Differentiation of bacterial gram type via rRNA detection

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ABSTRACT

The differentiation between gram negative and gram positive bacteria was investigated based on the identification of specific sequences in the 16S rRNA of pathogenic bacteria. The rRNA sequences were aligned and detection probes were identified using the AlleleID software. Subsequently, the specificity of the probes was checked using the basic local alignment sequence tool of the NCBI data bank. One 23 nt long probe sequence was identified that is able to bind to 79% of the selected gram negative bacteria where as the gram positive probe can only detect 64% of the selected gram positive bacteria. Unfortunately, it was impossible to identify a second probe that was specific and common to all gram negative and even less for all gram positive bacteria. Further experiment will be conducted to test the sensitivity of the probes and their limits of detection.

Acknowledgments

I would like to thank my advisor Professor Antje Baeumner for all the help and support she granted me with throughout my research and my thesis write up. I had the privilege of working with a great person and professor like her. I would also like to thank the Department of Biological and Environmental Engineering with all its professors and staff for fulfilling my undergraduate and graduate expectations and for being a real second home for me when I was far away from home. With certainty I confess that my experience at Cornell University was one of the greatest one can have with all the facilities, research, help, consultation, and student services and its wonderful campus. Lastly, I would like to thank my family and friends who were always there for me in happy and sad moments.

Finally, I would like to dedicate this work and my diploma to my parents Hilda and Sleiman Loubnan and to my sisters Vilma, Stephanie, Pamela and Anna-Maria and to a very special and unforgettable person who made this happen. There is no better joy and pride than having such a loving, caring and supporting family who sacrificed without questioning or complain to lead me to where I am every step of a long way where the best is yet to come.

Biography

Georgette Loubnan is from Lebanon where she was born and raised. Georgette came to the United States after completing a French-Lebanese Baccalaureate in 2002. She received both a bachelor of science and a master of engineering degree in Bioengineering from Cornell University, Ithaca, NY. Her major interests and concentration were in Tissue Engineering and Biosensor Design and their influence and applications in the medical and pharmaceutical field. Georgette Loubnan feels most happy around family and friends who surround her with passion and love.

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Chapter 1: Introduction

1.1 Bacterial Characterization

The tremendous diversity of prokaryotes has made bacterial speciation a very difficult task. From an evolution point of view, the high rate of recombination that resulted from genes acquisition, loss or transfer between bacteria has created various phenotypes and DNA sequences[9]. This genetic exchange happens through bacterial transformation, plasmid-mediated conjugation and virus-mediated transduction[6]. Therefore, determining relationships among bacteria and bacterial identification have become a major concern because of its primordial and direct impact on ecological, sanitary and environmental factors as shown below.

Bacteria are characterized in several ways each with certain limitations as shown in chapter 1.3. Categorization of prokaryotes is based on phenotypes, strain similarities, biochemical traits, habitat and chemical similarities. *In vivo*, bacteria have growth preferences such as the pH, the optimal growth temperature, the substrate utilization and the salt concentration in the medium. In addition, grouping decisions are made by characterizing bacteria based on their ability to sporulate, their fermentation and enzymatic products, their motility and flagellar orientation. Phenotypically, cell wall composition such as types of fatty acids present, peptidoglycan layer, presence of teichoic acids and presence of an outer membrane along with cellular biochemical components

have helped differentiating bacterial groups[10]. Along with morphological and biochemical characterization, antibiotic resistance susceptibility provides additional taxonomic criteria[8].

Genotypically, 16S rRNA is used to find the relatedness between bacterial species and to construct phylogenetic trees also called evolutionary trees or trees of life. Building those trees starts with aligning bacterial RNA then calculating the genetic distance between different bacterial genomes[13]. To assess the relatedness among bacteria and their placement on the tree of life three different methods are currently being used each serving a different purpose but all satisfying only one. The first method which is called Maximum Likelihood evaluates a hypothesis about evolutionary history in terms of the probability that the proposed model and the hypothesized history would give rise to the observed data set and the topology with the highest maximum probability (likelihood) is chosen[12]. The second method is called Bayesian Phylogenetic Inference which takes into account a priori beliefs about the expected results of a test (called the prior probability), and gives a revised estimate of probabilities based on the results of a test (posterior probabilities)[53]. The last method of using RNA as a bacterial characterization tool is Maximum Parsimony which is a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length[54].

1.2 Bacterial Impact on Environment and Humans

Bacterial infections cause a dilemma because of their variability and the limitation of present identification and curability tools. They originate from different sources and

cause serious illnesses. Some infections are foodborne, others are caused by bioterrorism or an epidemic, and many originate from water contamination or air pollution. In all these cases bacterial identification is crucial for providing the right help. For instance foodborne illnesses affect 81 million persons in the United States each year and cost the US economy $8-10 billion dollars a year[52]. Because screening bacterial presence in food at early stages is hard since there is a lack of rapid methods of detection manufacturers release product in the market directly after production without allowing for some time to run tests[43, 48, 49, 50, 51.] According to the CDC report of May 2007 and June 2007 a total of 73480 foodborne cases are caused by contamination with the pathogenic strain of *E- coli* a predominant gram negative bacterium in foodborne pathogens as well about 1.5 million case causes by infection with Salmonellosis, 51000 cases of Streptococcal disease where 3000 types of *Streptococcus* strains are drug resistant, 984 cases of Influenza caused by the *Haemophilus influenzae*, 475 cases of Meningococcal disease caused by the *Meningitidis* species, 2.5 million cases of infection by *Campylobacter* strains, 27000 cases of infection by *Bacillus* species, 8000 cases of infection by the *Vibrio* species, 96000 cases of infection by the *Yersinia* strains therefore a total of approximately 5 million cases of foodborne illnesses seen in 2007 in the US. On the other hand, biological weapon are causing lots of monetary and health damages. Losses are estimated between $478 million and $26 billion per 100000 exposed[44] and less than 55% survival rate[48, 49, 50, 51.]

1.3 Bacterial Identification and Detection

Developing efficient bacterial detection methods and devices has been a major concern and a difficult one because of limitation of detection issues associated with each device or method as shown later in this chapter. Bacteria's panel is very broad where it becomes a real challenge to reach one detection tool that can identify the entire pool. And as shown from an evolution perspective, bacterial recombinations are very frequent and new strains are created every day so the identification task becomes harder. Many attempts have been made to reach this goal as briefly discussed in this chapter.

Bacterial identification is based on their specific characteristics as outline in chapter 1.1. Microbiological and biochemical methods identify phenotypes of the bacteria and based on a cascade of experiments enable the identification of bacteria species and often times subspecies. It is a lengthy procedure taking several days and involves the culturing of the bacteria in special media[30]. One of the most basic identification tools is the Gram staining method and it is almost the first tool used in the identification of bacteria. According to University of Pennsylvania Health System, UPHS, this method is based on the retention of the crystal violet dye by the cell wall of the bacterium. And because gram negative and gram positive bacteria have different cell wall composition they react differently in the presence of the stain. Gram positive bacteria retain the stain and gram negative don't.

Antibodies directed against epitopes on the outer surface of bacteria that are specific for a certain strain or subspecies have found frequent use in the last three decades ([45, 46, 47]. Here, antibodies can be labeled with I^{125} (radiation molecule) [42], fluorescein[41] or bound to other antibodies to allow for detection.

In addition to the staining method and the immunological methods molecular biological techniques are very important as they provide high specificity. It is so because they target the bacterial DNA or RNA directly. In most cases, small segments of the DNA or RNA are amplified using the polymerase chain reaction (PCR) [33, 34, 37] prior to detection. Bacterial identification via their 16S rRNA gene has been studied extensively [10, 29, 31, 34, 35, 37]. In order to improve the specificity, often probe hybridization is required in addition to PCR amplification. Here, several approaches have been demonstrated including TaqMan, molecular beacon, sandwich hybridization and detection via fluorescence or liposome technology etc. [19, 33, 34, 36, 37, 39, 40].

1.4 Biosensors Technology

Biosensors are analytical devices that provide quantitative or sometimes also semiquantitative results in a rapid and simple manner[16]. Biosensors consist of a physico-chemical transducer and a biological recognition element. The biorecognition element binds to the analyte of interest. This binding event is measured by the transducer and transformed into an electrical or visual signal that can be quantified. (Figure 1).

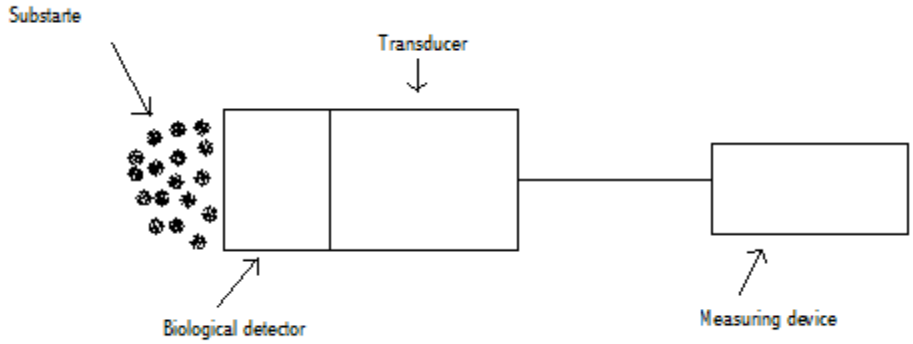

Fig 1: A schematic of a biosensor. The substrate in this work will be the 16S rRNA, the biological detectors are the reporter and the capture probes and the measuring device is a hand-held reflectometer.

Biological elements are the major selective component of the biosensor because they typically specifically bind to a substrate. They are divided in four major groups which are: enzymes, antibodies, nucleic acids and receptors.

The substrate binds to the prosthetic group of the enzyme and undergoes catalyses through an oxidation/reduction reaction[24]. Enzymes can be used in the pure form, or in microorganisms[27, 26] or in slices of intact tissue. The basic catalysis mechanism of an enzyme is shown below where S is the substrate, P is the product, E is the enzyme and k is the association or dissociation constant of the substrate-enzyme complex[16].

