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Lipid 654 R and S Isoforms in Bacteroidetes Known to Produce Lipid 654

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Lipid 654 R and S Isoforms in Bacteroidetes Known to Produce Lipid 654

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CHAPTER I: INTRODUCTION

BACKGROUND

Lipid 654

Lipid 654 is a serine dipeptide lipid originally identified as Flavolipin in Flavobacteria (Figure 1). Lipid 654 has been demonstrated to play a role in the development of two chronic inflammatory diseases, including periodontal disease and atherosclerosis. Most recently, studies have shown that Lipid 654 may be implicated in the development of multiple sclerosis[1]. While the link between infectious agents and autoimmune disease is still controversial, there have been numerous examples of gut microbes playing a role in systemic diseases such as Crohn's disease[2] and diabetes mellitus[3].

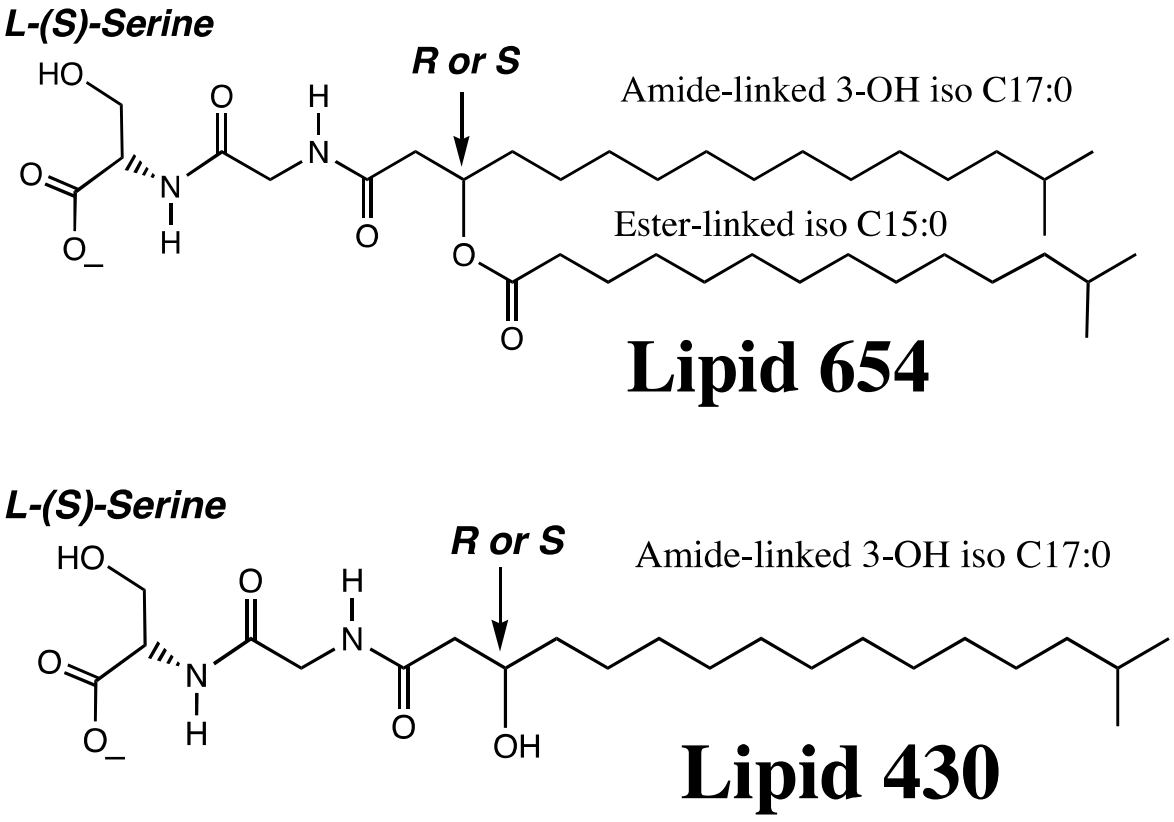


Figure 1. Structures of Lipid 654 and Lipid 430.

