T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOMOLECULAR SCIENCES

INVESTIGATION OF THE GLYCOSIDASE ACTIVITY AND RECOMBINANT PRODUCTION OF DIFFERENT *BACTEROIDES* SPECIES

MASTER OF SCIENCE THESIS

AYŞE BAYRAKTAR

Thesis supervisor:

Assoc. Prof. Dr. SERCAN KARAV

ÇANAKKALE- 2021

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ÇANAKKALE – 2021

ETHICAL STATEMENT

Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü Tez Yazım Kuralları'na uygun olarak hazırladığım bu tez çalışmasında; tez içinde sunduğum verileri, bilgileri ve dokümanları akademik ve etik kurallar çerçevesinde elde ettiğimi, tüm bilgi, belge, değerlendirme ve sonuçları bilimsel etik ve ahlak kurallarına uygun olarak sunduğumu, tez çalışmasında yararlandığım eserlerin tümüne uygun atıfta bulunarak kaynak gösterdiğimi, kullanılan verilerde herhangi bir değişiklik yapmadığımı, bu tezde sunduğum çalışmanın özgün olduğunu, bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi taahhüt ve beyan ederim.

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Ayşe BAYRAKTAR
26/08/2021

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Canakkale, August 2021

ÖZET

FARKLI *BACTEROİDES* TÜRLERİNİN GLİKOSİDAZ AKTİVİTELERİNİN İNCELENMESİ VE REKOMBİNANT OLARAK ÜRETİMİ

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26/08/2021, 43

Glikozilasyon hücrelerde en sık gözlenen translasyon sonrası modifikasyon çeşitlerinden bir tanesi olup, ökaryotik proteinlerin %50'sinden fazlası glikoprotein yapısındadır. Glikanlar, serbest oligosakkaritler veya bağlı oligosakkaritler (glikokonjugatlar) biçiminde bulunabilirler. Bağlı glikanlar temelde iki alt başlıkta sınıflandırılabilirler. *N*-bağlı glikanlar (*N*-glikanlar), çekirdek yapısında bulunan *N*-asetilglukozamin (GlcNAc) aracılığıyla, polipeptitlerde bulunan Asn-X-Ser/Thr dizisinin asparajin (Asn) yan zincirindeki karboksamit grubuna bağlanırlar. *O*-bağlı glikanlar ise polipeptitlerde bulunan serin veya treoninin OH grubuna glikosidik bağ ile bağlıdır. Glikan türlerinin glikoproteinlerin biyolojik fonksiyonlarına ne gibi katkılarda bulunduğu henüz anlaşılamamıştır. Bunun temel nedeni ise günümüzde kullanılan glikosidazların glikanları yeteri düzeyde ve seçicilikte ayıramamalarıdır. Glikobiyoloji alanında kullanılan enzimler aktif oldukları glikan türüne göre iki çeşittir; *N*-glikosidazlar ve *O*-glikosidazlar. *N*-glikanlar genel olarak 3 ana gruba ve *O*-glikanlar ise 8 gruba ayrılmaktadır. Günümüzde ticari olarak üretilen *O*-glikosidazlar, sadece 2 grup *O*-glikan (sınıf 1 ve sınıf 2) üzerinde aktivite göstermektedir.

Bacteroides'lerin potansiyel glikosidaz sahibi olduğunun araştırılması için bir strateji geliştirilmiştir. Bu yaklaşımın temeli; bakteriler proteinleri sindirebilecek gerekli enzimleri içermemektedir. Bu yüzden proteinleri karbon kaynağı olarak kullanamazlar. Glikoproteinler ise, protein ve glikan ikilisinden oluşmaktadır. Glikoproteinler üzerinde gözlemlenen gelişimin karbon kaynağı bu yüzden glikanlar olacaktır. Glikanların

glikoproteinlerden ayrılıp hücre içine alınabilmesi için ise bakterilerin önce glikosidazlar ile bu glikanları kesmeleri gerekmektedir. Bu bağlamda yapılan çalışmalar *Bacteroides fragilis* ve *Bifidobakteri infantis N*-glikan içeren laktoferrin, *O*-glikan içeren müsin ve hem *N*-glikan hem de *O*-glikan içeren fetuin glikoproteinleri üzerindeki gelişimleri gözlemlenmiştir. *Bacteroides fragilis*'in glikoproteinler üzerinde gösterdiği yüksek gelişimin ise genomlarında *O*- ve *N*- glikanları kesebilecek enzimlerinin olduğunu hakkında çok büyük ipucu vermektedir.

Anahtar Kelimeler: *Bacteroides*, Glikozidazlar, Glikoprotein, Moleküler Klonlama

ABSTRACT

INVESTIGATION OF THE GLYCOSIDASE ACTIVITY AND RECOMBINANT PRODUCTION OF DIFFERENT *BACTEROIDES* SPECIES

Ayşe BAYRAKTAR

Canakkale Onsekiz Mart University

School of Graduate Studies

Master of Science Thesis in Biomolecular Sciences

Advisor: Assoc. Prof. Dr. Sercan KARAV

26/08/2021, 43

Glycosylation is a type of post-translational modification observed in cells. Glycans may exist in the form of free oligosaccharides or linked oligosaccharides(glycoconjugates). *N*-linked glycans are linked to the carboxamide group in the asparagine side chain ofthe Asn-X-Ser / Thr sequence found in the polypeptides by means of *N*-acetylglucosamine. *O*-linked glycans are bound to the OH group of serine or threonine in the polypeptides by a glycosidic bond. The contribution of glycan species to the biological functions of glycoproteins is not yet understood. The main reason for this is that the glycosidases used currently are unable to release glycans at sufficient levels. The enzymes used in the field of glycobiology are two types *N*-glycosidases and *O*-glycosidases. Currently commercially produced *O*-glycosidases show activity on only 2 groups of *O*-glycans.

A strategy has been developed to investigate whether *Bacteroides* possess potential glycosidases. The basis of this approach; bacteria do not contain the necessary enzymes to digest proteins. The carbon source of development observed on glycoproteins will, therefore, be glycans.

In order for the glycans to be release from the glycoproteins and taken into the cell, bacteria must first release them with glycosidases. Studies in this context have observed the growth of *B.fragilis* and *B.infantis* on lactoferrin containing *N*-glycan, mucin containing *O*-glycan and fetuin glycoproteins containing both *N*-glycan and *O*-glycan. The high growth of

Bacteroides fragilis on glycoproteins gives us a hint of the fact that there are enzymes in the genome that can interrupt *O*- and *N*- glycans.

Keywords: *Bacteroides*, Glycosidases, Glycoprotein, Molecular Cloning

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SYMBOLS AND ABBREVIATIONS

Asn	Asparagine
Avr	Average
dH2O, dWater	Distilled water
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic acid
Dnase	Deoxyribonuclease I
e.g.	Exempli Gratia
et al.	Friends
GalNAc	*N*-acetylgalactosamine
GH	Glycoside hydrolase
GI	Gastrointestinal
HCl	Hydrochloric acid
HexNAc	*N*-acetylglucosamine
LB	Luria-Bertani
RCM	Reinforced Clostridial Medium
RBS	Ribosome binding site
ATG	Translation start site
Ori	The origin of replication
Kan	Kanamycin resistance gene
N-glycan	*N*-linked glycan
O-glycan	*O*-linked glycan
NaCl	Sodium chloride
Ni	Nickel
O.D	Optical density
PCR	Polymerase Chain Reaction
pH	Power of hydrogen
PNGase	Peptide-*N*-Glycosidase
Rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ser	Serine
TBE	Tris-Boric acid-EDTA
Thr	Threonine
g, G	Gram
M	Molar
°C	Degrees Celsius
%	Percent
Min	Minute
L	Liter
Kg	Kilogram
Mg	Miligram
Sec	Second

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CHAPTER 1
INTRODUCTION

1.1. Prebiotic Supply; Glycans

Glycosylation is a type of post-translational modification carried out in cells that is very common, with more than 50% of eukaryotic proteins being a glycoprotein. Glycans are present free or bound to proteins by *N*- or *O*- glycosidic bonds. There are two organelles in the cells the glycans can be attached to a protein that *N*-linked sugars are produced in the endoplasmic reticulum, and *O*-linked sugars are produced in the Golgi apparatus. *N*-Glycans are attached to protein covalently with the Asn (Asparagine) residue of proteins through a glycosidic bond but if where the Asn-X-Ser/Thr sequence exists. X shown in the middle of the specific amino acid sequence can be any amino acid except proline. Whereas *O*-linked glycans (*O*-glycans) are linked to the hydroxyl group of the amino acids that are serine (Ser) or threonine (Thr) found in protein by a glycosidic bond (Figure1). *O*-glycan structures contain eight different cores, whereas *N*-glycans contain only one common core including two HexNAc and three mannose residues (Figure 2). Different glycans are synthesized by adding monosaccharides such as fucose, mannose, etc. to the core structures with the help of glycosyltransferase and glycosidase enzymes (Ohtsubo et al., 2006). Glycans also play a role in cell to cell and cell to microorganism communication, folding, and stabilization of proteins.

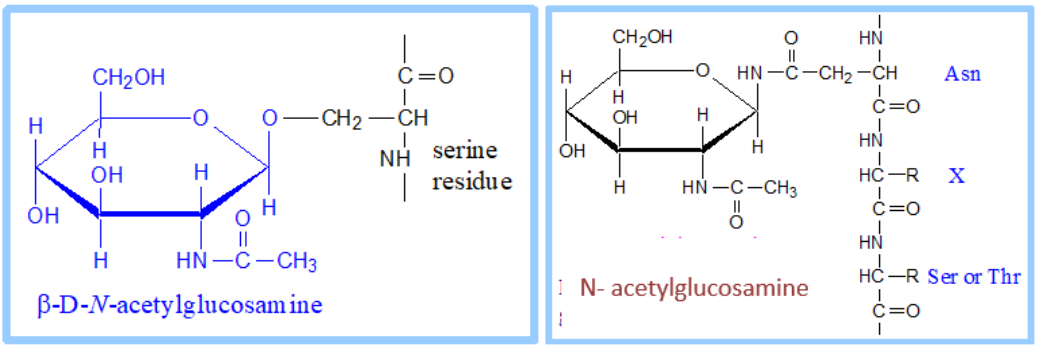


Figure 1: *N*-linked and *O*-linked oligosaccharides attachment to protein residues.

Especially considering the studies in recent years, glycans are of interest because of their ability to be the only class of compounds that have proven their ability to exhibit both prebiotic and antimicrobial activity. Therefore, clarifying the role of specific glycans is very

important for future studies with glycans. Glycans have many structural and biological properties, but their contribution to these properties in an individual sense is not yet fully known due to the inadequacy of past methods to release, separate, purify and characterize glycans bound to glycoproteins.

1.2. How Are Glycans Released from Glycoproteins Currently?

Deglycosylation processes first started with the application of chemical methods such as β-elimination and hydrazination. However, the biggest problem of these methods is that the residual sodium after the reaction inhibits the analysis of glycan in the mass spectrometer, the chemicals used have a carcinogenic effect (Choudhary et al., 1998) and the denaturation of the substrate necessitates the loss of the natural structure of the glycans and the remaining polypeptide (Patel et al., 1993). Therefore, these methods cannot be used in the food and health sector, as they alter the structure of glycoproteins, limiting the relationship of biological functions with glycans. Different enzymes such as PNGase F were used with these methods, but not enough glycosylation was achieved due to the lack of an active enzyme on all of the glycan species (Fucose bound to the *N*-glycan co-structure limits the activity of these enzymes) (Altmann et al., 1995). The major shortage of *N*-glycosidases was solved by the new *N*-glycosidase isolated from *Bifidobacteria infantis*. *N*-glycans can be released from different glycoproteins with no inhibition by this enzyme (Parc et al., 2015).

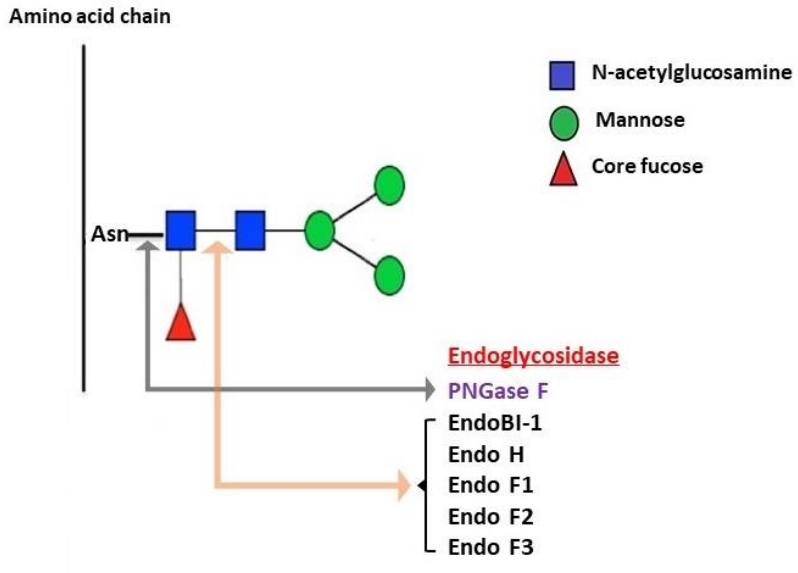


Figure 2: The core structure of *N*-glycan and activity of endoglycosidases

Deglycosylation Strategies:

There are several deglycosylation methods, that are chemical and enzymatically, to release glycans from glycoproteins.