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

The advantages of the enzymes being the sensing elements is that they bind to the substrate, they are highly selective, they have catalytic activity which improves the sensitivity of the biosensor and they react quickly because the catalysis reaction is fast

which decreases the response time (1- 5 min). On the other hand, they are very expensive and they may lose their activity after their immobilization on a transducer as it will be described a chapter to follow[16].

Antibodies are another type of sensing elements that bind specific antigens but have no catalytic effect. Here, an unknown antigen can be detected and quantified using labeled (fluorescence probes, radioisotopes, etc…) antibodies or by labeling the antigen itself. The affinity of the antibody to the antigen is K = [AgAb] / [Ag] [Ab]. So by measuring the ratio of the free antigen to the free to bound antigen at equilibrium, one can determine the total amount of ligand if the initial concentration of antibody added is known. The advantage of antibodies is their high selectivity and sensitivity when used in immunoassays particularly[16].

Nucleic acid is another powerful type of the biological component of the biosensor. Because every enzyme, protein, chemical substance in the organism is coded by a different stretch of nucleotides, DNA probes[17] can be used to detect specific sequences that are responsible for specific diseases, viral infections and cancer. Similar to labeling antibodies, DNA probes are labeled (radioactive, photometric, etc…) and a visual signal is detected then quantified using a transducer. DNA probes are either synthesized or cloned using genetic engineering methods[16]. This method is similar to the design that will be presented in this thesis.

Membrane bound receptors such as neuroreceptors and hormonal receptors are the body's own biosensors. When bound to a ligand, they trigger many biochemical changes such as opening of ion channel, activation of a second messenger system and enzymes activation. They are an interesting biological element because they can bind to a variety of molecules of similar structures. In the biosensor, receptors or ligands are tagged with florescence or labeled with radioactive material[16].

Transducers used for the detection of the biologically derived signal include electrochemical, optical, thermal and acoustic principles, with the two first ones being the most often used. The flow chart in Figure 2 by Eggins represents the general categories and subcategories of transducers[16].

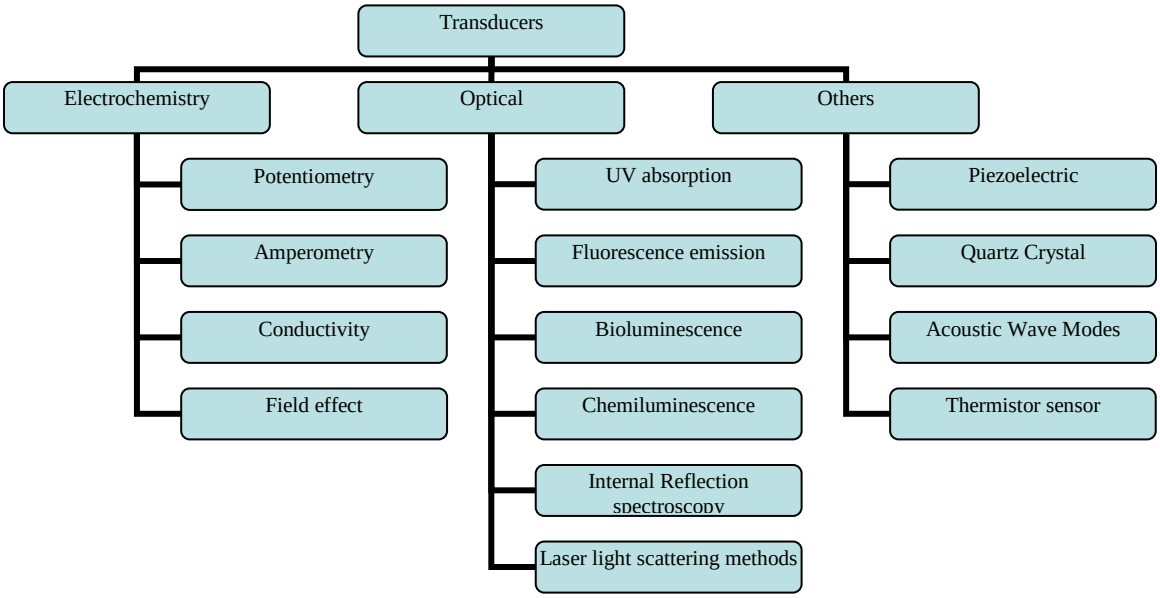

Fig. 2: Flow chart of different types and operations of transducers

1.43 Biosensor's Applications:

Biosensors' use spans a wide type of applications in health care, control of industrial processes and environmental monitoring[18]. From a health care perspective, biosensors are needed to monitor the metabolic state of a patient by performing measurements of blood, gases, ions and other metabolites. Biosensors are beneficial because their detection time is fairly short, a condition needed for patients in intensive care units and with extreme cases. In addition, biosensors are generally portable and light and efficiently used by patients at home. Industrially, biosensors are efficiently used to monitor the active components and products of the fermentation process or for analyzing pollutants and microbial contaminants. And because of their selectivity, biosensors provide accurate data when detecting an enzyme and immunological components in food and drinks using different types of biological sensing components and particular types of transducers as discussed in the previous chapter. Also the security industries have an urgent need for biosensors because of chemical and biological warfare[21]. Environmentally, water and air pollution gazes and contaminants, biological oxygen demand, pH, ions, pesticides[19] etc… are all in need of biosensors for detection.

Chapter 2: Design

The currently developed biosensors for bacterial identification have low limits of detection and provide reliable results as described in chapter 1.3. They have two disadvantages: (1) they require nucleic acid amplification either by cloning or by Polymerase Chain Reaction (PCR) which lead to additional costs and time consumption

and (2) the ones that use DNA probe hybridization as the sensing element can only detect few bacterial species with each probe therefore several probes need to be incorporated in the biosensor for larger bacterial identification which increases the probability of a false positive. Thus the main objectives of this thesis are (1) to design fewer probes (one for each bacterial gram) that can identify a larger pool of pathogenic bacteria and (2) to target the 16S rRNA without the need of amplification in order to reduce time and cost.

Experiments were performed to find genetic relatedness between pathogenic gram negative bacteria as well as pathogenic gram positive bacteria. For this purpose 16S rRNA genes were assembled and aligned to determine the most conserved region that would be a good detection target. After the alignment was performed the second step was to design a single probe for each gram type that would target the conserved region. Probes were selected based on their G: C ratio, length, melting temperature, non-existent self dimerization, and non-existing hairpin formation. The two latter conditions were judged based on calculation of the free energy of their occurrence. The most important feature of the probes design is their ability to bind, after they have met the former characteristics, to most of 16S rRNA of the pathogenic bacteria without any amplification, with very high sensitivity and high limits of detection. Therefore, theoretical studies were performed; experimental investigations were outside of the scope of this thesis. It is envisioned that liposome-lateral flow assay technology will be used to detect the 16S rRNA from gram positive and gram negative bacteria. The principle of the liposome-based detection is shown in Figure 3.

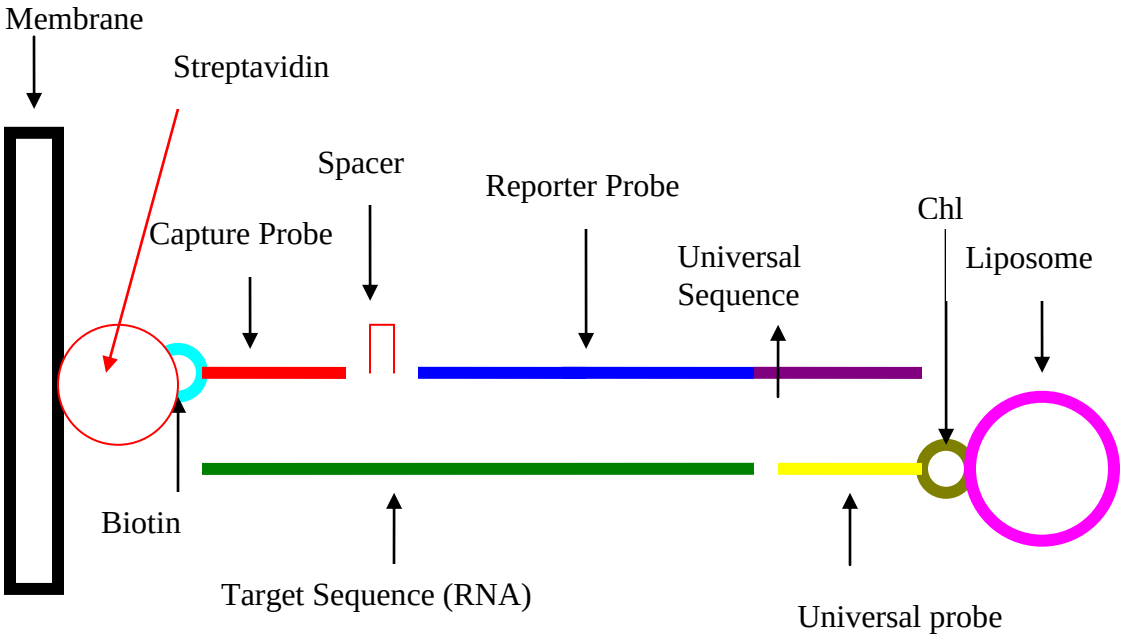

Figure 3. This is the hybridization assay where a DNA Capture Probe is immobilized on a membrane surface through a biotin molecule. The Capture Probe is then hybridized to the target sequence, 16S rRNA in this assay, and the latter is hybridized to a DNA Reporter Probe too. The hybridization is recognized by the binding of the Reporter Probe to a universal sequence that binds the liposome where a chemical reaction occurs and a signal is generated in a form of a dye. The spacer is the distance between the Capture and the Reporter Probe.

Chapter 3: Materials and Methods

3.1 Materials

DNA alignment and probe design were performed using the AlleleID 4.0 Software from PREMIER Biosoft International (Palo Alto, CA).