Lipid 654 is produced by Bacteroidetes species of the gut and oral cavity and can be detected in the serum of healthy individuals. Members of the Bacteroidetes phylum are considered to commensal organisms in the gut but some are considered to be pathogens when identified in subgingival plaque. While Lipid 654 was originally reported to be a TLR4 ligand,[4,5] it was more recently discovered that Lipid 654 acts by engaging TLR2 and TLR2 co-receptors including TLR6 and CD-14[6] as seen in Figure 2. In experiments by Clark et al., Lipid 654 was shown to activate TLR2-expressing HEK cells[6]. Furthermore, this activation could be inhibited by treatment with neutralizing anti-TLR2 antibody. When the same experiment was carried out on TLR4-expressing HEK cells, the cells failed to be activated by Lipid 654. It was also demonstrated that wild-type mice injected with Lipid 654 had increased serum levels of chemokine CCL2 when measured four hours later. The same Lipid 654 injections resulted in no increase in serum CCL2 when administered to TLR2(-/-) knockout mice.

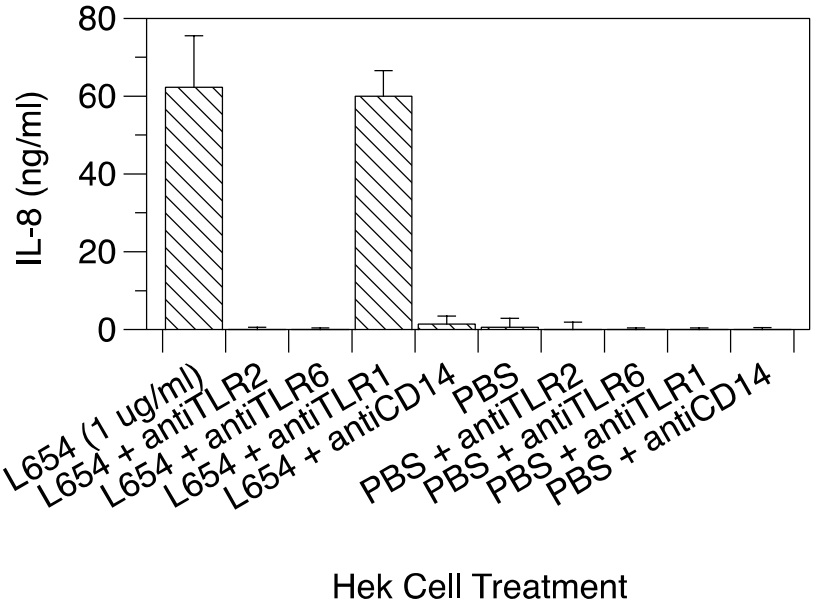


Figure 2. Lipid 654 (L654) effects on HEK cells stably transfected to express human TLR2. Cells were treated with either PBS (Controls) or *P. gingivalis* L654 (1 ug/ml) with or without the indicated neutralizing antibodies. This evidence shows that L654 engages TLR2 co-receptors and TLR6, in a manner similar to known diacylated lipoproteins (Pam2Cys but not Pam3 Cys).

Patients with multiple sclerosis are reported to have lower serum levels of Lipid 654.[1] These patients also show upregulation and increased expression of TLR2 on oligodendrocytes and peripheral blood mononuclear cells (PBMCs)[7,8]. Some authors have suggested that these two pieces of information, taken together, suggest that the presence of Lipid 654 in the circulation may act as a mechanism to mediate TLR2 tolerance[9]. Whether Lipid 654 plays a role in promoting inflammation or maintaining immune tolerance, there is a clear need to better understand the pathways through which Lipid 654 mediates the interaction between gut microbiome and host.

As it pertains to its presence in the oral cavity, Lipid 654 has also been implicated in the pathogenesis of periodontal disease. For example, Lipid 654 isolated from lyophilized *P. gingivalis* samples has been demonstrated to play a role in inflammatory disease via inhibition of osteoblast differentiation[10]. In these experiments, performed by Wang et al, osteoblasts were obtained from calvaria of wild-type or TLR2 knockout mouse pups that were genetically engineered to express Col2.3GFP transgene. Osteoblast function as well as osteoblast differentiation were then monitored by von Kossa stained mineral deposits and GFP fluorescence and gene expression respectively. These experiments showed that GFP expression, osteoblast gene expression, and mineral nodule formation were all significantly inhibited by Lipid 654. Furthermore, this inhibition was shown to be dependent on TLR2 engagement.

Despite the links established by a finding such as this, Lipid 654 extracts from gingival tissue samples showed relatively similar levels of Lipid 654 in both diseased and healthy tissues. If Lipid 654 were to be playing a key role in the disease process, one would expect to find increased levels in diseased tissues. At first, this might appear to indicate that Lipid 654 does not

play a role in periodontal disease. However, a closer look at other properties of Lipid 654 seem to explain this phenomenon.

