria and the final solution containing the designed plasmid and primers are being kept in storage for further experimentation in the fall of 2022. This will consist of locating specific gene functions to alter instead of randomly cutting three different locations of the 783 base pair gene sequence of LGT.

Introduction

From the discovery of the antibiotic penicillin to modern medicine, combating bacterial diseases has been imperative to mitigate diseases caused by infection of bacteria. Primarily, antibiotics were widely effective regarding the halt to infectious diseases. However, the overuse of antibiotics has caused bacteria to mutate, as a result, bacteria have developed ways to combat potential threats. According to researchers, “Since highly resistant bacteria are found among the first-step mutants, treatment with high concentrations is not effective in eliminating the whole population of bacteria present in an infection” (M. DEMEREC). Bacteria that have already mutated and developed defenses against antibiotics are not eradicated during the first-round treatment with antibiotics. In this research, a new way to lower pathogenicity, and reduce bacterial disease is being explored regarding the disruption of gene Prolipoprotein Diacylglyceryl Transferase (LGT) in *Streptococcus bovis*.

LGT is an enzyme that facilitates the synthesis of cell surface lipoproteins and aids in cellular growth and is located between the cell wall and the cell membrane (Pailler *et al*.). The direct function of LGT is not solidified, and there are few studies related to this enzyme. One of the only articles on LGT is research on E. coli. The disruption of this gene has not been done in *Streptococcus bovis*. Furthermore, the disruption of lipoprotein biosynthetic enzyme genes can be lethal to some gram-negative bacteria, however, studies have shown that gram-positive bacteria can withstand genetic alterations (A. Kovacs-Simon *et al*.). The bacteria chosen reflects this as *Streptococcus bovis* is a gram-positive organism.

Surface lipoprotein function is essential for virulence in bacterial species (Pailler *et al*.). Lipoproteins act as little antennas on the cell surface, and they allow the cell to facilitate numerous functions. More specifically, cell signaling or signal transduction. Cell signaling plays a substantial role in bacterial cell viability, such as cellular differentiation, defense from the host immune system, cellular growth, nutrient absorption, and colonization (A. Kovacs-Simon, *et al*.). The goal of this research is to hinder these functions by disrupting the source that aids in producing lipoproteins without killing the cell.

Streptococcus bovis is primarily found in gastrointestinal regions of farm animals (sheep, cattle, Oxen), and also make up a small percentage of the human’s normal flora in the intestinal tract (Sykes). Because the amount of this bacteria is so small in the human body, it is not able to cause disease. However, once infected with *Streptococcus bovis* from animal dung, the consequences can be problematic. Some of the diseases associated with *S. bovis* are, infective endocarditis, peptic-ulcer disease, diverticular disease, and is linked to colon cancer (Goldman).

Due to the rarity of contracting these diseases from this species of bacteria, there have been a reduced number of studies regarding this pathogen. Furthermore, the disruption of the LGT gene in this bacterium has not been accomplished. This study serves as the foundation to find new ways to combat bacterial diseases without the use of antibiotics.

Methods and materials

Location and verification of LGT gene on chromosomal DNA.

Primarily, the gene location of LGT in *streptococcus bovis* was not found, however, the complete genome of *streptococcus equinus* was used to find the sequence of LGT. The protein annotation of LGT and the coordinates for the gene were used to match it with the correct DNA sequence. Following this, the first 20 nucleotides on each side of the gene were used to design primers that were then ordered for further experimentation.

LGT gene extraction and gel electrophoresis

Streptococcus bovis bacteria arrived in a glass vial in powder form. Before experimentation could commence, the bacteria were revived in 5ml of lurea broth in a 37°C incubator. The overnight incubated bacteria broth was used to streak onto brain heart infusion media (BHI). Following this, the bacteria from the BHI plates were suspended in 1ml of 50% glycerol, then cryogenically frozen at -80°C. The bacteria left on the plate was stored at 4°C. To extract DNA from *Strep. bovis*, the rest of the overnight broth underwent miniprep DNA isolation procedures. Once the DNA was extracted, a PCR setup took place for LGT gene extraction and amplification. The primers were diluted and placed into separate centrifuge tubes. Once the rest of the PCR cocktail was synthesized, the samples were placed into four separate PCR tubes. Once the PCR was finished, a 1% gel for electrophoresis was made to confirm that the extracts gene segment was LGT.