3.2 Methods

3.2.1 Determination of list of gram positive and gram negative bacteria

The list of bacteria of both gram types was determined based on the research done by Greisen and his group who, in their work, intended designing probes and primers to

identify and differentiate between the most pathogenic gram positive and gram negative bacteria[31]. His probes were designed to target the 16S rRNA gene of most of the bacteria presented in this thesis. These bacteria include foodborne bacteria, bacteria used in biological weapons, bacteria causing environmental catastrophes and bacteria that are playing a primordial role in human health and widespread epidemics as described in chapter 1.3. The CDC database helped determining other pathogenic bacteria that weren't used in Greisen's research but are of a great importance because of their influence in some current infectious diseases (*Achromobacter xylosoxidans, Chryseobacterium meningosepticum, Lactobacillus brevi, Weissella paramesenteroides, Lactobacillus jensenii, Lactobacillus acidophilus, Bifidobacterium adolescentis, Streptomyces hygroscopicus, Streptomyces grisei, Finegoldia magna*). The list of bacteria used in this thesis is represented in Table 1 (Gram Negative) and Table 2 (Gram Positive) with their correspondent 16S rRNA gene length and C: G ratio and their accession number as stored in the NCBI database. No subspecies were selected because alignment of their 16S rRNA shows no difference in the sequence of nucleotides (data not shown) so it was assumed that only the major bacterial groups are needed to have a diverse enough selection of bacteria.

3.2.2 16S rDNA/RNA alignment

16S rDNA sequences available for the bacteria were downloaded from the NCBI database. In order to perform homology searches using the AlleleID software, the sequences had to be transformed into RNA sequences using the RC.exe program provided by Sam Nugen from Dr. Baeumner's research group by choosing the reverse

compliment option to obtain all the RNA sequences from 5' to 3' the way the software is programmed. All the sequences were saved in FASTA format to be correctly read by AlleleID then uploaded into the software. Then the sequences were aligned with the ClustalW program which is a component of the AlleleID software that is able to align such a high number of sequences. This process was repeated twice, one time for each gram type and the alignment results are shown in Table 3 (Gram Negative 16S rDNA) and Table 4 (Gram Positive 16S rRNA).

Table1: List of gram negative bacteria used in this research. For each bacterium, the 16S rDNA was found on NCBI with the correspondent accession code shown in table. Also the G: C ration was determined and shown.

Gram Negative Bacteria	NCBI Locus	Length of 16S rRNA	C Count	G Count	16S rRNA G C content, %
Achromobacter xylosoxidans	EF555462	1355	319	428	55.13
Acinetobacter calcoaceticus	AY568492	1259	286	280	44.96
Aeromonas hydrophila	AM262151	1350	438	309	55.33
Alcaligenes faecalis	AJ242986	1414	441	323	54.03
Bacteroides fragilis	C_003228	1533	446	327	50.42
Campylobacter fetus	NC_008599	1461	403	310	48.80
Campylobacter jejuni	NC_002163	1513	428	323	49.64
Chromobacterium violaceum	NC_005085	1474	473	340	55.16
Chryseobacterium meningosepticum	AJ704540	1450	423	307	50.34
Citrobacter freundi	NC_004464	1535	486	354	54.72
Derxia gummosa	AB089482	1449	460	339	55.14
Edwardsiella tarda	EF121756	922	285	208	53.47
Enterobacter cloacae	DQ988523	1498	478	342	54.74
Escherichia coli K12	ECORRNHK12	6134	1536	1608	51.26
Haemophilus ducreyi	NC_002940	1537	477	319	51.79
Haemophilus influenzae	AF224306	1499	475	307	52.17
Kingella kingae	AY551999	1482	454	320	52.23
Klebsiella rhinoscleromatis	AF009169	1088	357	239	54.78
Legionella pneumophila	NC_002942	1475	471	313	53.15

Continued Table 1: List of gram negative bacteria used in this research. For each bacterium, the 16S rDNA was found on NCBI with the correspondent accession code shown in table. Also the G: C ration was determined and shown.

Moraxella osloensis	DQ512759	815	255	159	50.80
Morganella morganii	AF500485	786	249	180	54.58
Neisseria gonorrhoeae	NC_002946	1545	492	358	55.02
Neisseria meningitidis	NC_003116	1545	484	356	54.37
Paracoccus denitrificans	NC_008686	1456	464	348	55.77
Proteus mirabilis	DQ768232	713	212	163	52.59
Providencia stuartii	AM040491	1478	467	324	53.52
Pseudomonas aeruginosa	NC_008463	1526	482	346	54.26
Pseudomonas putida	NC_002947	1518	478	342	54.02
Rahnella aquatilis	DQ298108	849	285	189	55.83
Rhodospirillum rubrum	NC_007643	1477	475	362	56.67
Salmonella typhimurium	NC_003197	1544	488	356	54.66
Serratia marcescens	AB061685	1532	486	349	54.50
Shigella dysenteriae	NC_007606	1542	486	353	54.41
Shigella flexneri	NC_004741	1541	488	351	54.45
Shigella sonnei	NC_007384	1542	486	354	54.47
Vibrio parahaemolyticus	NC_004605	1471	468	324	53.84
Yersinia enterocolitica	NC_008800	1489	472	335	54.20

Table2: List of gram positive bacteria used in this research. For each bacterium, the 16S rDNA was found on NCBI with the correspondent accession code shown in table. Also the G: C ration was determined and shown.

Gram Positive Bacteria	NCBI Locus	Length of 16S rRNA	C Count	G Count	16S rRNA G: C Ratio, %
Aerococcus viridans	AY707778	1419	417	321	52.01
Bacillus amyloliquefaciens	AY055221	500	159	120	55.80
Bacillus subtilis	NC_000964	1553	491	365	55.12
Bifidobacterium adolescentis	NC_008618	1534	524	388	59.45
Clostridium innocuum	DQ440561	1329	406	280	51.62
Clostridium perfringens	NC_008261	1518	463	335	52.57
Corynebacterium genitalium	X84253	1392	464	322	56.47
Corynebacterium jeikeium	C_007164	1527	511	348	56.25
Corynebacterium xerosis	AM233487	1374	470	329	58.15
Deinococcus radiopugnans	Y11334	1469	485	355	57.18
Enterococcus avium	DQ411811	1481	448	342	53.34

Continued Table 2: List of gram positive bacteria used in this research. For each bacterium, the 16S rDNA was found on NCBI with the correspondent accession code shown in table. Also the G: C ration was determined and shown.

Erysipelothrix rhusiopathiae	AB055905	1594	479	324	50.38
Finegoldia magna	AB109769	7317	1733	1318	41.70
Gardnerella vaginalis	DQ066447	508	176	118	57.87
Gemella haemolysans	AM157450	1517	448	321	50.69
Lactobacillus acidophilus	NC_006814	1572	483	359	53.56
Lactobacillus brevi	NC_008497	1563	463	345	51.70
Lactobacillus jensenii	AB289172	666	203	130	50.00
Lactococcus lactis	NC_008527	1548	465	332	51.49
Lactococcus lactis	NC_002662	1548	465	332	51.49
Listeria monocytogenes	NC_002973	1511	467	341	53.47
Micrococcus luteus	AB023371	1468	489	348	57.02
Mycobacterium bovis	C_002945	1537	523	366	57.84
Mycobacterium gordonae	DQ123634	294	105	71	59.86
Mycobacterium smegmatis	NC_008596	1528	522	366	58.12
Mycobacterium tuberculosis	NC_002755	1536	523	366	57.88
Mycoplasma genitalium	NC_000908	1519	409	284	45.62
Mycoplasma hominis	AY738737	823	197	151	42.28
Mycoplasma pneumoniae	NC_000912	1513	412	283	45.94
Pediococcus acidilactici	AY917122	588	139	163	51.36
Peptostreptococcus	PEP16SRNAS	1462	436	324	51.98
Propionibacterium acnes	NC_006085	1525	532	341	57.25
Propionibacterium lymphophilum	AJ003056	1502	492	352	56.19
Staphylococcus aureus	NC_007795	1555	453	341	51.06
Streptococcus agalactiae	NC_007432	1507	458	327	52.09
Streptococcus bovis	DQ256273	867	271	180	52.02
Streptococcus equinus	DQ232522	1469	444	320	52.01
Streptococcus intermedius	DQ232531	1477	450	329	52.74
Streptococcus mitis	AY005045	1478	453	328	52.84
Streptococcus mutans	NC_004350	1552	475	342	52.64
Streptococcus pneumoniae	NC_008533	1458	447	327	53.09
Streptococcus pyogenes	NC_002737	1335	401	300	52.51
Streptococcus sanguinis	DQ163032	485	143	91	48.25
Streptomyces grisei	AB184205	1477	497	367	58.50
Streptomyces hygroscopicus	AB045864	1485	507	373	59.26
Ureaplasma urealyticum	AF073452	1435	385	274	45.92
Weissella paramesenteroides	AY436633	388	109	81	48.97

Chapter 4: Results and Discussion

A single probe for each bacterial gram type was designed to target the 16S rRNA. These probes are intended for biosensor detection methods where they are anticipated to effectively bind the 16S rRNA at a low limit of detection and a very high sensitivity as it will be tested in future work. For this purpose, 16S rRNA of all the bacteria selected for this research were aligned in order to identify a conserved region that will be used as the detection target so that the probes will hybridize to it and identify the gram type present in the specimen. The bacteria were selected based on the work performed by Greisen group and the CDC reports (*Achromobacter xylosoxidans, Chryseobacterium meningosepticum, Lactobacillus brevi, Weissella paramesenteroides, Lactobacillus jensenii, Lactobacillus acidophilus, Bifidobacterium adolescentis, Streptomyces hygroscopicus, Streptomyces grisei, Finegoldia magna)* as mentioned in chapter 3.2.1. These bacteria are known to be some of the most pathogenic bacteria present in nature and are causing the major infectious diseases whether they are foodborne, environmental, or biowarfare and affecting the human health as seen in chapter 1.2. It is hypothesized that this selection of bacteria entails a wide range and a big variety of frequently identified bacteria. This selection might have not embraced other bacteria causing other frequently occurring diseases which could easily be incorporated in future work. A limitation of bacterial selection was the absence of a sequenced 16S rDNA for certain bacteria a reason for which they couldn't be selected and aligned. So once more bacterial genome sequencing is available more bacteria will be used in similar way to this research in order to identify a larger pool of pathogenic bacteria.