To understand why Lipid 654 levels may be similar in both diseased and healthy gingival tissues, it is important to note that two isoforms of Lipid 654 may exist in bacterial Lipid 654 extracts. These isoforms are enantiomers differing in the R and S configuration in the beta carbon of the 3-OH iso C17:0 fatty acid. The key difference between these isoforms is that the R form can be hydrolyzed to generate another important lipid: Lipid 430. As it turns out, this difference may be crucial to the pathogenicity of Lipid 654.

Lipid 430

Lipid 430 (Figure 1) is a de-esterified hydrolysis product of Lipid 654. Hydrolysis of Lipid 654 to Lipid 430 is reported to be enzymatically catalyzed by phospholipase A2 (PLA2) (see Figure 3). The fact that phospholipase A2 hydrolyzes Lipid 654 is a significant finding in itself due to the fact that Lipid 654 is not a phospholipid and does not contain a glycerol moiety (see Figure 1). Much like Lipid 654, Lipid 430 has been demonstrated to activate TLR2 expressing HEK cells, but not TLR4 expressing HEK cells[6]. The activation of the former can also be inhibited by anti-TLR2 antibodies. Lipid 430 also increases serum CCL2 levels of wild-type mice, but does not increase serum CCL2 levels when injected into TLR2 –deficient mice. Lipid 430 was also demonstrated to inhibit both osteoblast differentiation and function, just as Lipid 654. In the same experiments as those mentioned above, Lipid 430 was shown to inhibit osteoblast gene expression and mineral nodule formation[10]. Of great importance, Lipid 430 was also shown to stimulate TNF-α and RANKL gene expression in wild-type cells. Stimulation of TNF-α and RANKL gene expression did not occur in TLR2 knockout cells. These conclusions led

investigators to conclude that Lipid 654 and Lipid 430 have the ability to promote bone loss in a TLR2 dependent manner in periodontitis.

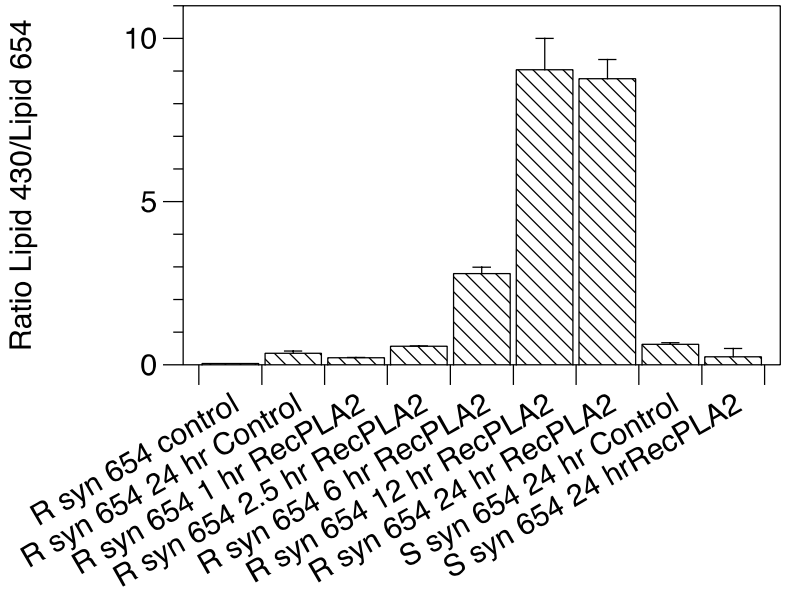


Figure 3. Hydrolysis of synthetic L654 by a commercially available preparation of human recombinant secretory PLA2. Enantioenriched R or S synthetic L654 preparations were prepared by Dr. Mike Smith and Mr. Chris Dietz of the Department of Chemistry at the University of Connecticut. Aliquots of synthetic R or S L654 (250 ng) were sonicated in buffer (1 ml of 10 mM Tris, pH 7.5, 2.5 mM CaCl and 150 mM NaCl) for 20 sec and after adding 5ug of human recombinant secretory PLA2 (Cayman Chemical, >95% purity), samples were stirred at 37°C for up to 24 hours. Controls received no enzyme. Samples were then acidified with 25 □l of acetic acid and extracted three times with chloroform. Extracts were pooled, dried and reconstituted in HPLC solvent. L654 and L430 were quantified using multiple reaction monitoring mass spectrometry (LC-MRM MS) as previously reported [1]. Figure 5 shows hydrolysis of only the R synthetic 654. We also evaluated honey bee venom PLA2 and porcine pancreatic PLA2 (Sigma) and demonstrated that both of these PLA2 enzyme preparations catalytically hydrolyze essentially only the R synthetic L654 (data not shown).