TOPO-TA Cloning into Escherichia coli

To have a stock of the LGT gene segment, the segment was placed into E. coli via TOPO-TA protocols from Thermofisher. Once the reaction was completed, a single agar plate was inoculated with the bacteria and labeled with numbers from 1-10. A small blot of bacteria was placed on each number. This was done to keep track of possible colonies that may not have received the LGT gene segment. After a 37°C incubation period, colonies from the first five blots of bacteria were taken up for PCR to verify if the LGT DNA sequence was taken up. The primers were added to the PCR cocktail, then placed into the PCR apparatus. After the PCR process was completed, a gel was made to make sure that the LGT gene segment was taken up. The DNA within the gel was then extracted and saved for further use.

Plasmid and primer selection

DNA plasmid PDSO5 was ordered within *Strep. bovis*. To effectively alter the LGT gene segment, it was decided to use this plasmid with BSA1 restriction sites with a GFP zone in between those sites (CRISPR-Cas9 plasmid) Because the specific gene alterations to the LGT gene segments were unknown, three sites, in the beginning, middle, and end of the gene segment were chosen to be disrupted. This was done because gene function was unknown. The primers were designed according to each gene segment that was altered. The three sections included a PAM site (nGG) to guide the Cas9 for cutting. Once the primers were designed, they were married together by matching each of the upper and lower DNA segments that corresponded to each other. A BHI media plate was inoculated with PDSO5 *Strep. bovis* at 37°C overnight. Along with the media, two test tubes containing 5ml of lurea broth and the plasmid were also placed in the 37°C incubator overnight. Once this was completed, the bacteria on the BHI plate were placed at 4°C. The two test tubes underwent miniprep DNA isolation and purification protocols.

Plasmid digest synthesis

The purified plasmid DNA was then used to create digests to cut out the BSA1 restriction sites. This was done with the addition of BSA1 restriction enzymes included in a cocktail with allowed the GFP region to be cut out of the plasmid. A 2% gel was then made to view the married upper and lower portions of the 3 primers (2 for each section), and the PDSO5 digest. This was done to view if the primers were correct and if there was plasmid from the plasmid bacteria colonies.

Marrying of primers for the addition to PDSO5

The upper and lower portions of the primers were then married altogether. Water was boiled in a glass container as each of the primers was pipetted into one single Eppendorf tube. PDSO5 was added along with DNA binding enzymes for primer attachment. The solution was placed into the hot water and left overnight. After leaving the primers in the water overnight, they were placed on a small rack to cool. During this step, the cas9 were guided by the primers to the selected PAM sites and the DNA was cut. The cooling process allowed the DNA to rejoin via non-homologous end joining. After this was completed, the solution was placed into the -20°C freezer for further experimentation.

Results

Location and verification of LGT gene on chromosomal DNA.

LGT gene in *Strep. equinus* was found to be 783 base pairs. The reverse transcribe LGT gene segment is as follows:

```
atgattaatccaattgcgattcaatttggcccttttgcgattcgatggta
cgctcttttcattgtgtcaggtatgcttcttgctgtttaccttgcgatgaaa
gaagcaccgcgtcgaaaaataattccagacgacattttagattttat
cttgattgcatttccgcttgccattatcggtgcgcgtctctattatgtgatt
tttgattttaactactatgctagtcagccatggacagaaatctttgctgt
ttggcatggcggtttagctatttatggcggcttgctgacaggtgccatt
gtcttgtttatcttttcatattatcatgcgattcatccgcttgattttcttgac
attgcagctcctggagtgatgcttgcgcaagctattggtcgctgggg
aaatttcgtcaaccaagaagcctatggtaaggctgtaaaaagctta
aactaccttccagattttattaaaaatcagatgtatatcgatggtagtt
accgtgttccgactttcttgtatgagtcaatgtggaatttgctcggtttta
```

tcattatcatgagcttaagacaccgcccacatttctttaaacaaggtg
aaattactttcttttacttgatttggtatggatgtggacgctttgtgataga
aggaatgcggacagatagtttgatgtttcttggcatgcgcgtgtcgc
agtggttatcagcaatcttggtcattatcggtattgtgatgattgtctgg
agacgaagacaaaaggatattccttattatcaaaattag

The highlighted portions of the DNA were the segments from *Strep. equinus* which were used to design primers to extract the gene from *Strep. bovis*

LGT gene extraction and gel electrophoresis.