Table 3: Alignment results of the 16S rDNA of selected gram negative bacteria. The sequence shown in red is the most conserved among all the bacterial 16S rDNA of the species below and constitutes a great target for probe design. The red sequence A to G is the most conserved 16S rDNA sequence. It is located at 1020 nt. in the 16S rDNA (black) and ends at the 1044 nt. in *E. coli* since the designed probe is of 24 nt. length a shown in table 5. The breaks between the sequences only exist for legibility of the sequences.

Pathogenic Gram (-) Bacterium	Target Sequence in red
	5′ 3′
Rhodospirillum rubrum	--GGGACACG----GTGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Paracoccus denitrificans	--GAGACCTG----TGGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCGTG---
Chromobacterium violaceum	--GGAGCCGT----AACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCGTG---
Kingella kingae	--GGAGCCGT----AGCAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG--**-
Neisseria meningitidis	--GGAGCCGT----AACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Neisseria gonorrhoeae	--GGAGCCGT----AACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Derxia gummosa	--GGAGCCGG----GACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Achromobacter xylosoxidans	--AGAACCGG----AACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**

Continued table 3: Alignment results of the 16S rDNA of selected gram negative bacteria. The sequence shown in red is the most conserved among all the bacterial 16S rDNA of the species below and constitutes a great target for probe design. The red sequence A to G is the most conserved 16S rDNA sequence. It is located at 1020 nt. in the 16S rDNA (black) and ends at the 1044 nt. in *E. coli* since the designed probe is of 24 nt. length a shown in table 5. The breaks between the sequences only exist for legibility of the sequences.

Pseudomonas putida	--GGAACTCT----GACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Pseudomonas aeruginosa	--GGAACTCA----GACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Legionella pneumophila	--GGAACACT----GATAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Vibrio parahaemolyticus	--GGAACTCT----GTGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Aeromonas hydrophila	--GGAATCAG----AACAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Yersinia enterocolitica	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Haemophilus influenzae	--GGAACTTA----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG--**-
Haemophilus ducreyi	--GGAACTAT----GTGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Proteus mirabilis	--GGAACGCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Edwardsiella tarda	--GGTACGCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCGTG---
Serratia marcescens	--GGAACTCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Providencia stuartii	--GGAACTCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Morganella morganii	--GGAACTCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Citrobacter freundi	--GGAACTCT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Salmonella typhimurium	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Klebsiella rhinoscleromatis	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Enterobacter cloacae	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Shigella dysenteriae	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Shigella sonnei	--GGAACTGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Escherichia coli K12	--GGAACCGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Shigella flexneri	--GGAACCGT----GAGAC**A----GGTGCTG------CATGGCTGTCG----TCAG**--CTCG**TG---**
Rahnella aquatilis	----------
Moraxella osloensis	----------
Bacteroides fragilis	--TCA**CCGCT----GTGA-A----GGTGCTG------CATGGTT**GTCG----------
U77658	TAGGAGCCATTCTCGAGACAT--GGGTGTTGTGCGGCCTTGGCTGCCGCG--TCAG---CTCGTG---
Chryseobacterium meningosepticum	--ACATT--**T----TTCA-A----GGTGCTG------CATGGTTG**TCG**----TCAG---CTCGTG---**
Alcaligenes faecalis	--ARAACCGG----AACACA----GGTGCTG------CATGGCTGTCG----TCAG---CTCGTG---
Campylobacter jejuni	--AGAACTTA----GAGACA----GGTGCTG------CATGGCTGTCG----TCAG---CTCGTG---
Campylobacter fetus	--AGAAAGTT----GAGACA----GGTGCTG------CATGGCTGTCG----TCAG---CTCGTG---

Table 3': Position of the reverse compliment of the red sequence (table 3) in the 16S rRNA where the reporter probe will bind (5'CTGACGACAGCCATGCAGCACCT 3')

Gram Negative Bacteria	Position of the reverse compliment of the red sequence in the 16S rRNA where the reporter probe will bind
Achromobacter xylosoxidans	n/a
Acinetobacter calcoaceticus	477
Aeromonas hydrophila	322
Alcaligenes faecalis	405
Bacteroides fragilis	n/a
Campylobacter fetus	n/a
Campylobacter jejuni	n/a
Chromobacterium violaceum	410
Chryseobacterium meningosepticum	n/a
Citrobacter freundi	471
Derxia gummosa	394
Edwardsiella tarda	428
Enterobacter cloacae	437
Escherichia coli K12	472
Haemophilus ducreyi	470
Haemophilus influenzae	433
Kingella kingae	431
Klebsiella rhinoscleromatis	121
Legionella pneumophila	405
Moraxella osloensis	n/a
Morganella morganii	358
Neisseria gonorrhoeae	474
Neisseria meningitidis	474
Paracoccus denitrificans	451
Proteus mirabilis	390
Providencia stuartii	447
Pseudomonas aeruginosa	471
Pseudomonas putida	469
Rahnella aquatilis	n/a
Rhodospirillum rubrum	472
Salmonella typhimurium	484
Serratia marcescens	468
Shigella dysenteriae	472

Continued Table 3': Position of the reverse compliment of the red sequence (table 3) in the 16S rRNA where the reporter probe will bind (5'CTGACGACAGCCATGCAGCACCT 3')

Shigella flexneri	472
Shigella sonnei	472
Vibrio parahaemolyticus	393
Yersinia enterocolitica	446

Table 4: alignment results of the 16S rRNA of the most pathogenic gram positive bacteria. The sequence shown in red is the most conserved among all the bacterial 16S rRNA of the species below and constitutes a great target for probe design. It is located at 994 nt. in the 16S rRNA and ends at the 1017 nt. in *Bacillus subtilis* since the designed probe is of 23 nt. of length a shown in table 5. The black sequences represent the rest of the DNA sequence. The breaks between the sequences only exist for legibility of the sequences.

Pathogenic Gram (+) Bacterium	Target Sequence in Red
	5' 3'
Streptococcus sanguinis	---GGG----------ATCG-----AA----------CCGCTGA------
Mycoplasma hominis	-CGAGG---------CTTATCGCAGGTAA---------TCACG----------TCCT TCATCGA-
Lactococcus lactis	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTCC---
Lactobacillus brevi	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGG---
Enterococcus avium	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGG---
Listeria monocytogenes	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGG---
Staphylococcus aureus	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGG---
Bacillus subtilis	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTG**G---**
Bacillus amyloliquefaciens	-**CGCGG---------CTGCTGGCACG**TAG----------**TTAGCCGTGG**---
Weissella paramesenteroides	----------
Lactobacillus jensenii	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGA---
Lactobacillus acidophilus	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGTGA---
Deinococcus radiopugnans	-CGCGG---------CTGCTGGCACGGAG----------TTAGCCGGTG---
Gardnerella vaginalis	-CGCGG---------CTGCTGGCACGGAG----------TTAGCCGGTG---
Bifidobacterium adolescentis	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---
Streptomyces hygroscopicus	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---
Streptomyces grisei	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---
Micrococcus luteus	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---
Mycobacterium smegmatis	-CGCGG---------CTGCTGGCACGTAG----------TTGGCCGGTC---
Mycobacterium gordonae	----------
Mycobacterium tuberculosis	-CGCGG---------CTGCTGGCACGTAG----------TTGGCCGGTG---
Mycobacterium bovis	-CGCGG---------CTGCTGGCACGTAG----------TTGGCCGGTG---
Corynebacterium genitalium	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---
Corynebacterium jeikeium	-**CGCGG---------CTGCTGGCACGTAG----------TTAG**CCGGTG---