Lipid 430 is a Hydrolysis Product of Lipid 654

The sections above have highlighted a few questions that need to be addressed: 1) If Lipid 430 is driving periodontal disease, would it be important to examine bacterial Lipid 430 levels to assess

pathogenicity? 2) If Lipid 654 is driving periodontal disease, why do healthy gingival tissues and diseased gingival tissues have similar levels? The answer to the first of these questions is found in the fact that, as noted above, Lipid 430 is a hydrolysis product of Lipid 654. Lipid 430 itself is found in extremely low levels in bacterial lipid extracts. This can be seen clearly in Figure 4 where Lipid 430/Lipid 654 ratios are assessed in multiple oral bacteria. Interestingly, Lipid 430/Lipid 654 ratios are much higher in tissue samples (see Figure 5).

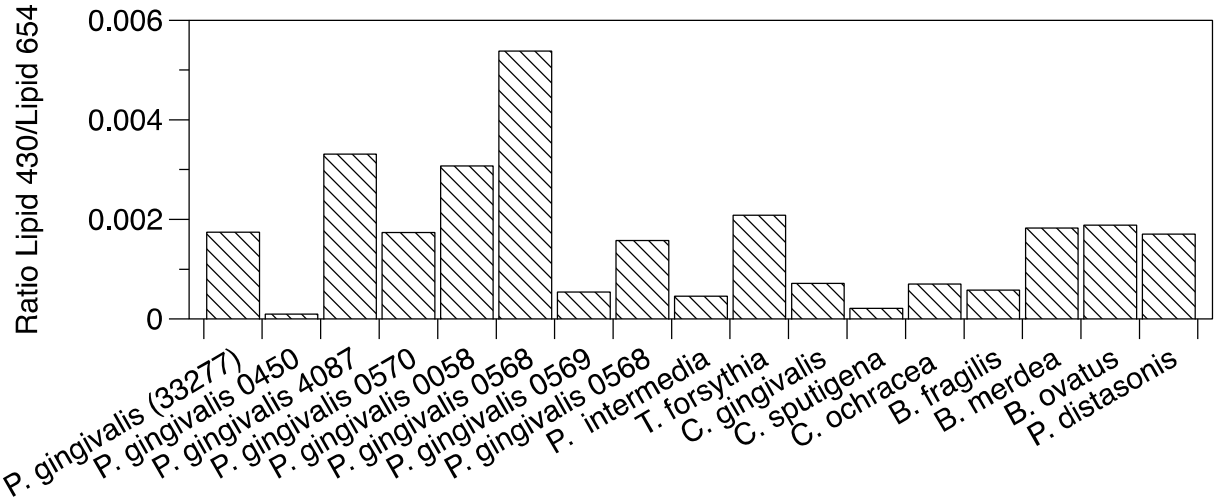


Figure 4. L430/L654 ratios in lipid extracts from oral and gastrointestinal Bacteroidetes. Bacteria samples were obtained from cultured organisms or from donated samples. The *P gingivalis* samples except for the ATCC strain and *T. forsythus* were obtained from the Forsyth Institute, and the intestinal Bacteroidetes were obtained from Dr. Sid Finegold. Bacterial samples were extracted using the Bligh and Dyer procedure and the extacted lipids were analyzed by MRM-MS [1].