• Hydrazine treatment is cannot use for food-related applications since it is carcinogen (Choudhary et al., 1998).

•*N*-Glycosidase F (PNGase F)

• Endoglycosidases F1, F2, and F3

•(EndoBI-1) Endo-β-*N*-acetylglucosaminidase isolated from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (Karav, German, et al., 2017).

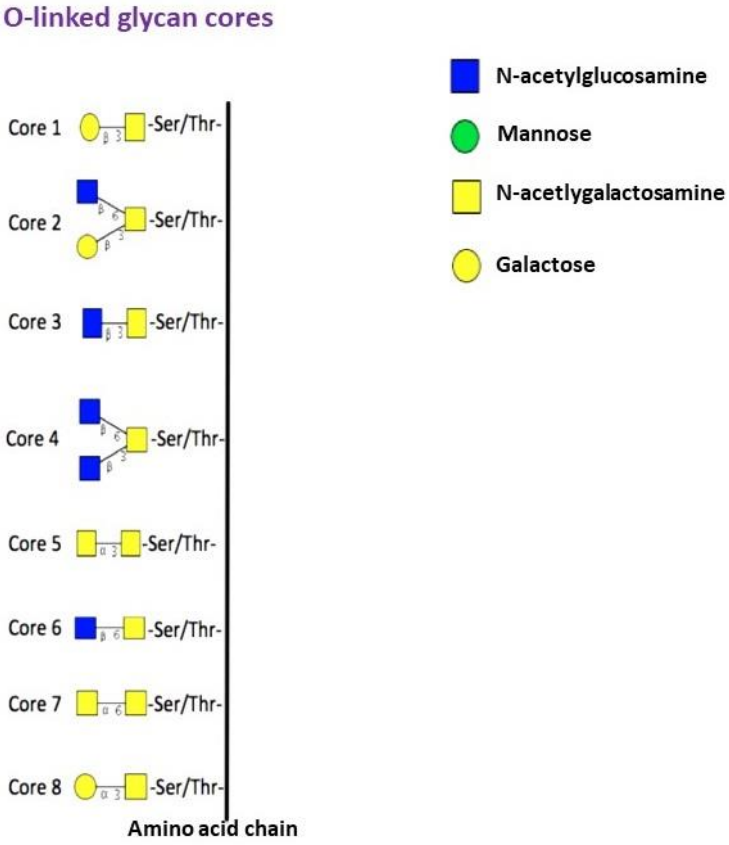


Figure 3: *O*-glycan core structures

Currently, many biotechnology companies have *O*-glycosidases in their sales product profiles. However, no *O*-glycosidase is commercially capable of achieving sufficient *O*-glycan release. The types of *O*-glycans shown in Figure 3 can only be released from core 1 and core 2 containing *O*-glycans. Therefore, there is a very serious problem in the release of

glycoproteins from *O*-glycans. Commercially produced *O*-glycosidases were isolated from *Enterococcus faecalis* and *Streptococcus pneumonia*. This thesis will target *Bacteroides* species that contain much more glycosyl hydrolase and transfer genes (about 20 percent of the genome) than these microorganisms (H. M. Wexler, 2007).

1.3. Intestinal Microflora

Glycans, which can be used by bacteria as the sole carbon source in the intestinal microflora where vital competition is high, do not allow the direct growth of pathogens. Recent studies have shown that glycans cannot be digested by humans throughout the GI (Gastrointestinal) tract and they reach the large intestine and promote the growth of beneficial bacteria here and that the metabolism of glycans by the intestinal microflora can be achieved by their specific enzymes (Karav et al., 2016). Intestinal microbiota can colonize more than 1000 species. This diversity can vary between individuals and is subject to life-long endogenous and exogenous factors. Besides, a person's sex, age, level of antibiotic use, birth shape, geographic location, and feeding type are highly effective on microbiota (Conlon et al., 2015). When *in-vivo* and *in-vitro* studies are taken into consideration in recent years, it has been shown that the survival of microorganisms in intestinal microflora is directly related to the richness of the enzyme groups in their genomes (Carding et al., 2015). A commonly found normal healthy human flora is subdivided into six classes of bacteria. These are *Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia* (Yatsunenko et al., 2012). *Firmicutes* and *Bacteroidetes* suppress intestinal microbiota by 90 percent among these classes. *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Ruminococcus, Peptostreptococcus, Fusobacterium, Escherichia, Enterococcus, Enterobacter, Klebsiella, Lactobacillus,* and *Proteus* were found to be examined at the class level. These microorganisms compete for survival in the intestinal microbiota. The most important strategy they use in this struggle is to colonize the environment by using different genomic equipment.

1.4. How Does *Bacteroides* Dominate Intestinal Microbiota

Bacteroides known as opportunistic pathogens are gram-negative and rod-shaped bacteria (H. M. Wexler, 2007). *Bacteroides* species are non-spore-forming, independent, or

linked to something. Also, these bacteria are very common in the human intestine and are a group of bacteria that have a symbiotic relationship with humans (A. G. Wexler et al., 2017). *Bacteroides* have an important role in the production of useful compounds and the production of the energy the body needs, particularly by helping to break down foods. They are known to be involved in many metabolic processes in the human body (Duan et al., 2010). These; fermentation of carbohydrates, digestion of nitrogen compounds and bile acid and have important roles in the bio transfer of other steroids (Ochoa-Repáraz et al., 2010). They also use much better-developed pump systems than other microorganisms to remove toxins from the cell. *Bacteroides* also have very high antibiotic resistance. *Bacteroides*, which have the strongest antibiotic resistance among anaerobes, are not affected by antibiotics such as cefoxitin, clindamycin, metronidazole, carbapenems, and fluoroquinolones (Vedantam, 2009).

1.4.1. The Nutrient Source of *Bacteroides*

Carbohydrate fermentation of *Bacteroides* and other intestinal microorganisms produces free nutrients, which can be reabsorbed in the large intestine. These absorbed compounds make up the majority of our daily energy needs. *Bacteroides* species can use highly developed nutrients compared to other microorganisms. These species have an advantage over other microorganisms by using simple and complex sugars and polysaccharides found in the intestine as the sole carbon source. In particular, the *Bacteroides thetaiotaomicron* species can use starch with multiple gene regions responsible for binding and digestion (Xu, 2003). In addition, such a gene map is known as the species containing the most glycosyl hydrolases among the identified prokaryotes so far.

1.5. Aim of the Study

The overall aim of this thesis is to discover new glycosidases from *Bacteroides* species, to perform specific glycan release by manipulating the activities of these enzymes.

- To determine the growth of *Bacteroides* species on glycoproteins containing different *O*- and *N*-glycans,
- Quantification of expression increases of candidate genes in growing *Bacteroides* species by quantitative PCR,

- Transferring the identified *Bacteroides* genes from the genomic DNA of the bacteria to an *E. cloni 10G* Chemically Competent Cells using a pRham Vectors and producing them recombinantly,
- Thus, novel glycosyl hydrolase enzymes will be introduced to the literature to pioneer different glycan profiling.

Objectives to Be Achieved:

1) To produce recombinant Bacteroides glycosidases which will be used for deglycosylation incorrect gene sequencing and high purity, and to bring enzymes that have never worked before to the field of glycobiology. To be able to produce active *O*-glycosidases on all *O*-glycan nuclei and to pave the way for new projects by filling the gap in the field of *O*-glycosidases, especially in the field of glycobiology.

2) For the first time, to achieve a selective release of desired glycans between different fusion tags and glycans, thereby developing a whole new approach to both tests the individual functions of different types of glycans and determine how they affect the biological roles of the glycoproteins to which they are bound,

3) Establishing new libraries of glycan data in this field, by detailing which types of glycans can be released from glycoproteins by mass spectrometry, and filling a large gap in the field of glycobiology. To shed light on the researchers working in this field in these libraries and their projects,

4) When all these studies are concluded, it is aimed to fill the gap on specific glycan release in the literature, to publish the results and to contribute to the development of our country through a new method and ideas to be developed.

CHAPTER 2
PREVİOUS STUDIES

2.1. Deglycosylation Strategies for the Study of Protein-Bound Glycans

Release of glycans from the polypeptide chain is a necessary step in the current generation of analytical methods used to characterize the glycans. There are different deglycosylation processes, which are chemical and enzymatic methods to release glycan. Chemical methods such as β-elimination and hydrazineation prevent glycan analysis in mass spectrometry of sodium remaining after the reaction, and the chemicals used have carcinogenic effects (Choudhary et al., 1998). The chemical method also causes denaturation of substrate and alteration of the natural structure of glycans and remaining polypeptides, thereby, limited to studying of biological functions of glycans. These methods are not suitable for food-related studies and also can not be used in the food and health sector. On the other hand, there are different enzymes for deglycosylation such as PNGase F and EndoBI-I (Karav, Cohen, et al., 2017; Karav, German, et al., 2017; Parc et al., 2015) These enzymes are active on the *N*-glycan core shown in Figure 2. However, there is a serious problem in the release of glycoproteins from *O*-glycans. There are 8 different *O*-glycan cores (Figure 3). The currently used *O*-glycosidases originate from *Enterococcus faecalis* and *Streptococcus pneumonia* are active on only 2 of the 8 *O*-glycan species. *Bacteroides* species that contain many glycosyl hydrolase genes (about 20 percent of the genome).

A strategy has been developed to investigate whether Bacteroides possess potential glycosidases. The basis of this approach; bacteria do not contain the necessary enzymes to digest proteins. Therefore, they cannot use proteins as carbon sources. Glycoproteins are composed of protein and glycans. The carbon source of development observed on glycoproteins will be glycans.

CHAPTER 3
MATERIAL METHOD

3.1. Materials

3.1.1. Chemicals, Kits, Culture Media, and Essential Items

In Table 1, the chemicals, kits, and essential items for given studies were listed.

Table 1

List of chemicals, kits, culture media, and essential items

Name of Materials	Codes	Items
Reinforced Clostridial Medium (RCM)	M105411.0500	Bacteroides culture media
Brain Heart Broth	1.10493.0500	Bacteroides culture media
Anaerocult A	1.32381	For creating an anaerobic environment
Anaerotest	1.15112	Atmosphere control test strip.
Anaerobic Jar	1.13681.0001	Anaerobic condition for bacteroides
Colostrum Whey	University of California, Davis	Glycoprotein
Phusion Hot Start II High-Fidelity PCR Master Mix	F-565S	Amplification of target genes
High Capacity cDNA Reverse Transcription kit	4368814	Real-time PCR
AMPIGENE® qPCR Green Mix Hi-ROX	ENZ-NUC104-1000	Real-time PCR
DNase I	EN0521	DNA degredation
EconoTaq PLUS 2X Master Mix	30035-1	Amplification of target genes

Safe Red Loading Dye	G108-R	DNA Gel electrophoresis
1kb plus DNA ladder	D011-500	DNA Gel electrophoresis
50 bp DNA ladder	SM0371	DNA Gel electrophoresis
6x DNA loading dye	R0611	DNA Gel electrophoresis
Agarose	16500-500	DNA Gel electrophoresis
SYBR Safe DNA gel stain	2009284	DNA Gel electrophoresis
Boric Acid	100165	DNA Gel electrophoresis
EDTA	EDT001.500	DNA Gel electrophoresis
Kanamycin Monosulfate	K-120-5	Antibiotic for non esistance bacteria
Expresso Rhamnose Cloning and Expression System Kit- N-His	49011-1	Cloning kit
Qubit™ dsDNA BR Assay Kit	Q32850	Measurement of DNA concentration
Glycerol Solution	GLY001.500	Bacterial stock
LB Broth (Luria-Bertani)	A507002-0250	Bacterial culture media
PureLink RNA Mini kit	12183018A	RNA isolation kit
Lysozyme, Egg White	0663-5G	RNA isolation chemical
İdexx realPCR DNA/RNA spin column kit	99-56103	RNA isolation kit

3.1.2. Substrates

Bacterial strain were used for study listed as follow;

Bacteroides vulgatus ATCC 8482

Bacteroides thetaiotaomicron VPI-5482 (ATCC29148)

Bacteroides fragilis NCTC 9343 (ATCC25285)

Also colostrum whey (University of California, Davis) were used as a glycoprotein

3.1.3. Laboratory Equipment Used in Molecular Analysis

All laboratory equipment required for the thesis studies in the research lab of the Molecular Biology and Genetics Department at the Canakkale Onsekiz Mart University

(COMU) and ULUOVA dairy was used and maintained according to the user's manuals. The devices used in this study are listed in Table 2.