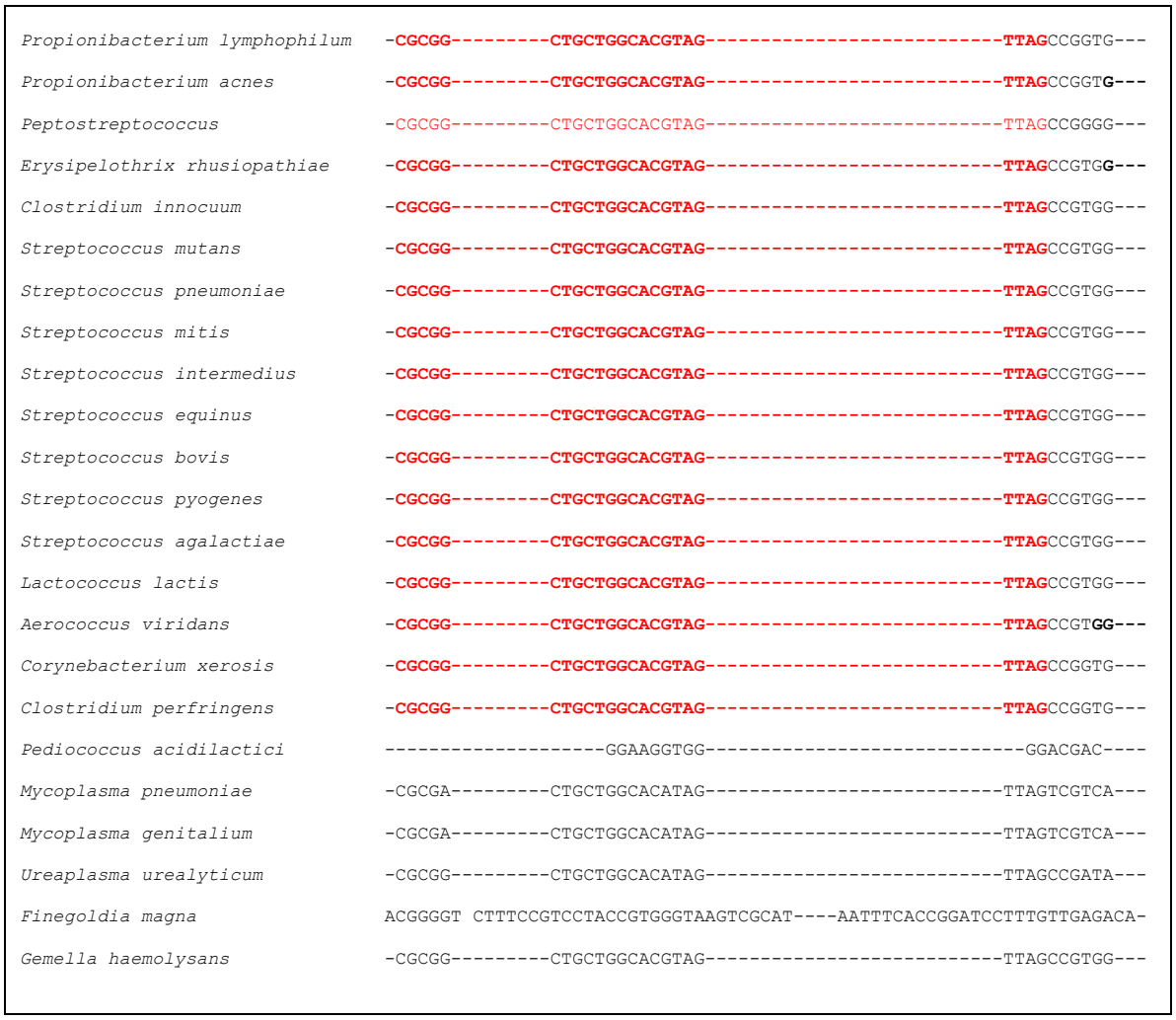

Table 4': Position of the red sequence (table 4) in the 16S rRNA where the reporter probe will bind (5'CGCGGCTGCTGGCACGTAGTTAG 3')

Gram Positive Bacteria	Position of the red sequence in the 16S rRNA where the reporter probe will bind
Aerococcus viridans	935
Bacillus amyloliquefaciens	9
Bacillus subtilis	1016
Bifidobacterium adolescentis	1019
Clostridium innocuum	822
Clostridium perfringens	1012
Corynebacterium genitalium	906
Corynebacterium jeikeium	1017
Corynebacterium xerosis	945

Continued Table 4': Position of the red sequence (table 4) in the 16S rRNA where the reporter probe will bind (5'CGCGGCTGCTGGCACGTAGTTAG 3')

Deinococcus radiopugnans	n/a
Enterococcus avium	962
Erysipelothrix rhusiopathiae	1067
Finegoldia magna	n/a
Gardnerella vaginalis	3
Gemella haemolysans	1005
Lactobacillus acidophilus	1022
Lactobacillus brevi	1015
Lactobacillus jensenii	172
Lactococcus lactis	1011
Listeria monocytogenes	978
Micrococcus luteus	995
Mycobacterium bovis	n/a
Mycobacterium gordonae	n/a
Mycobacterium smegmatis	n/a
Mycobacterium tuberculosis	n/a
Mycoplasma genitalium	n/a
Mycoplasma hominis	n/a
Mycoplasma pneumoniae	n/a
Pediococcus acidilactici	n/a
Peptostreptococcus	955
Propionibacterium acnes	1025
Propionibacterium lymphophilum	1023
Staphylococcus aureus	1018
Streptococcus agalactiae	978
Streptococcus bovis	358
Streptococcus equinus	968
Streptococcus intermedius	968
Streptococcus mitis	971
Streptococcus mutans	1015
Streptococcus pneumoniae	937
Streptococcus pyogenes	903
Streptococcus sanguinis	n/a
Streptomyces grisei	1004
Streptomyces hygroscopicus	1005
Ureaplasma urealyticum	n/a

Continued Table 4': Position of the red sequence (table 4) in the 16S rRNA where the reporter probe will bind (5'CGCGGCTGCTGGCACGTAGTTAG 3')

Weissella paramesenteroides	n/a

4.1 Reporter and Capture Probes Design

Table 3 represents the alignment of the 16S rDNA of pathogenic gram negative bacteria. Since AlleleID can only read DNA sequence and the purpose of this work is aligning the 16S rRNA all the uracils in the RNA sequences were replaced with thymidines so that the program would read the "U" as a "T" and this switch of bases was proven to have no effect on the probes design. In order to verify this statement, all the 16S rRNA were converted to their reverse complement using the RC.exe software developed by Sam Nugen from Baeumner's research group and the DNA sequences that resulted were aligned and the AlleleID software came up with the same probe result (method used in table 3). The most conserved sequence is shown in red in Tables 3 (16S rDNA) and 4 (16S rRNA) in the 5' to 3' direction. This result allows the design of a probe which is exactly the red sequence in the case of the gram negative bacteria (because DNA was aligned) and the reverse compliment of the red sequence in the case of the gram positive bacteria (because RNA was aligned) so it will hybridize to it. But since every 16S rRNA/DNA has different characteristics (%GC, length) the software calculated all the variables that describe the effectiveness of the probes and the results are shown in Tables 6 and 7. The reporter probe is a DNA probe that is theorized to bind to the 16S rRNA of the pathogenic gram negative bacteria shown in Table 1 in the direction of 3'-5' on the RNA. It is anticipated to perform experiments with actual bacterial rRNA in a liposome-

based lateral flow biosensor to test this hypothesis. So since the red sequence is a DNA sequence it was therefore adopted as the reporter probe.

As for the gram positive bacteria the method used was the same the one performed for the gram negative bacteria except that the aligned sequences in Table 2 are the 16S rRNA of the selected gram positive bacteria. Shown in Table 4 is the alignment of the 16S rRNA of gram positive bacteria and in red is the most conserved sequence (5'- 3') among the positive gram. So since the red sequence is an RNA sequence its reverse compliment was therefore adopted as the reporter probe. In order to verify that aligning 16S rRNA would give the same result as aligning 16S rDNA, all the 16S rRNA of gram positive bacteria were converted to DNA again using the RC.exe software developed by Sam Nugen from Baeumner's research group. A new alignment was then performed and the probe results are shown in table 7 which are the same as the previously selected probe from RNA alignment. Like for the gram negative probes designed, gram positive probes will be tested in later experiments to verify their ability to bind 16S rRNA and identify the bacterial gram type.

Capture probe sequences were generated using the AlleleID software taking the reverse compliment of the sense primer for the gram negative bacteria since the aligned sequences were 16S rDNA as shown in tables. As for the gram positive bacteria the sense primer was taken as it is since the aligned sequences were 16S rRNA. Unfortunately, it was impossible to find a capture probe that was specific and common to all gram negative and even less for all gram positive bacteria.

4.2 Hybridization and Cross-Hybridization results

Tables 6 and 7 show for each selected bacteria the corresponding reporter and capture probes with their characteristics such as the Gibbs Free Energy for hairpin formation, for self and cross dimerization, %GC, length of the probes and their optimal temperature. The reporter probes colored in red are the selected ones (they are all the same sequence) and as hypothesized they will bind to the corresponding bacterial species selected. The sequences represented in blue are the selected capture probes and as seen, only one capture probe will bind many gram negative bacterial RNA but almost for each gram positive bacteria there is a different capture probe as described in more detail below.

The results for how many bacteria each selected probe can bind to are summarized in Table 8. A blast search was performed on the probes at the end of the design to make sure there would not be any cross-hybridization between gram types and it was concluded that gram positive probes will not bind 16S rRNA of gram negative bacteria and vice-versa using AlleleID blast search option and therefore no expected false signal to be generated in the biosensor. Specifically, for gram negative bacteria the reporter probe selected in red (5' GGTGCTGCATGGCTGTCGTCAG 3') can detect 79% of selected gram negative bacteria which corresponds to 31 bacteria out of the 39 bacteria shown and the capture probe shown in blue (5' CGTGTTGTGAAATGTTGGGTTAAG 3') can bind to 44% (17 out of 39). For the gram positive bacteria of the reporter probe selected in red (5' CTAACTACGTGCCAGCAGCCGCG 3') can detect 64% of the selected gram positive bacteria which corresponds to 30 bacteria out of the 47 bacteria shown and the capture probe is different for every bacteria.

For the bacterial 16S rRNA sequences that wouldn't hybridize to the resulted probes only few mismatches took place. In the gram negative bacteria, 4 nt. mismatches occurred in *Bacteroides fragilis*, 1 nt. in *Chryseobacterium* species, 1 nt. in *Alcaligenes* species and 1 nt. mismatch in the *Campylobacter* species. In the gram positive bacteria, 7 nt. mismatches occurred in *Mycoplasma hominis*, 1 nt. in *Deinococcus radiopugnas*, 1 nt. in *Gardnerella vaginalis*, 1nt. in the *Mycobacterium* species, 15 nt. in *Pediococcus acidilactici*, 2 nt. in the *Mycoplasma pneumoniae* and *Mycoplasma genitalium* and 1 nt. in *Ureoplasma urealyticum*.

Table 5: The sequences suggested to be tested with *E. coli* (gram negative) and *B. subtilis* (gram positive) are listed in Table 5 below. Reporter probes will be tagged at their 3' end with the universal sequence to allow hybridization with the universal liposome. Capture probes will be biotinylated at their 5' end as shown in Figure 5 and 6.
The universal probes were tagged onto the liposomes and bind the universal sequence attached to the Reporter probe in solution. The capture probes were immobilized on the nitrocellulose membrane through Biotin-Streptavidin interaction.