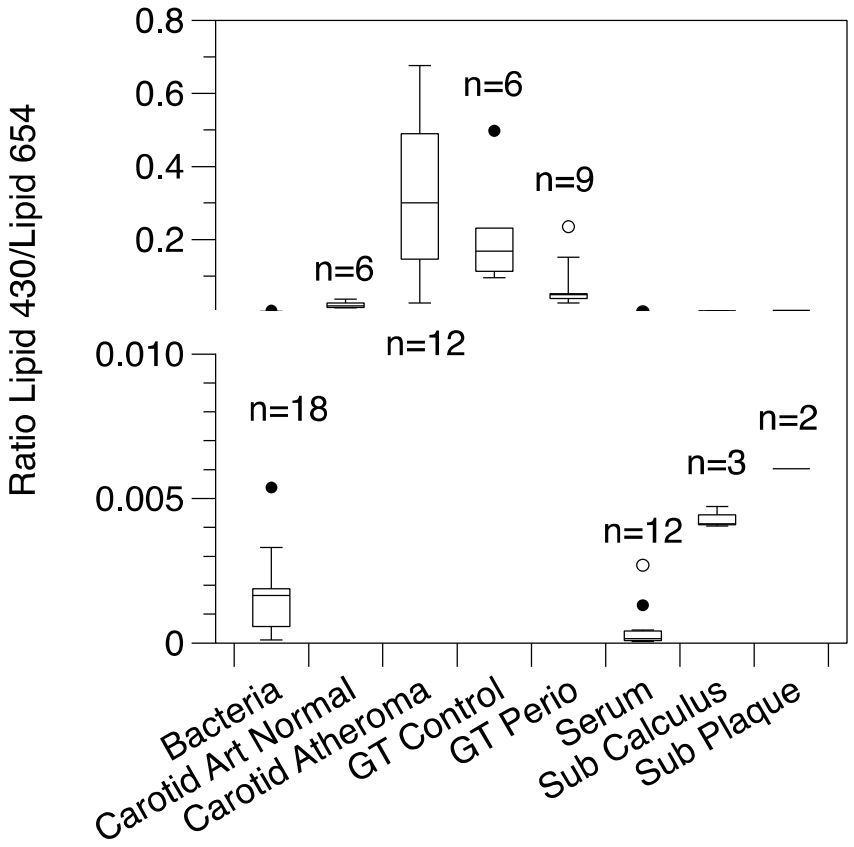


Figure 5. L430/L654 ratios in lipid extracts from bacteria, carotid artery, gingival tissue, plaque and calculus samples. Bacteria samples shown in Figure 3 were averaged and depicted at left. Tissue samples were obtained at the time of surgical excision and were frozen until the time of processing. Subgingival calculus samples were obtained from extracted teeth and plaque samples were obtained with endodontic paper points from severe periodontitis sites of two subjects. The control carotid artery samples were obtained from the National Disease Research Interchange (Philadelphia, PA). Gingival tissue samples were obtained from either healthy (GT Control) or chronic periodontitis (GT Perio) sites. All samples were extracted using the Bligh and Dyer procedure and the extracted lipids were analyzed by MRM-MS for quantification of L430 and L654 [1].

These findings suggest that Lipid 654 is hydrolyzed to Lipid 430 within tissues. As for the second question posed – the answer can be found by revisiting one of the properties of Lipid 654 noted above. Specifically, Lipid 654 may be synthesized as two isoforms differing in the R and S configuration in the beta carbon of the 3-OH iso C17:0 fatty acid. Because serine is recovered in Lipid 654 as the L enantiomer, Lipid 654 containing *(R)* and *(S)* isoforms represent diastereomers.

The important distinction between these two forms that has not yet been brought up, is that only the R form appears to be hydrolyzed by lipase enzymes, specifically PLA2. Preliminary work using a new mass spectrometric analytic approach utilizing differential ion mobility MRM suggested the presence of two isoforms of Lipid 654 in a purified preparation of Lipid 654 from *P. gingivalis* (ATCC 33277). (see Figure 6). At the same time, inactive Lipid 654 samples have equal levels of the R and S isoform. It can also be seen that treating the active Lipid 654 samples with phospholipase A2 will hydrolyze the excess R isoform, and only the R isoform, until both forms are present in equivalent amounts. These results form the basis for suggesting that R Lipid 654 is preferentially hydrolyzed by PLA2. Therefore, differences in the recovery or R versus S Lipid 654 in various isolates of bacterial Lipid 654 could account for the differences in biological activity between these preparations.