Table 2

List of Laboratory Equipment

Machine names	Brand name
Sensitive scales	Shimadzu
Autoclave	NUVE
Incubator	Indem Nüve EN 400
Incubator	SETRA
-80 °C freezer	Operon
-20 °C freezer	Bosh
-20 °C freezer	Arçelik
Refrigerator	Bosh
Pure water system	Millipore
Pure water system	NUVE
Gel Imaging System	Vilber Lourmat, France
Gel Imaging System	Thermofisher iBright
Horizontal electrophoresis	Cleaver
Power supply	Cleaver
Power supply	Biorad
UV-vis spectrometer	Thermo scientific Nanodrop one
Mini centrifuge	witeg Wisd
Mini centrifuge	LLG
Shaker	Benchmark
Centrifuge	Beckman Allegra X-15R
QubitTM 3 Fluorometer	Invitrogen
Vortex	Vortex Genie 2
Vortex	LLG

Microwave	Bosh
Microwave	Arçelik
pH meter	IsoLab
Thermometer	IsoLab
Ice Flakes Machine	İZMAK
Cooled centrifuge	Hettich MIKRO 200 R
Cooled centrifuge	Eppendorf™ 5427 R
Fume cupboard	SETRA
Biological safety cabinet (class II)	Nücleon
Thermal Cycler (PCR)	Thermo scientific MiniAmp
Real time PCR	Applied Biosystems
Ultrasonic water bath	Elmasonic
Speedvac evaporator	Labconco
Thermo Shaker	INOVIA

3.2. Methods

3.2.1. Anaerobic Growth Conditions of *Bacteroides* Species

Reinforced Clostridial Medium (RCM) and/or Brain Hearth Broth (BHB) were used for the growth of *Bacteroides* into anaerobic condition. To provide an anaerobic environment 2.5 liters of Anaerobic jar (Merck) and Anaerocult A (Merck) were used to keep the internal oxygen, and Anaerotest (Merck) was used to show that internal oxygen is depleted (Figure 4). The inoculation was started by adding a piece from each *bacteroides* stocks into 50 mL falcon tubes containing RCM medium. The cells were cultured at 37 °C for about 48 hours (Figure 5). Then new stock cultures were prepared with a concentration of 15% glycerol (1.5 mL liquid culture + 0.5 mL 60% glycerol) from each growth culture.



Figure 4: Anaerocult A, Anaerobic jar, and Anaerotest used for anaerobic condition

3.2.2. Primer Design for Real Time PCR

Real time PCR primers were designed using NCBI primer designing tool. Chosen target genes from Cazy.org were also checked by NCBI and the Primer-BLAST settings were used for generating real-time PCR primers.

Table 3

Real time PCR primers

Microorganism	Protein Reference Accession Number	Working codes	Real Time primers
	ABR37815.1	Vul1	Forward primer: AGCTGTTTCCCCCGCAAATA Reverse primer: AGAAATGGCGGGATACGTCC
Bacteroides vulgatus ATCC 8482	ABR38247.1	Vul2	Forward primer: ACTCCCATCAGCAAGGAACG Reverse primer: ATCAGGGACCGCAATGGTTT

	ABR38452.1	Vul3	Forward primer: AAGCCAGCCCGATGGAATAG Reverse primer: ATGCAAATGGGAAACCGCAG
	16S Rrna Vulgatus	Vulref	Forward primer: GACTGCCCTATGGGTTGTAAA Reverse primer: CGGAGTTAGCCGATCCTTATTC
Bacteroides fragilis NCTC 9343 (ATCC25285)	CAH08790.1	Fra1	Forward primer: TCGAAAGTGCGAGCTTGAGT Reverse primer: TCCGGTGATTTGCGGTACAA
	CAH06100.1	Fra2	Forward primer: CGCTTTGGAGGGGGAGATAC Reverse primer: ATTACTTCATCCGCCGTGCT
	CAH06414.1	Fra3	Forward primer: AGGGGCATTTGTACCGACTG Reverse primer: TCACAATCATCGGCCAGCAT
	CAH07299.1	Fra4	Forward primer: CGGGCGCTTTGAAGTATGTG Reverse primer: CCGTATGTGGTCGAATCGGT
	16S rRNA Fragilis	Fraref	Forward primer: AGG TTG TTG GTG AGG TAA Reverse primer: CCAATATTCCTCACTGCT

3.2.3. *Bacteroides* Growth and Glycoprotein Adding into Growth Medium

BHB media was prepared for *Bacteroides* growth. Approximately 45 mL media was put into falcon tubes and labelled as Sample and Control (Table 3) taken a piece of cell with the help of a loop while stocks were not thawed. The inoculation was done from *Bacteroides* stocks into 50 mL falcons and put into Anaerobic jar. For the anaerobic condition, 2.5 liters of Anaerobic jar (Merck) and Anaerocult A (Merck)that were used to keep the internal oxygen, and Anaerotest (Merck)that was used to show that internal oxygen, were used

(Figure 5). This system was put into an incubator and The cells were incubated at 37°C for about 48 hours.



Figure 5: Anaerobic growth for *Bacteroides*

After cell growth took place, 5mg whey(colostrum) was dissolved in 2 mL pure water for glycoprotein induction. The anaerobic jar was taken from the incubator and open falcon tubes one by one. 2 mL dissolved and filtered proteins were put into each sample media. On the other hand, water was put into control groups.The jar incubated at 37°C for about 1 hour after the indicator (anaerotest) was converted blue to white.

3.2.4. RNA Isolation

RNA isolation was carried out with a commercially purchased kit, idexx RNA/DNA kit. 200 µL media was transferred into 1.5 mL eppendorf tubes and added 195 µL lysis buffer, 5 µL Carrier RNA, and 5 µL proteinase K which were obtained from the kit. After serial washing steps, total nucleic acid elution was done according to the kit's instructions. The concentrations of RNA samples were determined using Nanodrop one which is a UV-

Vis spectrophotometer. In order to control for RNA degradation, isolates were also loaded into an electrophoresis gel and was visualized if there was any degradation.

Table 4

Amount of reagent for cDNA synthesis and Real-time PCR

Reagent	Amount	Explanation
10X DNAse buffer	1 µL	DNase treatment for degradation of DNA contamination
DNase I	1 µL	
Calculated RNA amount	X µL (0.4µg)	
RNase free water	Y µL	
Total	10 µL	
Random primers	2 µL	DNAse inhibition
dNTP	0.8 µL	
25 mM EDTA	1 µL	
10X RT Buffer	2 µL	cDNA synthesis
Reverse Transcriptase	1 µL	
RNase free water	3.2 µL	
Master Mix	5 µL	Real time PCR
Forward Primer	0.3 µL	
Reverse Primer	0.3 µL	
RNase free water	0.4 µL	
cDNA (diluted)	4 µL	

3.2.5. cDNA Synthesis

cDNA synthesis carried out by a commercially purchased kit, High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific). In the cDNA synthesis, firstly DNase treatment was carried out if there was any DNA contamination. The beginning RNA amount was determined according to the concentration of RNA samples. All reagents shown in the Table 4 were put into PCR tubes and after a short spin they were incubated at 37°C for 30 minutes.

DNase Inhibition

All reagents shown in the Table 4 were added into cDNA synthesis tubes. Then the mixtures were incubated at 65°C for 10 minutes. In this step DNase was inhibited.
Finally all reagent shown in Table 4 put into cDNA synthesis tubes and started PCR amplification according to following procedure:

25°C; 10 min.
37°C; 120 min
85°C; 5 min
4°C; | ∞

3.2.6. Real Time PCR

Designed real time primers shown in Table 3 were dissolved and diluted as 1:10 to get 10µM working concentration. Amplified cDNA was also diluted as 1:4 for the real time analyses.

All reagents shown in the Table 4 were put into the reaction plate and PCR amplification was run according to Table 5.

Table 5
PCR amplification conditions

95°C	10 min.	Holding Stage
95°C	15 sec.	

55°C	30 sec.	Cycling stage
72°C	1 min	40 cycle
95°C	15 sec.	Melt curve stage
60°C	1 min.	
95°C	15 sec.	

3.2.7. Molecular Cloning of *Bacteroides* Genes

Lucigen Expresso Rhamnose Cloning and Expression System kit was used for molecular cloning (Figure 6). There are a variety of cloning models such as conventional and *in vivo* cloning systems. This kit is an *in vivo* cloning system which means cloning is performed into the cell. The vector provided from the kit has 18 nucleotide long ends which provide strong binding with the amplified gene. The primers required for the amplification of target genes were designed according to these ends. PCR products can be cloned instantly, with no enzymatic treatment therefore this strategy allows for fast cloning.

E. cloni 10G Chemically Competent Cells provided from the kit are an *E. coli* strain optimized for high-efficiency transformation during molecular cloning. The *E. cloni* 10G cells are excellent for cloning and also, they can produce high yield plasmid DNA because of endA1 mutation.

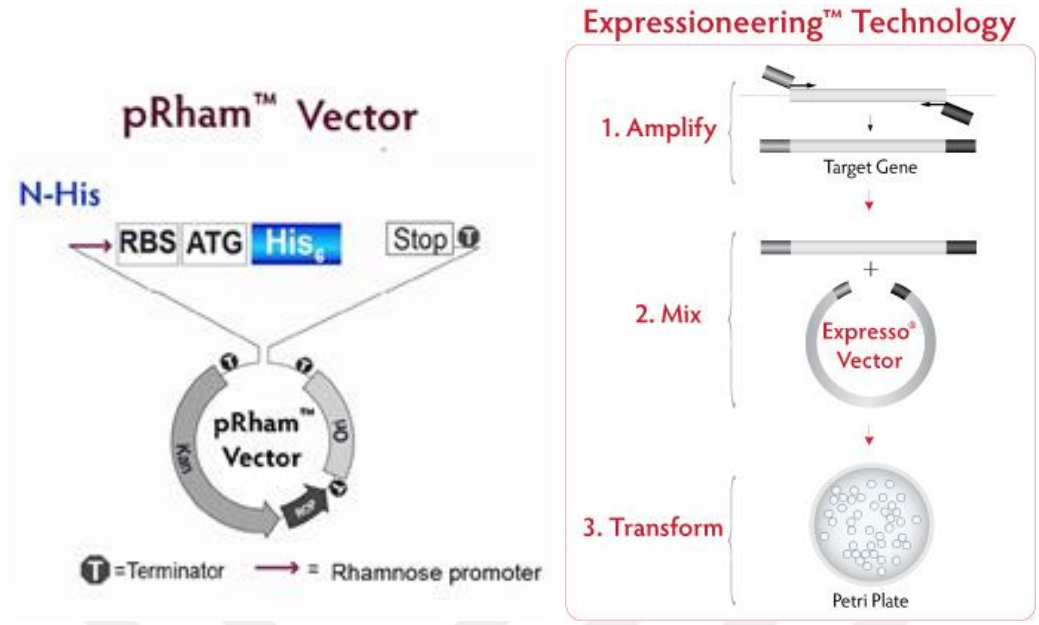


Figure 6. Vector and Molecular cloning strategy

The pRham Vectors are supplied in a pre-linearized format for an instant, directional insertion of the target. The vector provided from the kit has a variety of regions such as Ori and Kan. The meanings of the regions in the vector are as follows:

RBS, ribosome binding site;

ATG, translation start site;

Stop, translation end site;

Kan, kanamycin resistance gene; as a marker for antibiotic resistance

ROP, Repressor of Priming (for low copy number);

Ori, the origin of replication.

3.2.8. Primer Design of Target Genes for PCR Amplification

The primers were designed following the kit's instructions. Briefly, some of the designed primers bind to the relevant part of the vector, while the other half are annealed to the targeted gene part which is shown in Figure 7.