The bacteria was primarily grown on a lurea broth agar. However, the bacteria showed signs of little to no growth. After this, it was decided to use BHI media agar plates- which worked exponentially better for Strep. Bovis bacterial growth. After DNA extraction from *Strep. bovis*, The DNA isolated was a yield of 15.04ng/μL and 10.009ng/μL. After PCR including LGT gene extraction, the gel showed that the gene extracted had about 780 base pairs which roughly matches the 783 base pair desired sequence.

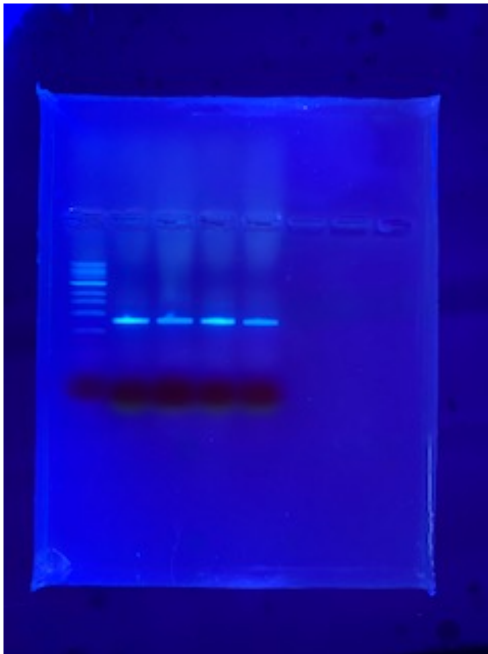

Figure 1: Agarose gel examining presence of LGT gene in *Streptococcus bovis*

TOPO-TA Cloning into Escherichia coli.

TOPO-TA cloning of LGT into *E. coli* was successful (*Figure 2*). The gel indicated that the gene segment of LGT was within the *E. coli*.

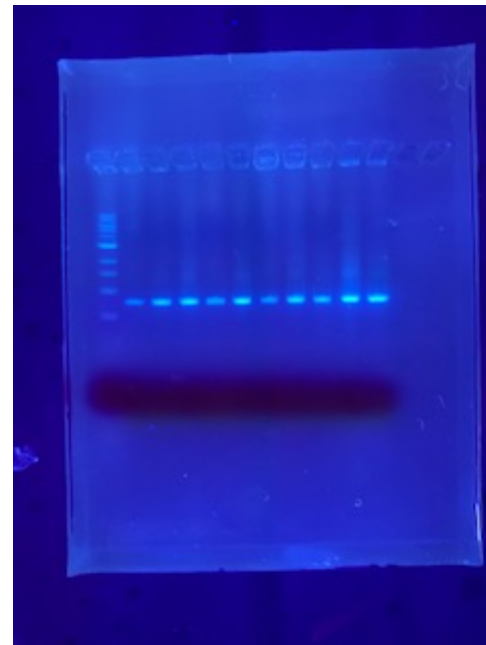

Figure 2: Agarose gel examining presence of LGT gene following TOPOTA cloning into *E. colI*

Plasmid and primer selection.

The plasmid selected was the CRISPR-Cas9 plasmid (PDSO5) which allowed for the cutting of the GFP gene (fluoresces green when active) (*Figure 3*).