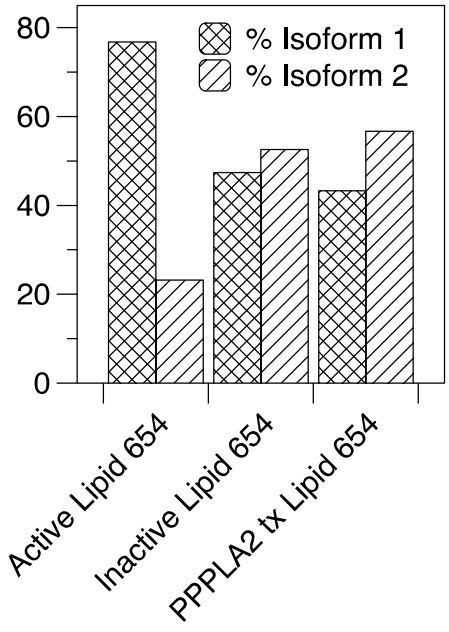
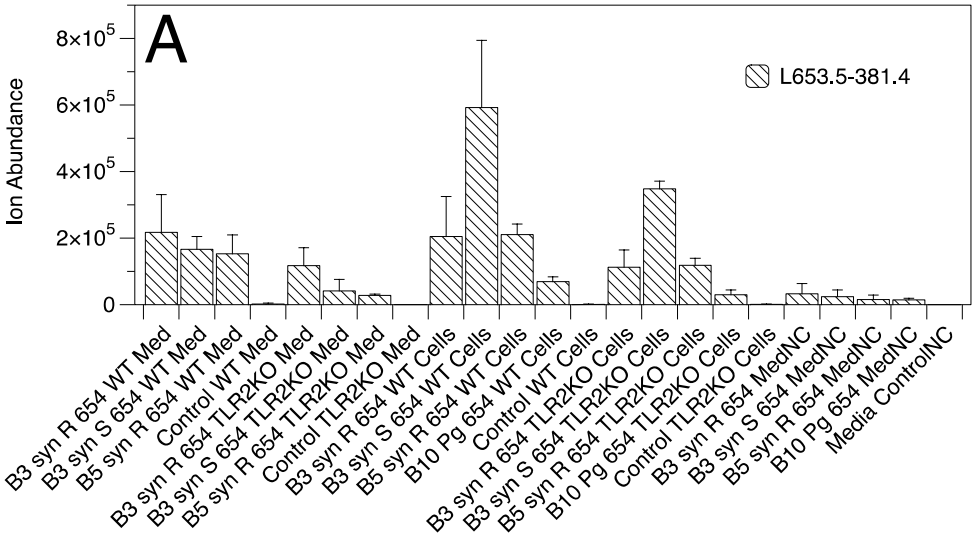


Figure 6. Relative levels of R and S isoforms of Lipid 654 in active, inactive and PP PLA2 treated Lipid 654 samples.

To examine the biological relevance of this hydrolysis, synthetic R and S Lipid 654 preparations were analyzed for catalytic hydrolysis by bone marrow macrophages. A shown in Figure 7, recovery of Lipid 654 and lipid 430 is frequently less in TLR2 knockout cells compared with wild type cells. The ratio of Lipid 430 to Lipid 654 was substantially less in the TLR2 knockout cells than the wild type cells. This suggests that cell-mediated hydrolysis of Lipid 654 is at least partially enantioselective, similar to that observed with PLA2 hydrolysis of these synthetic standards.