Function	Sequence 5'-3'	Length	Binding Location in 16S rRNA (*E-coli* and *Bacillus subtilis*)
Gram (-) Reporter Probe	AGGTGCTGCATGGCTGTCGTCAG	23	472
Gram (-) Capture Probe	CGTGTTGTGAAATGTTGGGTTAAG	24	422
Universal Sequence	GGGGGTGGGGGTGGGGGTGG	20	N/A
Universal Probe on the liposome	CCACCCCCACCCCCACCCCC	20	N/A
Gram (+) Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	23	1014
Gram (+) Capture Probe	GCCCTTTACGCCCAATAATTCC	22	942

Table 6: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram negative bacteria. These probes will bind the 16S rDNA. So they were converted to their reverse compliment in order to bind the 16S rRNA as shown in table 5.

Pathogenic Gram Negative Bacteria	Sequence	Tm	GC %	Hairpin ΔG	Self Dimer ΔG	Cross Dimer ΔG	TaOpt
		ºC		kcal/mol	kcal/mol	kcal/mol	ºC
Providencia stuartii							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-1.8	55.9
Capture Probe	ACCGAACATCTCACGACACG	58.4	55	0	0		

Continued Table 6: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram negative bacteria. These probes will bind the 16S rDNA. So they were converted to their reverse compliment in order to bind the 16S rRNA as shown in table 5.

Chromobacterium violaceum							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	56.1
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Neisseria meningitidis							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.7	57
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Neisseria gonorrhoeae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.7	57
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Kingella kingae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.7	56.1
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Derxia gummosa							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-3.7	57
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Alcaligenes faecalis							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-3.7	55.6
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Alcaligenes denitrificans							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-3.7	55.3
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Vibrio parahaemolyticus							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Aeromonas Hydrophilia							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.7	56.5
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Edwardsiella tarda							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-2.1	56.3
Capture Probe	GTAGCGGGACTCAACCCAAC	58.7	60	-2	-2		
Proteus mirabilis							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.7
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Morganella morganii							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Providencia stuartii							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Yersinia enterocolitica							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.7
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Serratia marcescens							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.5

Continued Table 6: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram negative bacteria. These probes will bind the 16S rDNA. So they were converted to their reverse compliment in order to bind the 16S rRNA as shown in table 5.

Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Citrobacter freundii							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Salmonella typhimurium							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Klebsiella pneumoniae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.1
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Enterobacter cloacae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Shigella sonnei							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Escherichia coli O157H7							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Shigella dysenteriae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.7
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Shigella flexneri							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Escherichia coli K12							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.6	55.5
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Haemophilus influenzae							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Haemophilus ducreyi							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	53.8
Capture Probe	CTTAACCCAACATTTCACAACACG	58.2	41.7	0	-0.9		
Pseudomonas putida							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-2.1	56.1
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Pseudomonas aeruginosa							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-3.8	55.2
Capture Probe	TAACCCAACATCTCACGACACG	59.1	50	0	0		
Legionella pneumophila							
Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-0.9	54.5
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Rhodospirillum rubrum							

Continued Table 6: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram negative bacteria. These probes will bind the 16S rDNA. So they were converted to their reverse compliment in order to bind the 16S rRNA as shown in table 5.

Reporter Probe	CTGACGACAGCCATGCAGCACCTG	67.1	62.5	-1.2	-3.5	-2.1	57.1
Capture Probe	ACCCAACATCTCACGACACG	58.4	55	0	0		
Eikenella corrodens							
Reporter Probe	AACGCAGTTCCCAGGTTAAGCCCG	66.4	58.3	-0.7	-0.9	-1.8	55.6
Capture Probe	CCTCTGACACACTCTAGCTATCC	58.2	52.2	0	-4.5		
Rahnella aquatilis							
Reporter Probe	CCCCACTTTGCTCTTGCGAGGTCA	66.1	58.3	-1	-1	-1.5	55.4
Capture Probe	TAATCCCATCTGGGCACATCC	57.8	52.4	-2	-3.5		
Enterobacter aerogenes							
Reporter Probe	AACAGAGCGAGACAGCCATGCAGC	66.7	58.3	0	-3.5	-1.5	54.2
Capture Probe	GGATAAGGGTTGCGCTGTTG	57.6	55	0	-5.3		
Campylobacter jejuni							
Anti-Reporter Probe	AAACCCTGACGCAGCAACGCCGC	69.7	65.2	-1.2	-1.2	-3	56.1
Capture Probe	ACGCTCCGAAAAGTGTCATCC	59.2	52.4	0	0		
Moraxella osloensis							
Reporter Probe	ACGCTCGCACCCTCTGTATTACCG	65.5	58.3	0	-0.3	-1.5	55.4
Capture Probe	CACCTACACTCGCTTTACGC	57.1	55	0	0		
Alcaligenes faecalis							
Reporter Probe	AACCATGCAGCACCTTCACAGCGG	67.1	58.3	0	-3.5	-1.3	54.8
Capture Probe	ACTTAAGCCGACACCTCACG	58.1	55	0	-4		
Chryseobacterium meningosepticum							
Reporter Probe	AACACCTCACGGCACGAGCTGACG	68.3	62.5	-1	-3.1	-2	54.3
Capture Probe	AACTAGTGACAGGGGTTGCG	58	55	-0.7	-4.6		
Campylobacter fetus							
Anti-Reporter Probe	AAACCCTGAAGCAGCAACGCCGC	67.7	60.9	-1.2	-1.2	-3	55.4
Capture Probe	ACGCTCCGAAAAGTGTCATCC	59.2	52.4	0	0		

Table 7: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram positive bacteria. These probes are DNA probes that will bind the 16S rRNA of the pathogenic gram negative bacteria.

Pathogenic Gram Positive Bacteria	Sequence	Tm	GC %	Hairpin ΔG	Self Dimer ΔG	Cross Dimer ΔG	TaOpt
		ºC		kcal/mol	kcal/mol	kcal/mol	ºC
Erysipelothrix rhusiopathiae						-1.7	55.3
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CTCCCTTTACGCCCAATAATTCC	58	47.8	0	-1.2		
Clostridium innocuum						-1.9	56.6
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CAGACTTAGTACGCCACCTACG	59	54.5	-0.3	-2.1		

Continued Table 7: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram positive bacteria. These probes are DNA probes that will bind the 16S rRNA of the pathogenic gram negative bacteria.

Streptococcus mutans						-0.3	56
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CTCCCTTTACGCCCAATAAATCC	58	47.8	-0.9	-0.9		
Streptococcus pneumoniae						-4.4	56.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCACAGCCTTTAACTTCAGAC	58	50	0	-0.9		
Streptococcus mitis						-4.4	56.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCACAGCCTTTAACTTCAGAC	58	50	0	-0.9		
Streptococcus intermedius						-0.3	55.4
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TCGCTTTACGCCCAATAAATCC	58	45.5	-0.9	-0.9		
Streptococcus equinus						-4.4	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TTAAGCCACTGCCTTTAACTTCAG	58	41.7	-1.2	-1.2		
Streptococcus bovis						-4.4	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TTAAGCCACTGCCTTTAACTTCAG	58	41.7	-1.2	-1.2		
Streptococcus pyogenes						-2	56.4
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TTGAGCCAATGCCTTTAACTTCAG	59	41.7	-0.9	-0.9		
Streptococcus agalactiae						-2.6	56.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCACTGCCTTTAACTTCAGAC	58	50	-1.2	-1.2		
Lactococcus lactis						-2	56
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	ACACCAGACTTAATAAACCACCTG	58	41.7	-1.2	-1.2		
Lactococcus lactis (sub-species)						-2	56
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	ACACCAGACTTAATAAACCACCTG	58	41.7	-1.2	-1.2		
Lactobacillus brevis						-2.4	55.9
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CCGAAGGCTTTCACATCAGAC	58	52.4	-0.6	-0.6		
Enterococcus avium						-1.7	55.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TCGCTTTACGCCCAATAAATCC	58	45.5	-0.9	-0.9		
Aerococcus viridans						-1.7	54.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CTCCCTTTACGCCCAATAAATCC	58	47.8	-0.9	-0.9		
Listeria monocytogenes						-2	56.4
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GGGCTTTCACATCAGACTTAAAAG	57	41.7	-1.7	-1.7		
Staphylococcus aureus						-2	55.9
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	CGTGGGCTTTCACATCAGAC	57	55	-1.3	-1.3		

Continued Table 7: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram positive bacteria. These probes are DNA probes that will bind the 16S rRNA of the pathogenic gram negative bacteria.

Bacillus subtilis						-1.7	55.8
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCCTTTACGCCCAATAATTCC	59	50	0	-1.2		
Lactobacillus jensenii						-3.1	54.6
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TCGCTTTACGCCCAATAAATCC	58	45.5	-0.9	-0.9		
Lactobacillus acidophilus						-3.1	54.6
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TCGCTTTACGCCCAATAAATCC	58	45.5	-0.9	-0.9		
Bifidobacterium adolescentis						-2	55.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCCTTTACGCCCAATAATTCC	59	50	0	-1.2		
Streptomyces hygroscopicus						-2.9	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	AGCTCTTTACGCCCAATAATTCC	58	43.5	0	-3.1		
Streptomyces griseinus						-2.9	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	AGCTCTTTACGCCCAATAATTCC	58	43.5	0	-3.1		
Micrococcus luteus						-2.9	55.7
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	AGCTCTTTACGCCCAATAATTCC	58	43.5	0	-3.1		
Corynebacterium xerosis						-1.2	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCTCTTTACGCCCAGTAATTCC	58	50	-1.5	-1.5		
Corynebacterium genitalium						-2.3	57
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	AAGCTGCGGTATTACACAAACG	58	45.5	0	-3.1		
Corynebacterium jeikeium						-1.5	56.4
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCTCTTTACGCCCAGTAATTCC	58	50	-1.5	-1.5		
Propionibacterium lymphophilum						-2.5	56.1
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	AAGCTCTTTACGCCCAATAATTCC	59	41.7	0	-3.1		
Propionibacterium acnes						-4.2	56.2
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	GCCCTTTACGCCCAATAAATCC	59	50	-0.9	-0.9		
Clostridium perfringens						-3.5	56.6
Anti-sense Reporter Probe	CTAACTACGTGCCAGCAGCCGCG	67	65.2	0	-5.3		
Capture Probe	TTTCACATCCCACTTAATCATCCG	58	41.7	0	-0.9		
Streptococcus sanguinis						-4.3	53.3
Capture Probe	AGTGCCAAGGCATCCACCGTGCG	69	65.2	-2	-3.9		
Capture Probe	AGGCATTTCGTCGTTTGTCAC	58	47.6	0	0		
Streptococcus dysgalactiae						-2	58.2
Anti-sense Reporter Probe	ACGGCCACACTGGGACTGAGACAC	67	62.5	-1.5	-4.4		
Capture Probe	CGTTGCTCGGTCAGACTTCC	59	60	0	-1.2		

Continued Table 7: Reporter Probe (Red) and Capture Probes (Blue) results as exported from AlleleID for gram positive bacteria. These probes are DNA probes that will bind the 16S rRNA of the pathogenic gram negative bacteria.