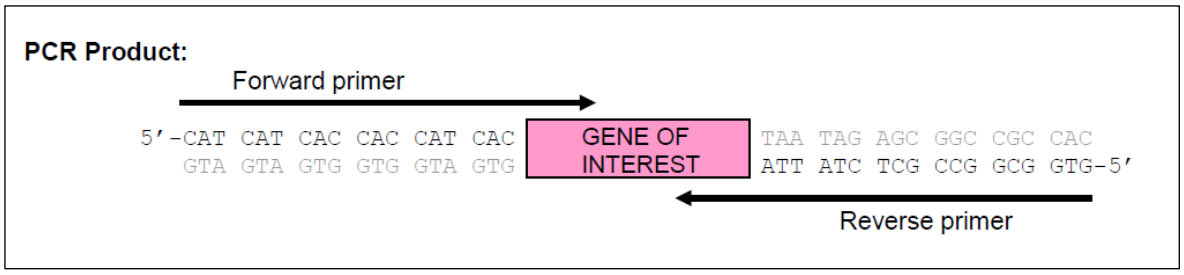


Figure 7: The general illustration of primer designing

Fusion to an N-terminal 6xHis tag (pRham™ N-His Kan Vector):

Forward primer (defined vector sequence includes 6 His codons):

5'-CAT CAT CAC CAC CAT CAC XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

(XXX represents codons 2 through 8 of the target coding region).

Reverse primer (defined vector sequence includes Stop anticodon):

5'-GTG GCG GCC GCT CTA TTA XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6} XXX_{n-7} (XXX_n – XXX_{n-6} represents the sequence complementary to the last 7 codons of the target coding region).

Figure 8: The general explanation of how to design primers according to pRham™ N-His vector

Table 6

Designed primers for pRham™ N-His vector for molecular cloning

Microorganism	Cloning primers
Bacteroides vulgatus ATCC 8482	Forward primer 5'-CAT CAT CAC CAC CAT CAC ACC AAA TGT ACA CAT AAG CAG Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG CGC ATA GAG ATG CGT GAA
Bacteroides thetaiotaomicron VPI-5482 (ATCC29148)	Forward primer 5'-CAT CAT CAC CAC CAT CAC AAA AGT CTT CGA ATC TTT TTG Reverse primer 5'-GTG GCG GCC GCT CTA TTA CAT TTT CTT TTC CGG AGA ATA
Bacteroides fragilis NCTC 9343 (ATCC25285)	Forward primer 5'-CAT CAT CAC CAC CAT CAC GTC CCA TTT ATA ATT TGT ACC Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG AAA TAC CTG ATT TTA ATA

3.2.9. PCR Amplification of Genes of Interest with the Designed Primer Sets for Different Strains of *Bacteroides*

The designed primers were prepared by dissolving them in sterile DNase-free water according to the company's instructions and were obtained as 100 µM. The 10 µM working concentration used for PCR amplification of target genes was prepared by dilution of stock which was 100 µM.

The PCR amplification was carried out according to the following Table 7. The bacterial cell was taken from stock culture approximately 2 µL without thawing. The final primer concentration was 0.2 µM and PCR amplification was run according to Table 8.

Table 7

Amount of reagent for PCR amplification

Master mix (Phusion High-Fidelity PCR Master Mix)	25 µL
Forwerd primer 10 µM	1 µL
Reverse primer 10 µM	1 µL
Template DNA	2 µL
dWater	21 µL
Total	50 µL

Table 8

PCR condition for amplification

95°C	5 min.	First denaturation step for cells explode to release genomic DNA	1 cycle
95°C	30 sec.	Initial denaturation	40 cycle
60°C	30 sec.	Annealing step	

72°C	1 min.	Elongation step	
72°C	10 min.	Final extension	1 cycle
4°C	∞	Hold	

3.2.10. Agarose Gel Electrophoresis

After PCR amplification, to control the PCR product base pair length was performed gel electrophoresis. %1 agarose gel was prepared using a red-safe gel dye. The PCR products were mixed with gel loading dye and put into the prepared gel with the DNA ladder. The loading samples were run at 100V for about 60 minutes. Then the loaded PCR product was visualized under the gel documentation system which was ST4 1100 (Vilber Lourmat, France). On the other hand, the PCR concentrations were determined with Qubit 3 Fluorometer by using dsDNA assay kit before the molecular cloning step.

3.2.11. Preparation of Growth Media for Cloning

In order for *E.cloni* 10 G cells to be successfully cultured, the appropriate media that is LB, known as Lysogeny broth was used. The LB medium is a nutrient-rich broth that is suitable for culturing *Escherichia coli*. Kanamycin which is an antibiotic was added into the media as a selective marker to select a successful transformant. 1.2% LB agar medium containing 30ug/mL kanamycin was prepared. The required LB and agarose were dissolved with the sterile water. Autoclave sterilization was carried out at 121°C for 20 minutes. The selective marker kanamycin was dissolved into 1mL distilled water and added by filtered into cooled media. Approximately 25 mL LB medium was poured into plates and stored at 4°C. Liquid LB media was also prepared for colony PCR. Kanamycin, a selective marker, was used in liquid media.

3.2.12. Transformation of His-tagged PCR Products and pRham Vectors into *E. cloni* 10G Cells by Heat Shock

All culture tubes were put onto ice for pre-chilled before use. The *E.cloni* 10 G cell, chemically competent cells provided from the kit, was completely thawed onto ice. On the other hand, the recovery medium also provided from the kit was put into an incubator (37°C) to bring to room temperature fast. The purpose of this recovery medium was a help to rapid healing of cells after transformation.

Since the concentration values of the PCR products were within the concentration range required that is 25-100 ng/µL for cloning, 1 µL of PCR product was enough for each strain. In sterile conditions, 2 µL pRham vector (25ng) and 1 µL PCR (25-100 ng/µL) product were put into *E.cloni* 10 G cells tube. The pipette tip was used for stirring this mixture without introducing air bubbles and warming the cells. The mixture of cells and DNA were transferred into a pre-chilled 15 mL falcon tube.

The heat shock was applied as follows:

The falcon tube was incubated on ice for 30 minutes. Then, heat shock was applied for 45 seconds in a preheated water bath set to 42°C. The vector and DNA were transferred into the cell through the cell pores opened during the thermal shock process. Finally tubes again quickly were placed on ice and incubated for 2 minutes to close cell pores. 960 µL of room temperature Recovery Medium was added to the cells in the culture tube to the rapid repair of the cells. Culture cells were incubated 37°C for about 1 hour at 250 rpm. Before plating transformed cells on LB agar plates containing 30 µg/mL kanamycin, the culture was concentrated. For this, all cultures were centrifuged at 400 rpm for 10 min. The supernatant part of cultures was removed and dissolved pellets with the 100 µL recovery medium. all 100 µL samples were spreaded on LB agar plates and incubated at 37°C overnight. The *E.cloni* 10 G cells containing no gene insertion (no transformation) were also spread on plate as a negative control because of selective marker kanamycin. All plates were incubated at 37°C overnight.

3.2.13.Verifying of Transformants by Colony PCR

To determine the successful colonies which contain the gene of insert Colony PCR was carried out. Randomly selected colonies were amplified with the sequencing primers supplied with the kit (pRham Forward Primer and pETite Reverse Primer) to confirm their transformants. These primers amplify from the ends of the region where the vector contains the gene of interest (Figure 10). If there was no insertion, the sequencing primers would not be able to amplify any region, so the band would not appear on the agarose gel.

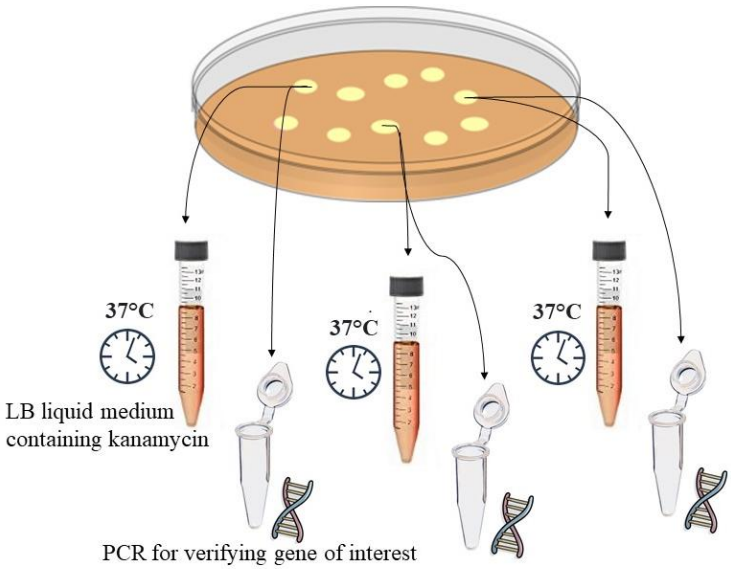


Figure 9: Schematic diagram for colony PCR

There were many colonies on overnight incubated plates. The good growth colonies were chosen and carried out colony PCR. Half of the chosen colony was picked and transferred into a PCR tube and the remaining colony was put into LB liquid medium containing kanamycin (shown in Figure 9) for the overnight incubation. PCR amplification was carried out using the master mix (EconoTaq Plus 2X Master Mix) and the sequencing primers provided with the kit (pRham Forward Primer and pETite Reverse Primer). All the reagents to was used in the PCR were performed as in the previously mentioned step 3.2.9. and in the same amount as in Table 7, except Template DNA amount. The amplification condition was 95°C for 5 minutes, then 40 cycles at 95°C, 30 seconds at 60°C, 1 minute at

72°C, and finally 72°C for 10 minutes. After PCR amplification, the gene of interest was controlled by %1 gel electrophoresis. The successful colonies according to the gel image were stocked.

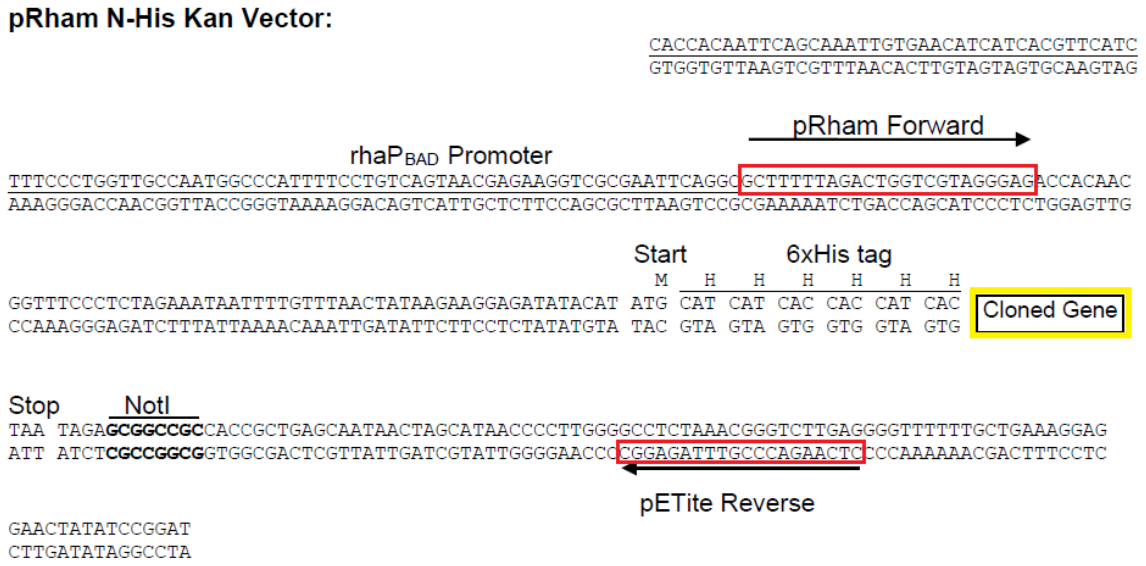


Figure 10: Part of the vector where the sequencing primers recognize on.

3.2.14. Sequencing of Target Genes After Cloning

PCR products amplified using the sequencing primer (pRham Forward Primer and pETite Reverse Primer) were sequenced. Genomic DNA sequences taken from NCBI database and sequenced gene of interest results were analyzed using the Geneious Prime 2020.2 program. Some regions which were low reading quality in sequencing results were trimmed to obtain more accurate results.

CHAPTER 4
RESEARCH FINDINGS

Anaerobic conditions could be achieved, and it was detected with the help of an anaerotest indicator that turns into white when oxygen is not available.

4.1. Anaerobic Growth of *Bacteroides* Species

Bacteroides Vulgatus and *Bacteroides Fragilis* were grown at anaerobic condition well however *Bacteroides Thetaiotaomicron* were not. The 15% glycerol stocks of *B. Vulgatu*s and *B.Fragilis* were prepared. However, Genomic DNA of *B.Thetaiotaomicron* was used for cloning.

4.2. Quantification of Expression Increases of Candidate Genes in Growing *Bacteroides* Species by Quantitative PCR

B.vulgatus and *B.Fragilis* were used for expression increases of candidate genes. These two strains were firstly grown on Brain Heart Broth and added 2.5mg/mL colostrum whey (California) which was rich in glycoproteins. RNA isolation was carried out using idexx DNA/RNA spin column kit. The concentrations of RNA isolates was shown in Table 9 and the gel image of these isolates were also shown in Figure 11.