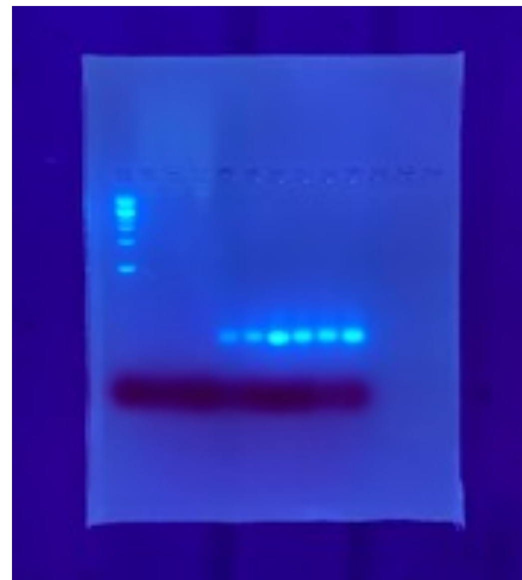

Figure 3: Agarose gel examining presence of plasmid PDSO5 presence (first three wells), and selectors for LGT gene cutting

Three PAM sites of the LGT gene were chosen as sites of disruption. From these, the designed spacers are as follows:

LGT spacer 1 upper:
gggtatatgagaccactactatgctagtcagccaggtctcggtttggg
LGT spacer 1 lower:
cccatatactctggtgatgatacgatcagtcggtccacagccaaaccc
LGT spacer 2 upper:
gggtatatgagacctacgctcttttcattgtgtcggtctcggtttggg
LGT spacer 2 lower:
cccatatactctggatgcgagaaaagtaacacagccacagccaaacc
c
LGT spacer 3 upper:
gggtatatgagacccgcccacatttctttaaacaggtctcggtttggg
LGT spacer 3 lower:
cccatatactctgggcgggtgtaaagaaatttgtccacagccaaaccc

These sites acted as a guide for the Cas9 cutting.

Plasmid digest synthesis.

The synthesis of the plasmid digest and the coupling of spacers were successful after DNA quantification and gel electrophoresis (*figure 4*).

Figure 4: Designed plasmid including Bsa1 restriction sites for the exercise of GFP gene

However, throughout this phase, miniprep of the plasmid culture was producing extremely low yields of extracted DNA. Another BHI media plate was inoculated with the remaining plasmid. After DNA quantification of the new plasmid colony, the DNA extracted was still low, so it was decided to move on with the original plasmid miniprep.

Plasmid: 29.067ng/µL
Spacer 1 upper and lower: 76.698ng/µL
Spacer 2 upper and lower: 77.448ng/µL
Spacer 3 upper and lower: 61.61ng/µL

Marrying of primers for the addition to PDSO5.

Discussion and Conclusion

The experiments throughout this portion of the research were somewhat successful, but there are still many things that need to be figured out before the next phase of research. Primarily, it was not known whether or not the LGT gene was in the same location in *S. bovis* as it was found in *S. equinus*. However, the gene with about the same number of base pairs was found within *S. bovis*, and that is what the rest of the research was based on. Furthermore, it is hypothesized that the gene extracted was LGT.

When trying to culture the bacteria to obtain a good stock to come back to, the bacteria would not grow on lurea broth. After finding a study where this organism was being studied, it was found that the bacteria were grown on brain, heart infusion media. After trying this, *S. bovis*. was growing efficiently.

One of the main problems that were occurring was the minuscule amount of plasmid extraction from *S. bovis*. This step was repeated about five times. One of which came from a different plasmid culture that was made while this issue was occurring. After trying this from the new growth culture, there still was not a desired amount of plasmid extracted. The plasmid from the first culture made was decided to be used for the digest synthesis, and the new one was stored to account for problems in the future.

Altogether, this primary stage of the research consisted of synthesizing all of the components to then marry into the bacteria itself. The next phase of research that has yet to be done is to marry the plasmid digests and the appropriate primers into *Streptococcus bovis*. At this point, the primers have already been added to the plasmid digest and are in a -20°C storage freezer. The bacteria and the to-be-made solution will be married via golden gate cloning along with an erythromycin antibiotic selector. After this step is completed, the organism will be qualitatively observed while monitoring any changes that deviate from the wild-type bacteria. Further research may include locating specific gene functions within LGT and disrupting them accordingly. An area that is wished to be explored as a result of the implications of this research may include targeted gene therapies for bacterial infections. Meaning, that gene functions of structures crucial for cell viability can be found and disrupted to reduce the virulence and pathogenicity of pathogens.