Mycobacterium tuberculosis						-2	58.8
Reporter Probe	ACCTTCGACAGCTCCCTCCCGAGG	68	66.7	-1.8	-3.1		
Capture Probe	ACGGCTACCTTGTTACGACTTC	59	50	0	0		
Mycobacterium bovis						-2	58.8
Reporter Probe	ACCTTCGACAGCTCCCTCCCGAGG	68	66.7	-1.8	-3.1		
Capture Probe	ACGGCTACCTTGTTACGACTTC	59	50	0	0		
Bacillus amyloliquefaciens						-4.1	53.7
Reporter Probe	ACCGTCAAGGTGCCGCCCTATTTG	66	58.3	-1.5	-1.5		
Capture Probe	TAGTTAGCCGTGGCTTTCTGG	58	52.4	-1.8	-3.8		
Mycobacterium gordonae						-3.8	56.6
Reporter Probe	ACCCGTTCGCCACTCGTGTACCC	68	65.2	0	-2.1		
Capture Probe	CCAGGCTTATCCCGATGTGC	59	60	0	-0.4		
Mycoplasma hominis						-2	55.1
Reporter Probe	ACCAGTCCTACCTTAGGCGGTCGC	67	62.5	-2.1	-2.9		
Capture Probe	CCACGTTCTCGTAGGGATACC	58	57.1	-2	-3.4		
Pediococcus acidilactici						-2.9	56.6
Reporter Probe	AATCCGCCTGGGGAGTACGACCGC	69	66.7	-1.4	-2.1		
Capture Probe	GTTTCCGCCCTTCAGTGCTG	60	60	0	-1.2		
Mycobacterium smegmatis						-0.9	56.2
Reporter Probe	AAGGATTCGCTCCACCTCACGGCA	67	58.3	-1.4	-1.4		
Capture Probe	AGACCCCGATCCGAACTGAG	59	60	0	-2		
Peptostreptococcus anaerobius						-2.9	56.4
Anti-sense Reporter Probe	AACTGCCCACCAAGGCGACGATCA	68	58.3	-2.5	-2.5		
Capture Probe	ACCGTGTCTCAGTTCCAATGTG	59	50	0	0		
Deinococcus radiopugnans						-1.5	55.8
Reporter Probe	AACCACAGCCTAGACGCCTGCCT	67	60.9	-1.2	-1.7		
Capture Probe	CAGTTACCTTGTTACGACTTCACC	59	45.8	-0.9	-0.9		
Mycoplasma pneumoniae						-2.1	54.4
Reporter Probe	AACATGCTCCACCACTTGTGCGGG	67	58.3	-1.3	-2.3		
Capture Probe	CAAGGATGTCAAGTCTAGGTAAGG	57	45.8	0	-1.7		
Mycoplasma genitalium						-2.1	54.4
Reporter Probe	AACATGCTCCACCACTTGTGCGGG	67	58.3	-1.3	-2.3		
Capture Probe	CAAGGATGTCAAGTCTAGGTAAGG	57	45.8	0	-1.7		
Ureaplasma urealyticum						-1.5	54.5
Reporter Probe	AACACCGACTCGTTCGAGCCGACA	67	58.3	-1.8	-3.4		
Capture Probe	ACTACCCAGGCACATCATTTAATG	58	41.7	-0.6	-1.8		
Finegoldia magna						-2.5	55.2
Anti-sense Reporter Probe	AAACCCTGATGCAGCGACGCCG	67	63.6	-1.2	-3.5		
Capture Probe	AGCCGGAGCTTTCTTCTATGG	58	52.4	-1.8	-4.4		
Gardnerella vaginalis						-2	57
Anti-sense Reporter Probe	AAACCCTGACGCAGCGACGCC	67	66.7	-1.2	-2.9		
Capture Probe	AGCGGTTTACAACCCGAAGG	59	55	-2.7	-2.7		
Gemella haemolysans						-2.9	54.6
Reporter Probe	AAAAGCCGCCTTCGCCACTGGTG	67	60.9	-0.6	-1.5		
Capture Probe	CGCCTCAGTGTCAGTTACAGG	59	57.1	-1.4	-1.4		

In Table 5 the sequences to be used in a liposome-based biosensor assay are listed for *E. coli* representing gram negative and *B. subtilis* representing gram positive bacteria. In Figures 4 and 5, a biosensor set up with these sequences is given.

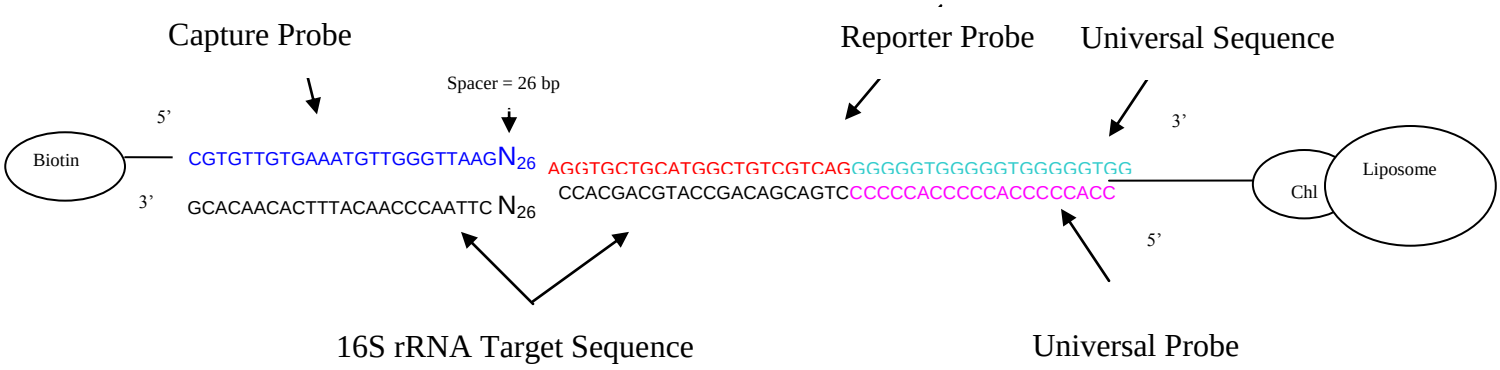

Figure 4: anticipated binding assay for the designed probes of gram negative bacteria. The capture probe is biotinylated and the liposome is tagged with a universal probe that will bind the universal sequence attached to the reporter probe.

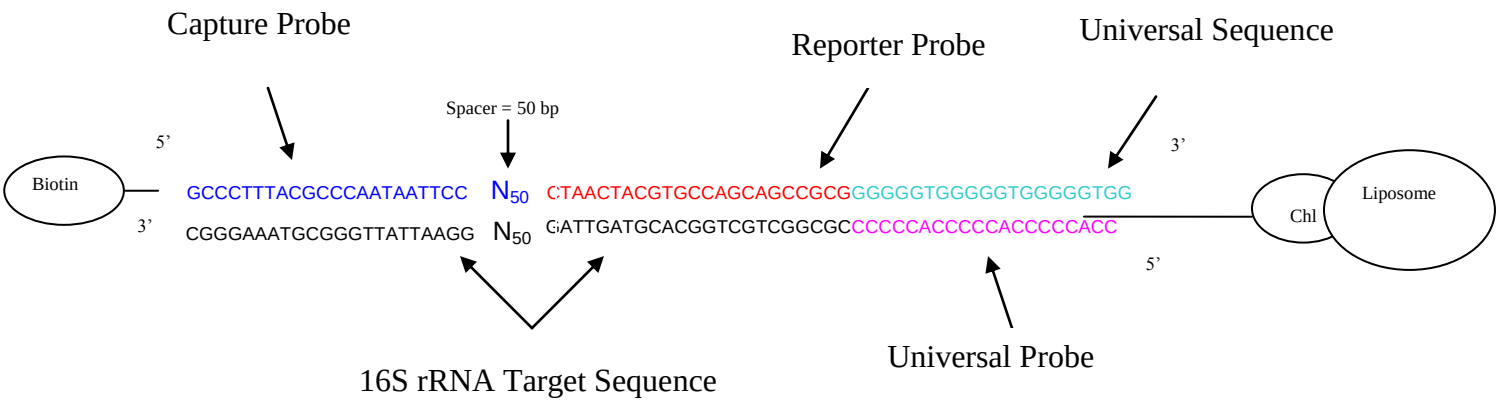

Figure 5: anticipated binding assay for the designed probes of gram positive bacteria. The capture probe is biotinylated and the liposome is tagged with a universal probe that will bind the universal sequence attached to the reporter probe

Table 8: Anticipated hybridization results.