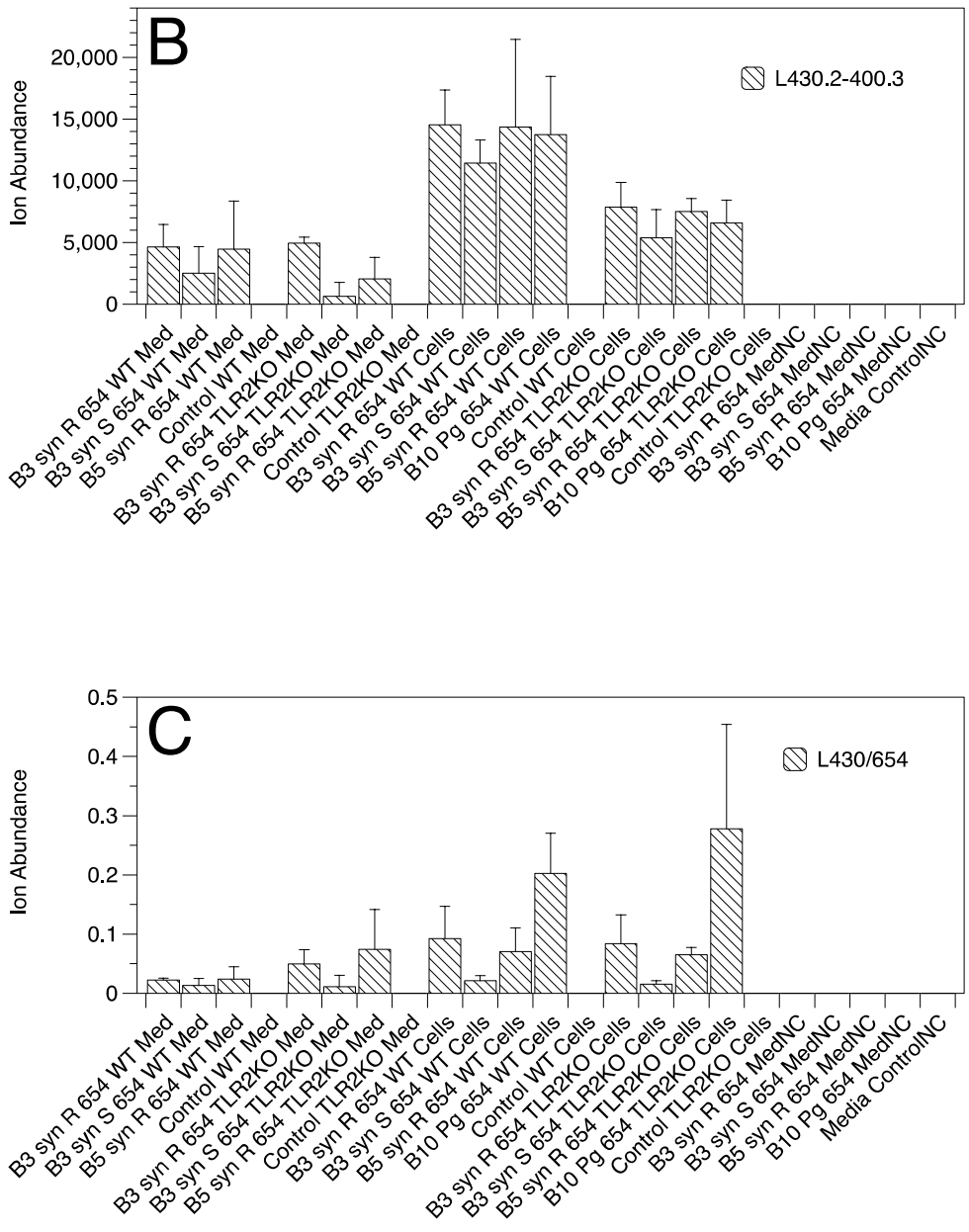


Figure 7A-C. Recovery of Lipid 430 and Lipid 654 in bone marrow macrophages and media samples after exposure to R or S Lipid 654 as well as bacterial Lipid 654. Fig. 7A shows the recovery of Lipid 654 and Fig. 7B shows the recovery of Lipid 430 in cells and media samples. Fig. 7C shows the difference between Lipid 430/Lipid 654 ratios when cells are treated with either R or S Lipid 654. Bone marrow macrophages were incubated for 24 hour with the indicated lipid preparations. Media samples were harvested and the cells washed twice with PBS. The cells were then scraped in PBS and transferred to glass tubes for lipid extraction. Both media and cell samples were acidified with acetic acid and extracted chloroform. The samples were dried and analyzed by MRM ESI as previously described.

Identification of Bacterial Taxa via 16S Ribosomal RNA Sequencing

As noted above, approximately one third of the bacteria that have been identified to reside in the human oral cavity are currently uncultivable[11]. These bacterial phyla have been identified through sequencing methods that allow investigators to uniquely identify bacterial phyla by the sequence of their 16S rRNA gene. The 16S rRNA gene codes for the 16S ribosomal RNA molecule. This is the portion of the 30S small subunit of the prokaryotic ribosome which binds to the Shine-Dalgarno sequence in order to initiate translation (see Figure 8).

This gene can be used to phylotype bacteria because portions of it have very slow rates of evolution. Carl Woese first developed the use of 16S rRNA gene sequences to phylotype bacteria by taking advantage of highly conserved regions within 16S rRNA introns[12]. Universal primer pairs which anneal to these highly conserved areas were first developed by Weisburg *et al*[13]. The primers developed by Weisburg were the 27F forward primer and 1492R primer which were used to amplify 1465 base pair amplicons. These amplicons contained highly variable regions of the 16S rRNA gene that could be used to uniquely identify bacterial taxa (see Figure 9). Sequencing of these highly variable regions, which uniquely identify bacterial phyla, is made possible by taking advantage of highly conserved regions within the gene which allow for sequencing primers to anneal. Because previous studies have associated bacterial phyla with their specific 16S rRNA gene sequences, the sequencing reads obtained from unknown bacterial populations can be identified using a Basic Alignment Search Tool (BLAST) to align the unknown sequence with one in a database – uniquely identifying the bacteria from which that sequence came.