Table 9

Concentration of RNA isolates

***Bacteroides* strain (Codes)**	**ng/uL**	**A260/A280**	**A260/A230**
B. fragilis sample (F1)	51,1	1,77	0,84
B. fragilis control (FC)	65,4	1,98	1,45
B.vulgatus sample (V1)	55,9	2,01	1,08
B. vulgatus control (VC)	121,6	2,12	1,47

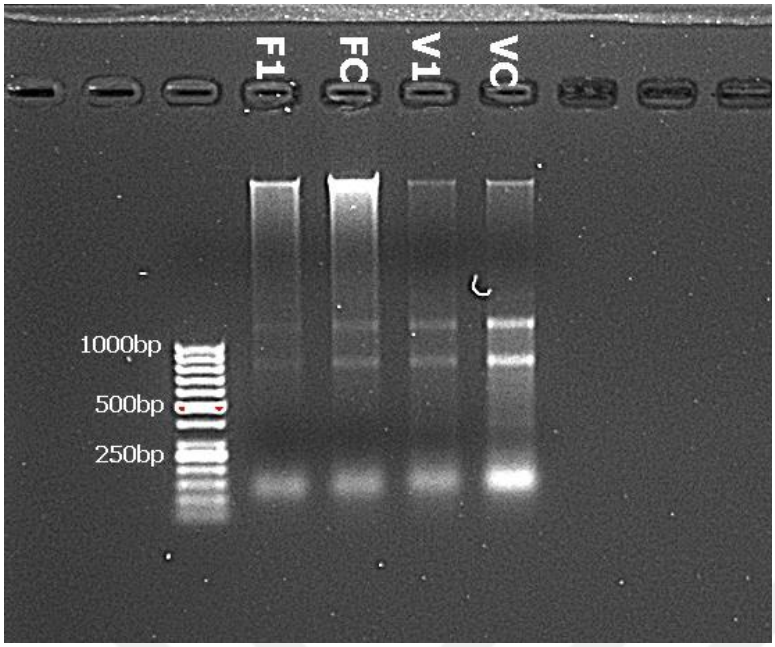


Figure 11: Gel image of RNA isolates. F1; *Bacteroides Fragilis* treatment group, FC; *Bacteroides Fragilis* control group, V1; *Bacteroides Vulgatus* treatment group, VC; *Bacteroides Vulgatus* control group.

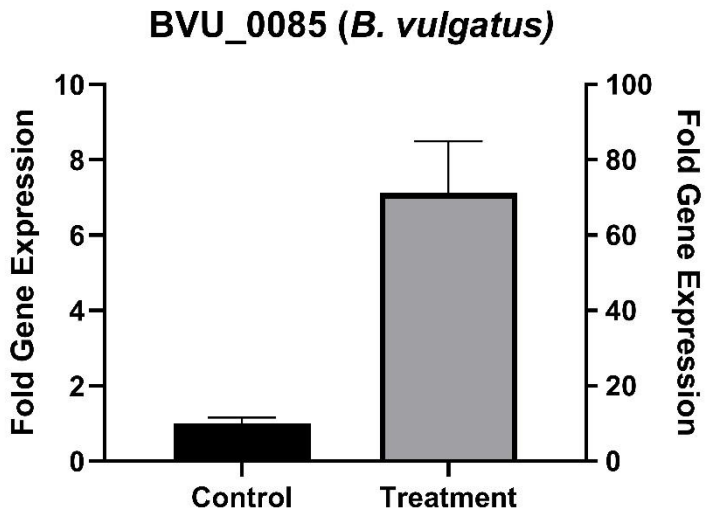
Real-Time PCR Ct values studied with 3 technical repetitions are shown in the Table 10.

Table 10

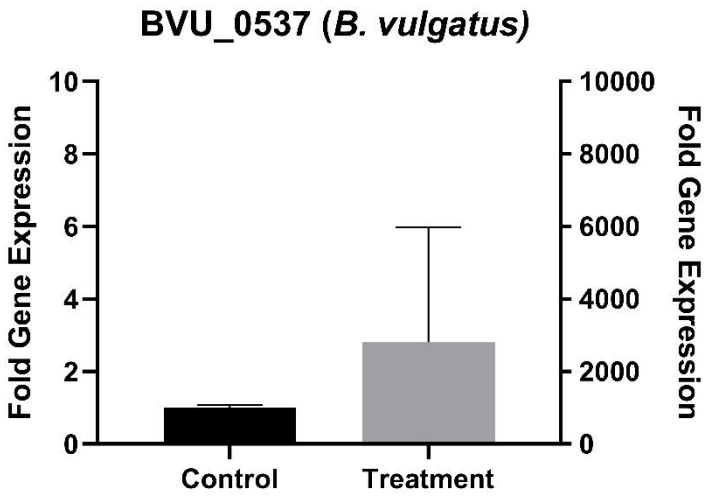
Ct values of RNA isolates and calculation of ΔΔCt

Gene	**Group**	**Ct**	**ΔCt**	**Avr**	**ΔΔCt**	**2^-(ΔΔCt)**		**Avr**
vul1	control	24,932	17,127		0,023	0,984039		
vul1	control	24,954	17,149	17,104	0,046	0,96883	control	1,00059
vul1	control	24,840	17,035		-0,069	1,048915		
vul1	treatment	16,850	10,958		-6,146	70,8204		
vul1	treatment	16,740	10,847		-6,256	76,43806	treatment	70,5820
vul1	treatment	16,985	11,093		-6,011	64,48758		
vul2	control	31,399	23,594		0,014	0,990576		
vul2	control	31,317	23,512	23,580	-0,068	1,048195	control	1,00062

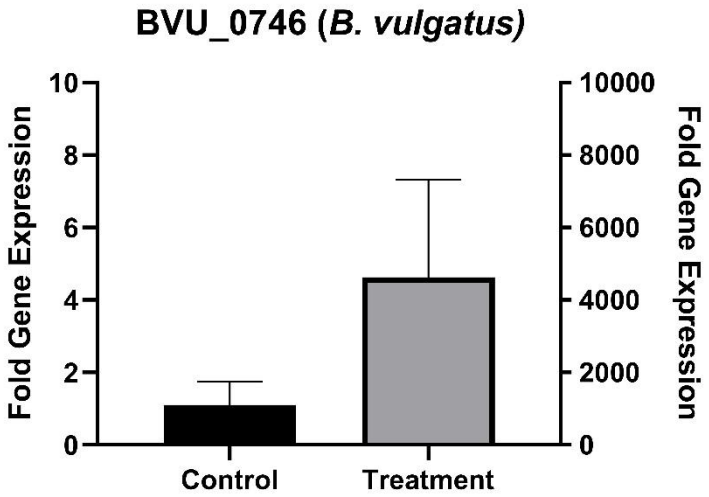
vul2	control	31,439	23,634		0,054	0,963097		
vul2	treatment	18,770	12,878		-10,702	1666,247		
vul2	treatment	17,011	11,118		-12,462	5640,31	treatment	2598,74
vul2	treatment	20,537	14,644		-8,936	489,6779		
vul3	control	33,950	26,145		0,332	0,794554		
vul3	control	34,035	26,230	25,814	0,416	0,749304	control	1,07450
vul3	control	32,870	25,065		-0,748	1,679648		
vul3	treatment	18,675	12,782		-13,032	8373,145		
vul3	treatment	20,210	14,318		-11,496	2887,418	treatment	4765,27
vul3	treatment	20,138	14,246		-11,568	3035,269		
v-ref	control	7,613						
v-ref	control	7,867	avr		7,805			
v-ref	control	7,935						
v-ref	treatment	5,776						
v-ref	treatment	6,076	avr		5,892			
v-ref	treatment	5,825						



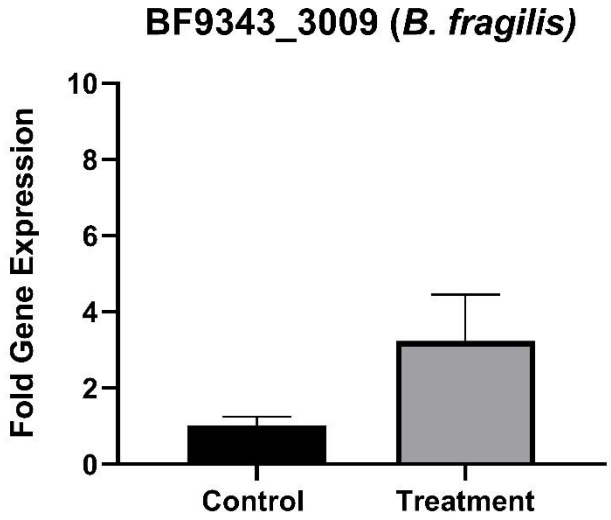
A


BVU_0537 (B. vulgatus)
Fold Gene Expression
10
8
6
4
2
0
10000
8000
6000
4000
2000
0
Fold Gene Expression
Control
Treatment

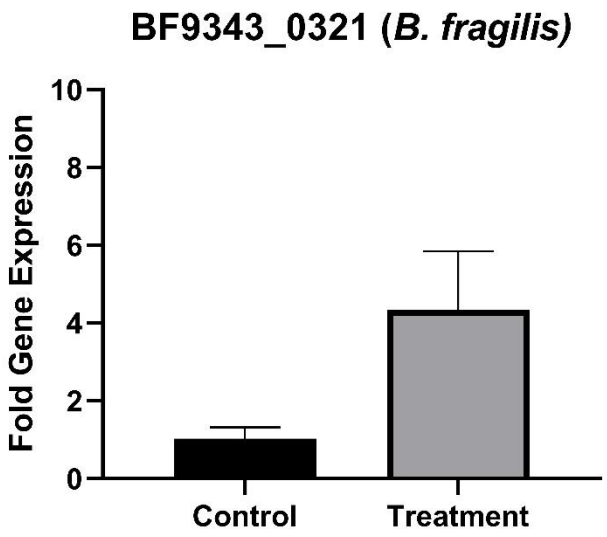
B


BVU_0746 (B. vulgatus)
Fold Gene Expression
10
8
6
4
2
0
10000
8000
6000
4000
2000
0
Fold Gene Expression
Control
Treatment

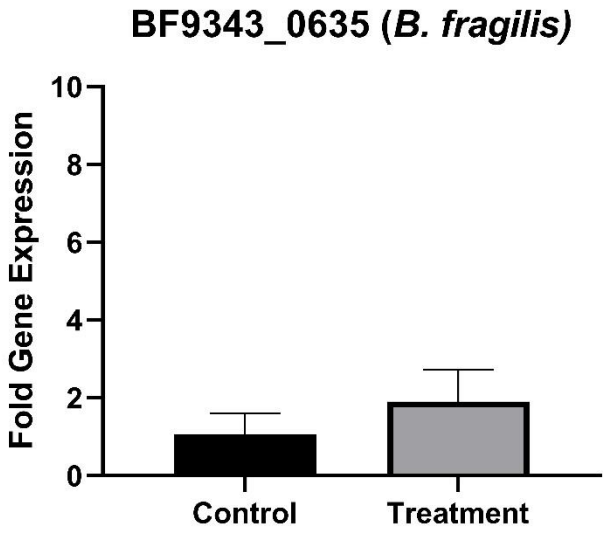
C


BF9343_3009 (B. fragilis)
Fold Gene Expression
10
8
6
4
2
0
Control
Treatment

D


BF9343_0321 (B. fragilis)
Fold Gene Expression
10
8
6
4
2
0
Control
Treatment

E


BF9343_0635 (B. fragilis)
Fold Gene Expression
10
8
6
4
2
0
Control
Treatment

F

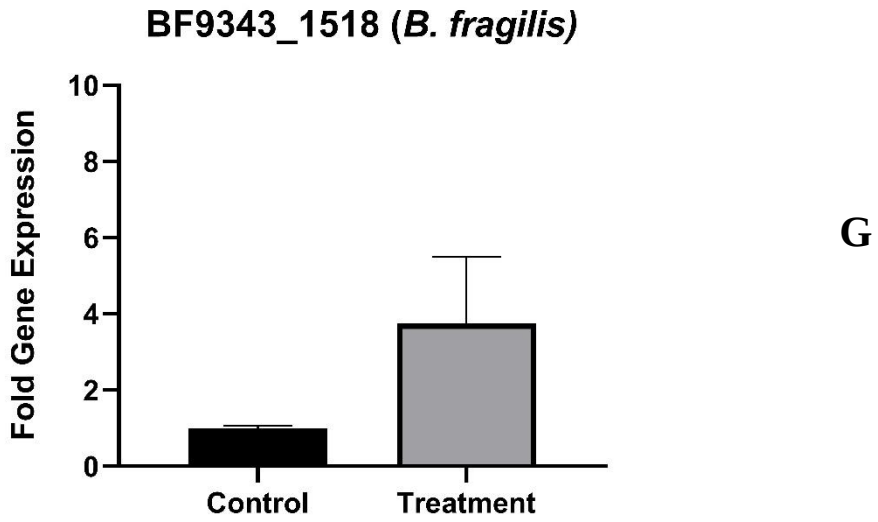


Figure 12: Represents increased expression in target genes with bar graph A,B,C,D,E,F,G represent BVU_0085, BVU_0537, BVU_0746, BF9343_3009, BF9343_0321, BF9343_0635, BF9343_1518 genes respectively.