Acknowledgments

Special thank you to my advisor Dr. Michael Wolyniak, to my lab-mate Connor Eickelman, and to the Office of Undergraduate Research for funding this research.

REFERENCES

Dekker, John P, and Anna F Lau. “An Update on the *Streptococcus Bovis* Group: Classification, Identification, and Disease Associations.” Journal of Clinical Microbiology, American Society for Microbiology, July 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4922088/.

Jans, Christoph, *et al*. “Genomics, Evolution, and Molecular Epidemiology of the Streptococcus Bovis/Streptococcus Equinus Complex (SBSEC).” Infection, Genetics and Evolution, Elsevier, 16 Sept. 2014, https://www.sciencedirect.com/science/article/abs/pii/S1567134814003451.

Kovacs-Simon, A, *et al*. “Lipoproteins of Bacterial Pathogens.” Infection and Immunity, American Society for Microbiology (ASM), Feb. 2011, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3028857/.

Libretexts. “Signal Transduction.” Biology LibreTexts, Libretexts, 14 July 2020, https://bio.libretexts.org/Learning_Objects/Worksheets/Biology_Tutorials/Signal_Transduction.

Mao, Guotao, *et al*. “Crystal Structure of *E. Coli* Lipoprotein Diacylglyceryl Transferase.” Nature News, Nature Publishing Group, 5 Jan. 2016,https://www.nature.com/articles/ncomms10198#:~:text=Phosphatidylglycerol%3Aprolipoprotein%20diacylglyceryl%20transferase%20(Lgt,to%20most%20Gram%2Dnegative%20bacteria.

N. Pailler, J. Aucher, W. Pires, & M. Buddelmeijer. “Phosphatidylglycerol::Prolipoprotein Diacylglyceryl Transferase (LGT) of *Escherichia Coli* Has Seven Transmembrane Segments, and Its Essential Residues Are Embedded in the Membrane.” Journal of Bacteriology, U.S. National Library of Medicine,https://pubmed.ncbi.nlm.nih.gov/22287519/.

Pompilio, Arianna, *et al*. “An Overview on *Streptococcus Bovis/Streptococcus Equinus* Complex Isolates: Identification to the Species/Subspecies Level and Antibiotic Resistance.” International Journal of Molecular Sciences, MDPI, 23 Jan. 2019, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6386949/.

Shah, Ishita M, and Jonathan Dworkin. “Microbial Interactions: Bacteria Talk to (Some of) Their Neighbors.” Current Biology : CB, U.S. National Library of Medicine, 25 Aug. 2009, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2892272/.

Shanan, Salah, *et al*. “Significant Association of *Streptococcus Bovis* with Malignant Gastrointestinal Diseases.” International Journal of Microbiology, Hindawi, 29 Oct. 2011,https://www.hindawi.com/journals/ijmicro/2011/792019/.

“*Streptococcus Bovis*.” *Streptococcus Bovis* - an Overview | ScienceDirect Topics, https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/streptococcus-bovis.

“*Streptococcus Bovis*.” *Streptococcus Bovis* - an Overview | ScienceDirect Topics, https://www.sciencedirect.com/topics/medicine-and-dentistry/streptococcus-bovis.

“*Streptococcus Equinus* Strain SHEEPZ001 Chromosome, Complete Genome – Nucleotide - NCBI.” National Center for Biotechnology Information, U.S. National Library of Medicine,https://www.ncbi.nlm.nih.gov/nuccore/CP075172.1?&withparts=on&expand-gaps=on.

Synefiaridou, Dimitra, and Jan-Willem Veening. “Harnessing CRISPR-Cas9 for Genome Editing in Streptococcus Pneumoniae.” 2020, https://doi.org/10.1101/2020.06.13.149682.

Synthego.Synthego,https://design.synthego.com/#/design/results/advanced?genome=streptococcus_equi_subsp_equi_gca_001175405&nuclease=cas9&direct=lgt.

Uniprot,https://www.uniprot.org/uniprotkb/A0Q200/entry.