Gram Negative Bacteria	Gram (-) Probe	Gram (+) Probe
Aeromonas hydrophila	+	-
Alcaligenes faecalis	-	-
Bacteroides fragilis	+	-
Campylobacter fetus	-	-
Campylobacter jejuni	-	-
Chromobacterium violaceum	+	-
Chryseobacterium meningosepticum	-	-
Citrobacter freundi	+	-
Derxia gummosa	+	-
Edwardsiella tarda	+	-
Enterobacter cloacae	+	-
Escherichia coli K12	+	-
Haemophilus ducreyi	+	-
Haemophilus influenzae	+	-
Kingella kingae	+	-
Klebsiella rhinoscleromatis	+	-
Legionella pneumophila	+	-
Moraxella osloensis	-	-
Morganella morganii	+	-

Continued Table 8: Anticipated hybridization results

Neisseria gonorrhoeae	+	–
Neisseria meningitidis	+	–
Paracoccus denitrificans	+	–
Proteus mirabilis	+	–
Providencia stuartii	+	–
Pseudomonas aeruginosa	+	–
Pseudomonas putida	+	–
Rahnella aquatilis	–	–
Rhodospirillum rubrum	+	–
Salmonella typhimurium	+	–
Serratia marcescens	+	–
Shigella dysenteriae	+	–
Shigella flexneri	+	–
Shigella sonnei	+	–
Vibrio parahaemolyticus	+	–
Yersinia enterocolitica	+	–
Gram Positive Bacteria		
Aerococcus viridans	–	+
Bacillus amyloliquefaciens	–	–
Bacillus subtilis	–	+
Bifidobacterium adolescentis	–	+
Clostridium innocuum	–	+
Clostridium perfringens	–	+
Corynebacterium genitalium	–	+
Corynebacterium jeikeium	–	+
Corynebacterium xerosis	–	+
Deinococcus radiopugnans	–	–
Enterococcus avium	–	+
Erysipelothrix rhusiopathiae	–	+
Finegoldia magna	–	–
Gardnerella vaginalis	–	–
Gemella haemolysans	–	–
Lactobacillus acidophilus	–	+
Lactobacillus brevi	–	+
Lactobacillus jensenii	–	+
Lactococcus lactis	–	+
Lactococcus lactis	–	+
Listeria monocytogenes	–	+

Continued Table 8: Anticipated hybridization results

Micrococcus luteus	-	+
Mycobacterium bovis	-	-
Mycobacterium gordonae	-	-
Mycobacterium smegmatis	-	-
Mycobacterium tuberculosis	-	-
Mycoplasma genitalium	-	-
Mycoplasma hominis	-	-
Mycoplasma pneumoniae	-	-
Pediococcus acidilactici	-	-
Peptostreptococcus anaerobius	-	-
Propionibacterium acnes	-	+
Propionibacterium lymphophilum	-	+
Staphylococcus aureus	-	+
Streptococcus agalactiae	-	+
Streptococcus bovis	-	+
Streptococcus equinus	-	+
Streptococcus intermedius	-	+
Streptococcus mitis	-	+
Streptococcus mutans	-	+
Streptococcus pneumoniae	-	+
Streptococcus pyogenes	-	+
Streptococcus sanguinis	-	+
Streptomyces griseinus	-	+
Streptomyces hygroscopicus	-	+
Ureaplasma urealyticum	-	-

Table 9: List of different results

G- bacteria that could bind both G- Rp and Cp	G- bacteria that could only bind G- Rp	G+ Bacteria that could only bind G+ Rp
Citrobacter freundi	*Aeromonas Hydrophilia*	*Aerococcus viridans*
Enterobacter cloacae	*Alcaligenes denitrificans*	*Bacillus subtilis*
Escherichia coli K12	*Alcaligenes faecalis*	*Bifidobacterium adolescentis*
Escherichia coli O157H7	*Chromobacterium violaceum*	*Clostridium innocuum*
Haemophilus ducreyi	*Derxia gummosa*	*Clostridium perfringens*
Haemophilus influenzae	*Edwardsiella tarda*	*Corynebacterium genitalium*
Klebsiella pneumoniae	*Kingella kingae*	*Corynebacterium jeikeium*
Morganella morganii	*Legionella pneumophila*	*Corynebacterium xerosis*
Proteus mirabilis	*Neisseria gonorrhoeae*	*Enterococcus avium*
Providencia stuartii	*Neisseria meningitidis*	*Erysipelothrix rhusiopathiae*
Salmonella typhimurium	*Providencia stuartii*	*Lactobacillus acidophilus*
Serratia marcescens	*Pseudomonas aeruginosa*	*Lactobacillus brevis*
Shigella dysenteriae	*Pseudomonas putida*	*Lactobacillus jensenii*
Shigella flexneri	*Rhodospirillum rubrum*	*Lactococcus lactis*
Shigella sonnei		*Lactococcus lactis (sub-species)*
Vibrio parahaemolyticus		*Listeria monocytogenes*
Yersinia enterocolitica		*Micrococcus luteus*
		Propionibacterium acnes
		Propionibacterium lymphophilum
		Staphylococcus aureus
		Streptococcus agalactiae
		Streptococcus bovis
		Streptococcus equinus
		Streptococcus intermedius
		Streptococcus mitis
		Streptococcus mutans
		Streptococcus pneumoniae
		Streptococcus pyogenes
		Streptomyces griseinus
		Streptomyces hygroscopicus

Since the purpose of this work is to design a single probe for the detection of each gram type, it was challenging deciding on the probe from the results shown tables 6 and 7. Each probe, as mentioned previously, was selected based most importantly on the fact that it will bind to most of the bacteria under a gram type with a good %GC, no hairpin formation, no self dimerization and a good length (18-35 nt.). In this thesis, theoretical results have shown that the designed probes would bind most of the selected conserved

sequence of the 16S rRNA as shown in chapter 4.3. More experiments will be conducted in order to verify this hypothesis such as testing whether these probes will actually bind to the 16S rRNA as shown theoretically, studying the limits of detection of those probes (how much RNA is needed to generate a signal) and finally eliminating any possibility for cross-reactivity where the gram negative probe will only bind to gram negative 16S rRNA and not to gram positive 16S rRNA and vice versa.

Other researches have also demonstrated some success in developing techniques to differentiate between gram negative and gram positive bacteria based on either RNA or DNA. One of the methods is the Nested PCR where gram –specific primers are designed to amplify the DNA of the species in the specimen and therefore differentiate between the two gram types based on the PCR results. For verification of this method, PCR results were run on an electrophoresis gel from where they were extracted, sequenced and blasted for comparison with databases available[37]. It is not surprising to learn about this research that there was a wide variation in the sensitivity of the gram positive specific primer pair which is one of the problems this thesis was dealing with because of the broad phenotypic and genotypic diversity in this group. Despite the success of the Nested PCR to differentiate between gram types, only 14 bacteria were detected, no foreseen cross-reactivity was mentioned and two rounds of PCR were needed one using general bacteria primers and another using gram-specific primers which is time consuming and financially challenging. In similar research Real-time PCR was used for the same purpose[33]. Different research developed DNA TaqMan probes to differentiate between the two different gram types by first amplifying the 16S DNA sequences using gram-specific primers and then designing gram-specific probes to detect the gram type[39]. This

fluorescence based genotyping procedure requires no gel electrophoresis, resolution of PCR products or visual assessment of bands like the previously described method. On the other it requires amplification and only target urinary tract infections (UTI). A similar research to the work performed in this thesis in the fact that it targets the 16S rRNA, was the design of primers and probes for the 16S rRNA genes and targets a wide variety of bacteria. Although this method succeeded to differentiate between gram positive and gram negative bacteria, it uses three series of oligonucleotide probes to detect the PCR product. The first series was developed to detect gram negative bacteria using two different probes and a universal probe and *Bacteroides*-specific probe. The second series was designed to detect seven other bacterial species causing meningitis using seven different probes and the third series target infection in the Cerebrospinal Fluid[31]. Clearly this method was able to achieve differentiation between gram types but that's because four different primers were used for amplification using PCR and 17 different probes were used for detection of several different species.

In contrast with the methods used previously, the detection method described in this thesis uses no DNA or RNA amplification, it requires less labor and time and uses only one probe for each gram type identification which increases the sensitivity of the biosensor a quality that will be tested in future work where a more in depth discussion will be conducted for the study and comparison of the sensitivity and detection limits of the design presented in this work

Chapter 5: Conclusion and Future Work

Two pairs of a reporter and a capture probe were designed to differentiate between gram negative and gram positive bacteria. Because the 16S rRNA is very conserved in bacteria, the purpose of this thesis was to target a commonly conserved 16S rRNA in each gram type in order to identify the latter. So all the selected sequences for each gram type were aligned and the most conserved sequence was selected as target of detection. Gram negative bacteria aren't as genotypically diverse as gram positive bacteria. For this reason the RP designed to detect the negative gram was able to hybridize to 79% (as shown in table 9) of the selected gram negative bacteria whereas the probe designed for identification of the positive gram was able to only detect 64% (as shown in table 9) of the selected gram positive bacteria. Under the same reasoning, the gram negative capture probe selected hybridized with 44% of the sequences where for each gram positive sequence a different capture probe was generated. For the purpose of testing the gram positive reporter probes the *Bacillus subtilis* CP was selected (Table 5). There is a big area of improvement in the design presented in this thesis. As seen in the precedent chapter, nt. mismatches in the 16S rRNA of the bacteria that didn't bind the probe were only few ranging between 1-7 mismatches so reducing the size of the probes (<24 nt.) could increases the number of bacteria detected without decreasing the specificity of the probes. More in depth studies will follow to test the possibility of designing shorter probes and conserving their gram-specific characteristics. Therefore in the work to follow, efficacy and sensitivity of the reporter probes designed will be tested with *E-coli K12* strain which will represent the gram negative type and with *Bacillus subtilis* which will represent the gram positive bacteria. Then the limits of detection of each probe will

be studied. This last issue is very important in this type of design because it doesn't require any RNA amplification, therefore having low limits of detection is the aim of this work.

Thank you,

Georgette Sleiman Loubnan

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