Table 11

Expression increase in target genes

Microorganism	Working Codes	Locus Tag	Increase In Expression
Bacteroides vulgatus ATCC 8482	vul1	BVU_0085	70,54
	vul2	BVU_0537	2597,13
	vul3	BVU_0746	4434,87
Bacteroides fragilis NCTC 9343 (ATCC25285)	fra1	BF9343_3009	3,01
	fra2	BF9343_0321	4,04
	fra3	BF9343_0635	1,67
	fra4	BF9343_1518	3,57

As a result of real time analysis, it was determined that the expression of the targeted genes increased when glycoprotein was added into the medium. In this case, it can be said that these genes take an active role in the glycan releases from glycoprotein.

BVU_0085(vul1) and BF9343_3009(vul2) were used for gene cloning. Also BT_456 (candidate gene of *Bacteroides thetaiotaomicron*) was used for cloning.

4.3.Molecular Cloning of Target Genes

4.3.1.Amplification of Target Genes

Cloning primers were designed for the target genes by following the kit's instructions. PCR amplification was successfully performed by adding reagent in the amounts shown in Table 7 and under the conditions shown in Table 8. DNA concentrations of PCR products were measured using the Invitrogen™ Qubit™ 3 Fluorometer before proceeding to the cloning step. Since the concentration of the PCR products were above the maximum concentration required for cloning (100 ng/µL), the samples were diluted in the required amounts (Table 12).

Table 12

The concentration of PCR products

Samples	**Concentration (ng/µL)**	**Diluted concentration (ng/µL)**
BVU_0085	509	85
BF9343_3009	408	82
BT_0456	365	91

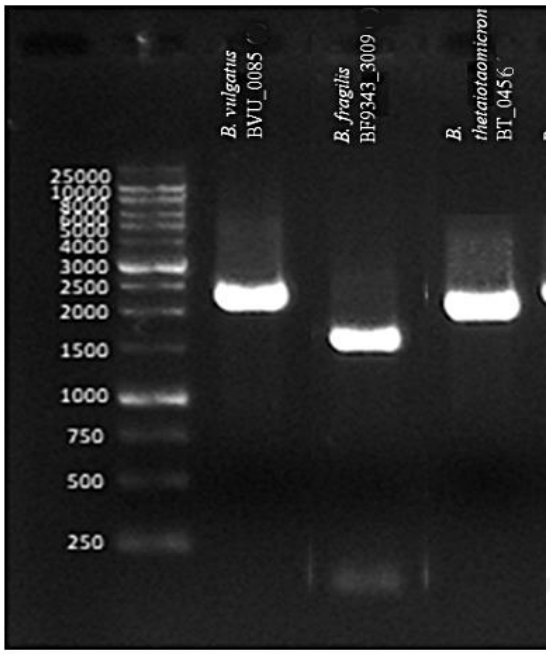


Figure 13: Gel image of target genes after PCR amplification

Table 13

Expected base lengths of cloned target genes

Microorganism Strain	Target Gene	Gene Length
Bacteroides vulgatus ATCC 8482	BVU_0085	2307 bp
Bacteroides fragilis NCTC 9343 (ATCC25285)	BF9343_3009	1557 bp
Bacteroides thetaiotaomicron VPI-5482 (ATCC29148)	BT_0456	2013 bp

4.3.2. Enzyme-free Molecular Cloning with the pRham™ Vectors

The vector and target gene, which were successfully transferred into *E.cloni* 10 G cells by heat shock, were incubated at 37°C overnight.

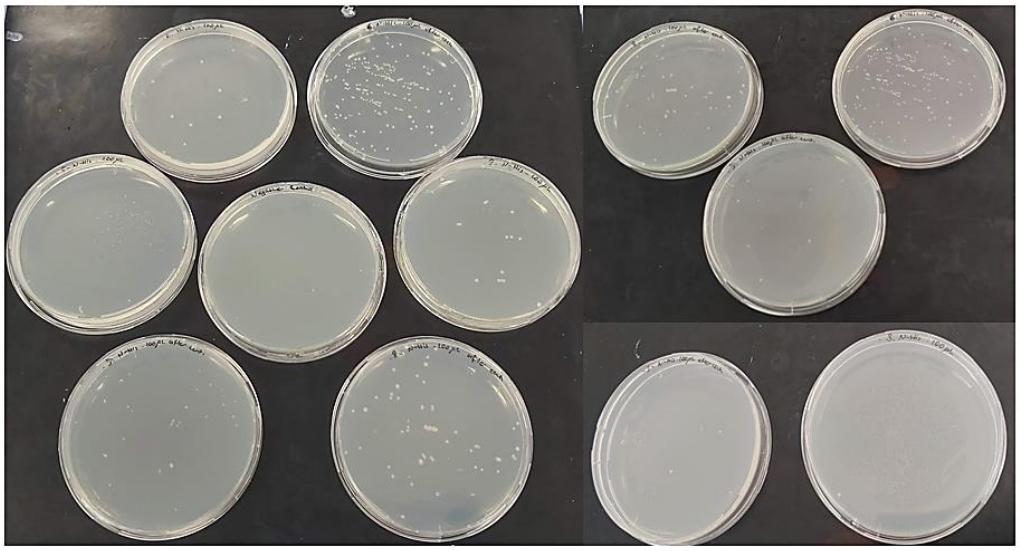


Figure 14: Colonies on LB agar containing kanamycin after transformation of N-His tagged target genes and vectors.

4.3.3. Confirmation of Transformants by Colony PCR

Colony PCR was carried out to check if selected colonies contain the gene of interest. Randomly selected colonies were amplified with the sequencing primers supplied with the kit (pRham Forward Primer and pETite Reverse Primer) to confirm their transformants. All chosen colonies were also transferred into LB medium for overnight incubation. The determined successful colonies according to gel images were stocked for further studies.

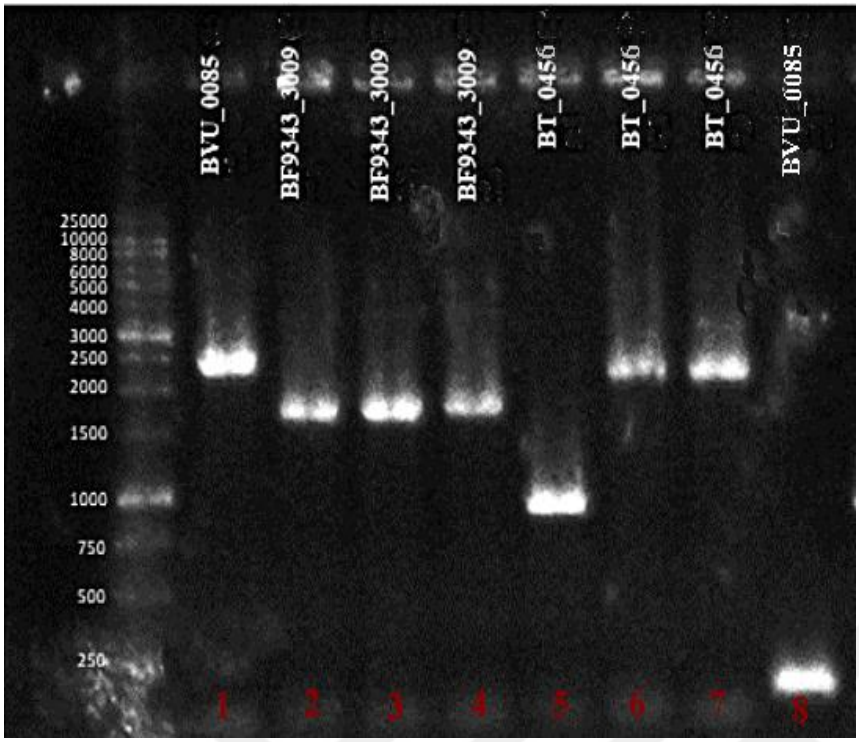


Figure 15: Gel image of N-His tagged target genes obtained after Colony PCR. Lanes 1and 8, 2-4, 6-7 represent BVU_0085, BF9343_3009, BT_0456 amplifications, respectively.
It was observed that one colony (Lane 1) was successful in the transformation of the target gene BVU_0085, and three colonies (Lanes 2,3, and 4) were successful in the transformation of the target gene BF9343_3009. Two out of 3 colonies were also successful in the transformation of the target gene BT_0546.

In this thesis, bands with expected base lengths for the 3 target genes from different *Bacteroides* strains were obtained. The cloning processes for 3 enzymes have been completed.

4.4. Verification of Sequenced Target Genes

The gene regions amplified with the pRham Forward Primer and pETite Reverse Primer were sequenced. The sequence results were blasted with the genomic sequence taken from the NCBI database. The chosen target genes are large in length. Therefore, the primer which was used for sequencing just read the beginning of the genes.

BVU_0085 (From *B.Vulgatus*)

The sequenced region of the BVU_0085 gene, which was sequenced with pRham Forward Primer and pETite Reverse Primer, matched 100% to genomic DNA from NCBI.

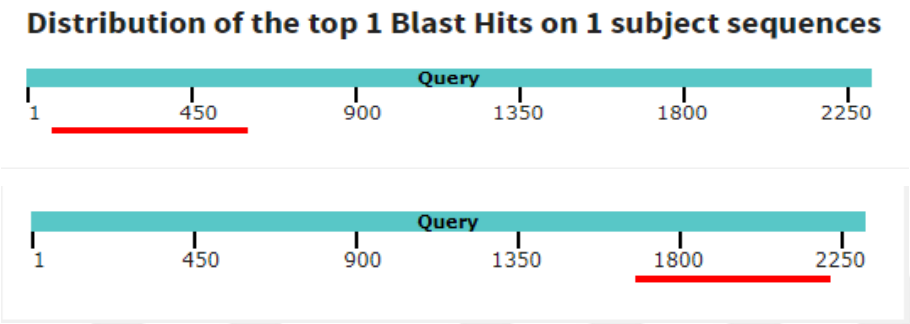


Figure16: General display of sequenced regions of the BVU_0085

Score	Expect	Identities	Gaps	Strand
990 bits(536)	0.0	536/536(100%)	0/536(0%)	Plus/Plus

```
Query  69   ATGTACCAGTACTCCCCCTGTAACAAAAGAGGTATCCATTGTACCCATAACCAACCATCT  128
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Sbjct  1    ATGTACCAGTACTCCCCCTGTAACAAAAGAGGTATCCATTGTACCCATAACCAACCATCT  60

Query  129  GGAGGAAACAAACGGTGCTTTTGTATTGAAAAGTAATACCTCCATCGGTGTTATTGACGC  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGAGGAAACAAACGGTGCTTTTGTATTGAAAAGTAATACCTCCATCGGTGTTATTGACGC  120

Query  189  CGAACTAATTCCCGCAGCCGAGTATTTGGCCGATATGCTTTCCGGTGCCACAGGCTACGA  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGAACTAATTCCCGCAGCCGAGTATTTGGCCGATATGCTTTCCGGTGCCACAGGCTACGA  180

Query  249  CCTTAAAGTAAAAGAAGGCGAAGGAACCATCACCCTTGCCTTGGGGGATGTTCAAGGAAA  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CCTTAAAGTAAAAGAAGGCGAAGGAACCATCACCCTTGCCTTGGGGGATGTTCAAGGAAA  240

Query  309  AGAAGGTGCCTATACCCTGACAGCCGAGAGCGATAAGGTCAACATTACAGGTAACTCTTA  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AGAAGGTGCCTATACCCTGACAGCCGAGAGCGATAAGGTCAACATTACAGGTAACTCTTA  300

Query  369  CGGAGGTGTCATTGCAGGTATCGAATCCCTCAGACAGCTGTTTCCCCCGCAAATAGAATC  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGGAGGTGTCATTGCAGGTATCGAATCCCTCAGACAGCTGTTTCCCCCGCAAATAGAATC  360

Query  429  TAAAGAAATTGTTAAAGGTACAGACTGGGCCATACCGGCCGTTAACATTCAAGATGCCCC  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TAAAGAAATTGTTAAAGGTACAGACTGGGCCATACCGGCCGTTAACATTCAAGATGCCCC  420

Query  489  CCGCTTTGAATGGAGAGGCATCATGCTGGACGTATCCCGCCATTTCTACACAATAGATGA  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CCGCTTTGAATGGAGAGGCATCATGCTGGACGTATCCCGCCATTTCTACACAATAGATGA  480

Query  549  GGTGAAAGAATTACTGGATGTCATGGCCCTTTACAAGATGAATAAATTCCACTGGC  604
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GGTGAAAGAATTACTGGATGTCATGGCCCTTTACAAGATGAATAAATTCCACTGGC  536
```

Figure 17: Sequenced region of BVU_0085 gene with the pRham Forward Primer

Score	Expect	Identities	Gaps	Strand
983 bits(532)	0.0	536/538(99%)	0/538(0%)	Plus/Minus

```
Query  1671  TGAAGGAACCATCAATGTAACTTGCCTTGACCCCACTGCGGAAATTCACTATACCACTGA  1730
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  538   TGAAGGAACCATCAATGTAACTTGCCTTGACCCCACTGCGGAAATTCACTATACCACTGA  479

Query  1731  TGGCAGTACTCCTACTTTACAATCTCCCGTATATGAAGGTCCCATCAAAGTGACTGAAAC  1790
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478   TGGCAGTACTCCTACTTTACAATCTCCCGTATATGAAGGTCCCATCAAAGTGACTGAAAC  419

Query  1791  CACAGACTTTACTTTCTGCACCTTCCGCCCTAATGGAAAGAAAGGTGACATAGCCAAGAC  1850
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418   CACAGACTTTACTTTCTGCACCTTCCGCCCTAATGGAAAGAAAGGTGACATAGCCAAGAC  359

Query  1851  ACGTTTCATCAAAAGTGAATATACCCCCTCTGTAACTGCCGCTCCTTCCAATCCGGGTTT  1910
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358   ACGCTTCATCAAAAGTGAATATACCCCCTCTGTAACTGCCGCTCCTTCCAATCCGGGTTT  299

Query  1911  GAAAGCTACATGGCACGAATTTAAAGGAAATAAATGCAGTGAAATAGAAAAAGCTCCGAT  1970
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298   GAAAGCTACATGGCACGAATTTAAAGGAAATAAATGCAGTGAAATAGAAAAAGCTCCGAT  239

Query  1971  CAATGGTACTTATCCAGTGGAAGACGTTATGATTCCTAAAAAAGTAAAAGGAAATATCGG  2030
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238   CAATGGTACTTATCCAGTGGAAGACGTTATGATTCCTAAAAAAGTAAAAGGAAATATCGG  179

Query  2031  ACTGATTATCAAAGGATATTTCAATGCTCCTCAAGACGATATCTACACTTTTGCACTCCT  2090
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178   ACTGATTATCAAAGGATATTTCAATGCTCCTCAAGACGATATCTACACTTTTGCACTCCT  119

Query  2091  GTCCGACGATGGTAGTACATTGACCATAGACAGTGAACAGATAGTAGACAATGACGGACC  2150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118   GTCCGACGATGGTAGTACATTGACCATAGACAGTGAACAGATAGTAGACAATGACGGACC  59

Query  2151  TCACGGACCTAAAGAAATAGTGGGACAAAAGGCATTGGCGAAAGGATATCATCCAATG  2208
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58    TCACGGACCTAAGGAAATAGTGGGACAAAAGGCATTGGCGAAAGGATATCATCCAATG  1
```

Figure 18: Sequenced region of BVU_0085 gene with the pETite Reverse Primer

BT_0456 (From *B. thetaiotaomicron)*

The sequenced region of the BT_0456 gene, which was sequenced with pRham Forward Primer and pETite Reverse Primer, matched 99% to genomic DNA from NCBI.

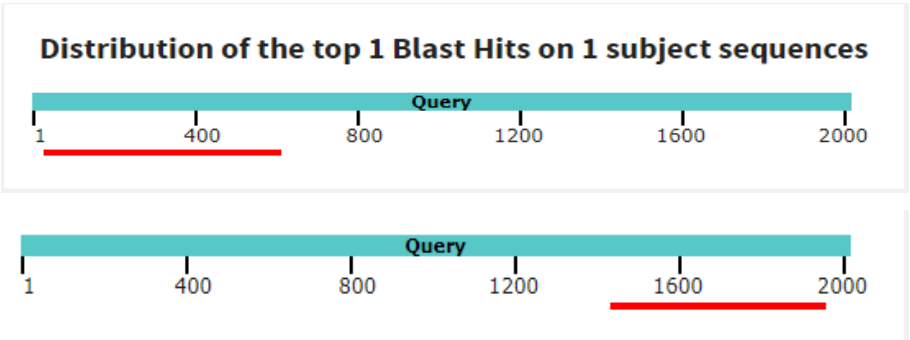


Figure19: General display of sequenced regions of the BT_0456

Score	Expect	Identities	Gaps	Strand
1062 bits(575)	0.0	580/582(99%)	1/582(0%)	Plus/Plus

```
Query  30   TGCATTTTTATTCCATACCTTATATATATTGGGGGCAGATCATTTGCGTCTGCTTCCCCA  89
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Sbjct  1    TGCATTTTTATTCCATACCTTATATATATTGGGGGCAGATCATTTGCGTCTGCTTCCCCA  60

Query  90   ACCTCAGCAATGTGTGCTTGCAAAAGGATATTTTATCGTGGGTAAAATGCAGCTTTCCAC  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ACCTCAGCAATGTGTGCTTGCAAAAGGATATTTTATCGTGGGTAAAATGCAGCTTTCCAC  120

Query  150  TCCTGTGCTTTCGCAGGAATGGAAACAGTTTGTGACGGAGATGGGAGGTACTCTGACTGA  209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCCTGTGCTTTCGCAGGAATGGAAACAGTTTGTGACGGAGATGGGAGGTACTCTGACTGA  180

Query  210  TCAGTCGGCATCCTCTATCAATATAAAATTGGTTGACGCGATTGACAATGTATCTGTCAA  269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCAGTCGGCATCCTCTATCAATATAAAATTGGTTGACGCGATTGACAATGTATCTGTCAA  240

Query  270  TAAGGAAGAGGCTTATCGTCTGACTATTACTCCAAAGGCAATTACTGTGGAGGCTGTAGC  329
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  241  TAAGGAAGAGGCTTATCGTCTGACTATTACTCCAAA-GCAATTACTGTGGAGGCTGTAGC  299

Query  330  AGAGCGCGGAGTATATTGGGCGATGCAGACGCTATATCAACTAAAAGAAGAAAAGGGAAA  389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  AGAGCGCGGAGTATATTGGGCGATGCAGACGCTATATCAACTAAAAGAAGAAAAGGGAAA  359

Query  390  GAAGAACCGCCTGCAATGTGCTACTATCACTGATTGGCCGGCTTTCCGGATTCGGGGTTT  449
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  360  GAAGAACCGCCTGCAATGTGCTACTATCACTGATTGGCCGGCTTTCCGGGTTCGGGGTTT  419

Query  450  CATGCAGGATGTAGGGCGTAGCTACTTGTCTTTGGAAGAATTGAAACGTGAGATAGCCAT  509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  CATGCAGGATGTAGGGCGTAGCTACTTGTCTTTGGAAGAATTGAAACGTGAGATAGCCAT  479

Query  510  ATTATCCCGATTTAAAATCAATACCTTTCATTGGCACTTGACGGAAAATCAGGCATGGCG  569
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Sbjct  480  ATTATCCCGATTTAAAATCAATACCTTTCATTGGCACTTGACGGAAAATCAGGCATGGCG  539

Query  570  TCTGGAGAGTAAAATCTTTCCGATGCTGAATGACAGTACGAA  611
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  540  TCTGGAGAGTAAAATCTTTCCGATGCTGAATGACAGTACGAA  581
```

Figure 20: Sequenced region of BT_0456 gene with the pRham Forward Primer

Score	Expect	Identities	Gaps	Strand
966 bits(523)	0.0	525/526(99%)	0/526(0%)	Plus/Minus

```
Query  1429  GAGGGGGATCTGACTAAAGTTTTTCCACCGGAAGAAGAGCTGAAAGATTCATATACCTAT  1488
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Sbjct  526   GAGGGGGATCTGACTAAAGTTTTTCCACCGGAAGAAGAGCTGAGAGATTCATATACCTAT  467

Query  1489  GAAGGGAAGCAATATGGAGTGCGTCCTGCCATAGGAGCCGGTATCTATCTTCGGCACGTA  1548
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Sbjct  466   GAAGGGAAGCAATATGGAGTGCGTCCTGCCATAGGAGCCGGTATCTATCTTCGGCACGTA  407

Query  1549  TGGGGTAAGATTGTGCCGGCTTTTTACAAAGATCCACAGGAGAATCATACTGCTTATGCT  1608
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  406   TGGGGTAAGATTGTGCCGGCTTTTTACAAAGATCCACAGGAGAATCATACTGCTTATGCT  347

Query  1609  TACACGTATGTCTATTCTTCTAAAACACAGGAAGTAGGCTTATGGGCTGAATTTCAGAAT  1668
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  346   TACACGTATGTCTATTCTTCTAAAACACAGGAAGTAGGCTTATGGGCTGAATTTCAGAAT  287

Query  1669  TATGGACGTTCAGAGAATGACCTGCCGCCATTGCCGGGAAAGTGGGATTATAAAGAAAGC  1728
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  286   TATGGACGTTCAGAGAATGACCTGCCGCCATTGCCGGGAAAGTGGGATTATAAAGAAAGC  227

Query  1729  CGTATTTGGATTAATGATCAGGAAATACTGCCCCCGGTATGGTCTGCTACTCATCTCGTG  1788
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  226   CGTATTTGGATTAATGATCAGGAAATACTGCCCCCGGTATGGTCTGCTACTCATCTCGTG  167

Query  1789  AAAAGCTCGGAAACAGCCTTAGGTAATGAGAATTGTGTGGCACGTCCGCCACTACGGGTT  1848
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  166   AAAAGCTCGGAAACAGCCTTAGGTAATGAGAATTGTGTGGCACGTCCGCCACTACGGGTT  107

Query  1849  CATTTGCATAAAGGCTGGAATAAGGTATTGCTGAAACTGCCTGTCGGCAAATTCTCAACG  1908
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  106   CATTTGCATAAAGGCTGGAATAAGGTATTGCTGAAACTGCCTGTCGGCAAATTCTCAACG  47

Query  1909  AATGAAGTACGATTGGTTAAATGGATGTTTACTGCAGTATTTGTGA  1954
             ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  46    AATGAAGTACGATTGGTTAAATGGATGTTTACTGCAGTATTTGTGA  1
```

Figure 21: Sequenced region of BT_0456 gene with the pETite Reverse Primer

BF9343_3009 (From *B.Fragilis*)

The sequenced region of the BF9343_3009 gene, which was sequenced with pRham Forward Primer and pETite Reverse Primer, matched 99% to genomic DNA from NCBI.

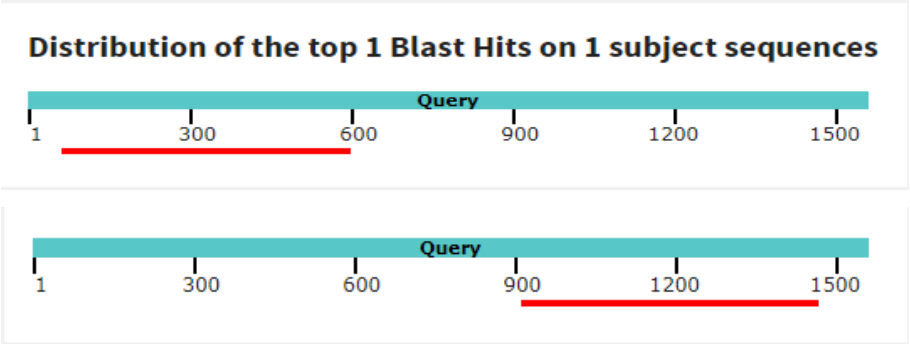


Figure 22: General display of sequenced regions of the BVU_0085

Score	Expect	Identities	Gaps	Strand
977 bits(529)	0.0	533/535(99%)	0/535(0%)	Plus/Plus

```
Query  63   GCTGTTCAAACCACAGTTGCTTAGAGAGTACTTTGCCATAAAACTCATCAAAATTTTCCG  122
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Sbjct  1    GCTGTTCAAACCACAGTTGCTTAGAGAGTACTTTGCCTTAAAACTCATCAAAATTTTCCG  60

Query  123  GATTGCCGGAATGCCACATCTGCTCGGCAAGTGCGAGAATACGCGGAAAGACCCGACGAT  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GATTGCCGGAATGCCACATCTGCTCGGCAAGTGCGAGAATACGCGGAAAGACCCGACGAT  120

Query  183  CGATCATGCTTTCCGTCACCCCGTCGTCCGTCCACAAGGCAGAGCTCATTCCGAGAATAA  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGATCATGCTTTCCGTCACCCCGTCGTCCGTCCACAAGGCAGAGCTCATTCCGAGAATAA  180

Query  243  GCGGATTTTCCTCCCGGGGCCTATAAGAAGGATTACGCAAATACACATCTTCCAGATCGA  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCGGATTTTCCTCCCGGGGCCTATAAGAAGGATTACGCAAATACACATCTTCCAGATCGA  240

Query  303  AAGTGCGAGCTTGAGTATATCCCTTCCAGGGGGTAAGCGGGAAGTTCAGATACGTATAAT  362
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  241  AAGTGCGAGCTTGAGTATATCCCTTCCAGGGGGTAAGCGGGGAGTTCAGATACGTATAAT  300

Query  363  AGTTTGTACCGCAAATCACCGGATAATTATGTCTGACGGCATTCTTCAAGGCCAGATCCC  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AGTTTGTACCGCAAATCACCGGATAATTATGTCTGACGGCATTCTTCAAGGCCAGATCCC  360

Query  423  GGTGTCCTCTCCAGTTCCACCACTGTATCACCACATTGTCCGGCAAGGAATAGCCGTCTT  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGTGTCCTCTCCAGTTCCACCACTGTATCACCACATTGTCCGGCAAGGAATAGCCGTCTT  420

Query  483  TGTAAATCACATCTCCCCAAAAGATGGCCTTCCTCCCTTTTTGTTTCAGATAATCGGCCA  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TGTAAATCACATCTCCCCAAAAGATGGCCTTCCTCCCTTTTTGTTTCAGATAATCGGCCA  480

Query  543  TCCGGGCCGAAAACCACAATTGTAGGTCATGACTGTCTTTTAGTTTTTCTTTTTC  597
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TCCGGGCCGAAAACCACAATTGTAGGTCATGACTGTCTTTTAGTTTTTCTTTTTC  535
```

Figure 23: Sequenced region of BF9343_3009 gene with the pRham Forward Primer

Score	Expect	Identities	Gaps	Strand
1026 bits(555)	0.0	555/555(100%)	0/555(0%)	Plus/Minus

```
Query  910   TTCTTGAGAGTAGAAGCCCTGCTGTTCCGGCCCCTGCCCTACAAAAGCTCCTATACGGGT  969
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555   TTCTTGAGAGTAGAAGCCCTGCTGTTCCGGCCCCTGCCCTACAAAAGCTCCTATACGGGT  496

Query  970   CAGGAACGGATAGCGTTTTATTTCGATGCGCCAGCCAAGTCCTTCGGTCAGATGCCAATG  1029
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495   CAGGAACGGATAGCGTTTTATTTCGATGCGCCAGCCAAGTCCTTCGGTCAGATGCCAATG  436

Query  1030  AAAGTAATTCATCTTCAGCATCGAAGCCATGTCGATATATTTCTTGATCGTAGACACTTT  1089
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435   AAAGTAATTCATCTTCAGCATCGAAGCCATGTCGATATATTTCTTGATCGTAGACACTTT  376

Query  1090  CTGATACTGGCGTCCGGAATCCAGCATAAAGCTGCGCCATTTCACCCGTGGAAAATCGGT  1149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375   CTGATACTGGCGTCCGGAATCCAGCATAAAGCTGCGCCATTTCACCCGTGGAAAATCGGT  316

Query  1150  GATCTCGACACAAGGAAATATCAGTCCTCTTTCCTCACCCGTGTTCCATTGCCTCAGACT  1209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315   GATCTCGACACAAGGAAATATCAGTCCTCTTTCCTCACCCGTGTTCCATTGCCTCAGACT  256

Query  1210  TTGAATGGCATAGATAAAGCCGGCATCATCCGCTGCCTCGACAATTATTCGCTCAGGCGT  1269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255   TTGAATGGCATAGATAAAGCCGGCATCATCCGCTGCCTCGACAATTATTCGCTCAGGCGT  196

Query  1270  CACACGGATGCGATAACCTTCTGTCGGCAAAGTAACATCTTTTTTCCAGGAGATGTGGCT  1329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195   CACACGGATGCGATAACCTTCTGTCGGCAAAGTAACATCTTTTTTCCAGGAGATGTGGCT  136

Query  1330  GTTTACCTCCTCTTTGCACCATTCCACAGCGCTTTCGGGCACCAGTTTTTCATAAAACAA  1389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135   GTTTACCTCCTCTTTGCACCATTCCACAGCGCTTTCGGGCACCAGTTTTTCATAAAACAA  76

Query  1390  ACGTATGAAAGAGCCGGGAGACTCCTCTGCGTACATTCGTATTTTACCTTGAAGCCGTAA  1449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75    ACGTATGAAAGAGCCGGGAGACTCCTCTGCGTACATTCGTATTTTACCTTGAAGCCGTAA  16

Query  1450  CCGGCCATCTGCCCT  1464
             |||||||||||||||
Sbjct  15    CCGGCCATCTGCCCT  1
```

Figure 24: Sequenced region of BF9343_3009 gene with the pETite Reverse Primer

Only the end regions of target genes could be sequenced because these genes' lengths are big. New primers should be designed and checked for if there are mutations in any region of genes

CHAPTER 5
RESULT AND RECOMMENDATIONS

Total RNA was isolated, and mRNA expression of the target genes were analyzed with the primer sets in Table 4 using the $2^{-\Delta\Delta Ct}$ method(Livak et al., 2001). The 16S rRNA ,housekeeping gene, transcription level was used to normalize the expression of the target genes (Pumbwe et al., 2006) .

Bacteroides have many glycosyl hydrolase enzymes in their genome. chosen target genes from these enzymes, the increase in gene expression levels was detected as we expected with real-time studies. Some selected target genes were transferred to *E.cloni* 10G cells by the molecular cloning method. Colonies, where the transfer was successful, were detected by colony PCR and these colonies were stocked for further studies. The cloned genes were sequenced for confirmation. It was determined that the genes were cloned correctly according to these sequences, but to check for any mutations, new primers must be designed and resequenced targeting the middle portion of the target genes.

The method we use in cloning is the *in vivo* cloning system and this system is faster and more reliable than other traditional methods. An antibiotic called kanamycin was used as the selective marker because the vector used for cloning contains the antibiotic-resistant gene region. Colony PCR was also performed to detect successful transformants. *E.cloni* 10G cells, which are competent cells provided with the kit, could take up the vector not containing the gene of interest during heat shock transformation (self-ligation). Therefore, it is checked that the gene of interest by colony PCR was inserted into the vector.

On the other hand, this cloning kit (lucigen) is provided to get 6xHis tagged proteins. In the further studies, this tag helps purification of produced enzymes using Ni-NTA resin.

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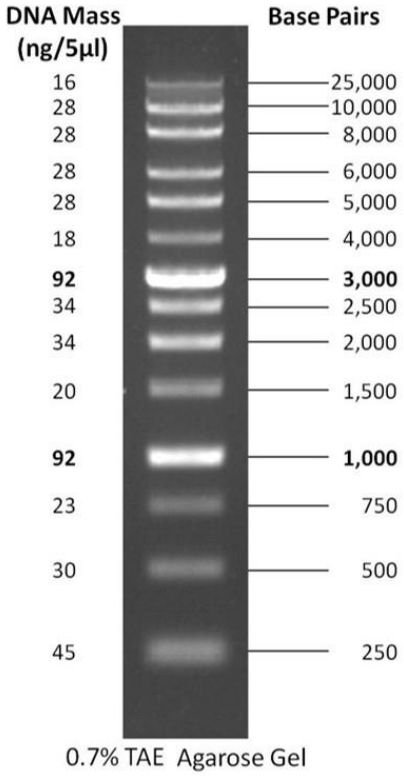
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APPENDICES

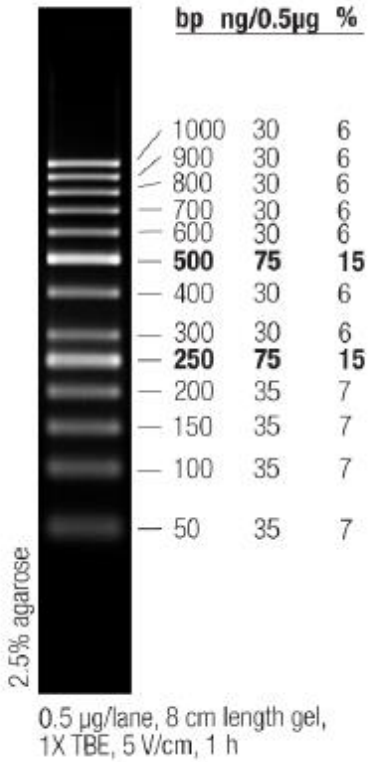
APPENDIX 1

GOLDBIO 1KB DNA LADDER USED AS MARKER



APPENDIX 2

GENERULER 50 BP DNA LADDER USED AS MARKER



APPENDIX 3

GENERAL TABLE OF *BACTEROIDES* TARGET GENES

Microorganism	GH family	Protein Reference Accession Number	Gene Name	Realtime primers	Cloning primers
Bacteroides vulgatus ATCC 8482	GH20	ABR37815.1	BVU_0085	Forward primer: AGCTGTTTCCCCCGCAAATA Reverse primer: AGAAATGGCGGGATACGTCC	Forward primer 5'-CAT CAT CAC CAC CAT CAC ACC AAA TGT ACA CAT AAG CAG Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG CGC ATA GAG ATG CGT GAA
	GH20	ABR38247.1	BVU_0537	Forward primer: ACTCCCATCAGCAAGGAACG Reverse primer: ATCAGGGACCGCAATGGTTT	Forward primer 5'-CAT CAT CAC CAC CAT CAC TTC TAT CAT TAC CTC ATC Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG AAA TAC ACC TGT TTG
	GH36	ABR38452.1	BVU_0746	Forward primer: AAGCCAGCCCGATGGAATAG Reverse primer: ATGCAAATGGGAAACCGCAG	Forward primer 5'-CAT CAT CAC CAC CAT CAC TTC TTC CAC TTT GAA AAT TGC Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG AAG AAT AAT TGG TTA ACT
Bacteroides fragilis NCTC 9343 (ATCC25285)	GH20	CAH08790.1	BF9343_3009	Forward primer: TCGAAAGTGCGAGCTTGAGT Reverse primer: TCCGGTGATTTGCGGTACAA	Forward primer 5'-CAT CAT CAC CAC CAT CAC GTC CCA TTT ATA ATT TGT ACC Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG AAA TAC CTG ATT TTA ATA
	GH20	CAH06100.1	BF9343_0321	Forward primer: CGCTTTGGAGGGGGAGATAC Reverse primer: ATTACTTCATCCGCCGTGCT	Forward primer 5'-CAT CAT CAC CAC CAT CAC CTA TCC GTT TTG TAA TAC GTC Reverse primer 5'-GTG GCG GCC GCT CTA TTA ATG AAA CGA ACC AAC AGA ACA
	GH20	CAH06414.1	BF9343_0635	Forward primer: AGGGGCATTTGTACCGACTG Reverse primer: TCACAATCATCGGCCAGCAT	Forward primer 5'-CAT CAT CAC CAC CAT CAC AAA TTT GTT AAC CGA TTC Reverse primer 5'-GTG GCG GCC GCT CTA TTA TTT CTT ATA AAC CTT CAG
	GH16	CAH07299.1	BF9343_1518	Forward primer: CGGGCGCTTTGAAGTATGTG Reverse primer: CCGTATGTGGTCGAATCGGT	Forward primer 5'-CAT CAT CAC CAC CAT CAC AAA CAC AAA AAC TTA TTG TTA G Reverse primer 5'-GTG GCG GCC GCT CTA TTA TTT TTT ATT TTC CGG TAA TTC
Bacteroides thetaiotaomicron VPI-5482 (ATCC29148)	GH20	AAO75563.1	BT_0456	Forward primer: GGAAAATCAGGCATGGCGTC Reverse primer: CTCTCTGGCTTCCTCCAACG	Forward primer 5'-CAT CAT CAC CAC CAT CAC AAA AGT CTT CGA ATC TTT TTG Reverse primer 5'-GTG GCG GCC GCT CTA TTA CAT TTT CTT TTC CGG AGA ATA