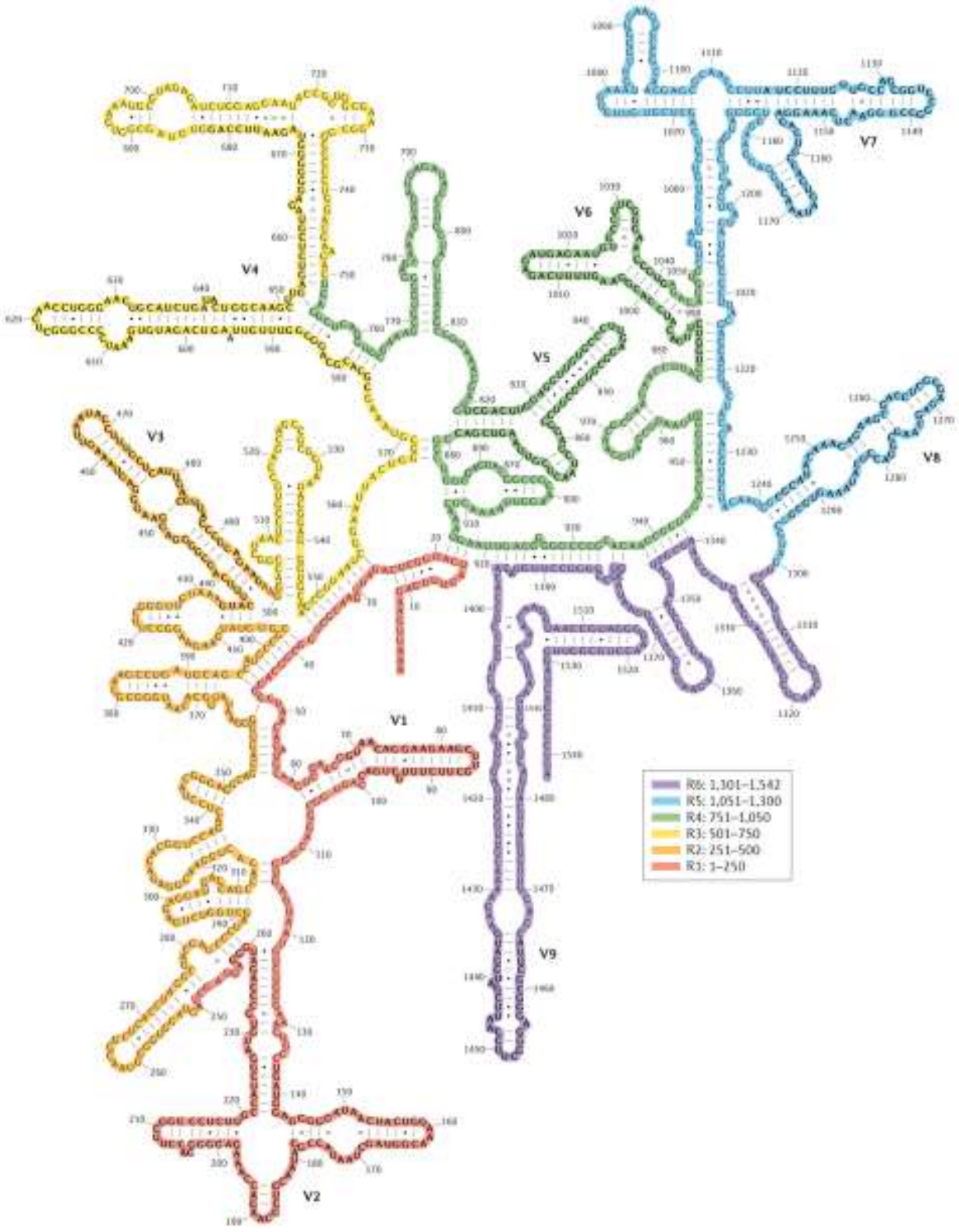


Figure 8. Secondary structure of 16S rRNA molecule[14].

Amplification and sequencing of the 16S rRNA gene can be used either to identify new bacteria (as phyla can be classified based on sequences) or to confirm the presence of bacteria in a sample population. In the case of this study, this sequencing technique will be utilized to confirm the purity of cultures grown up for lipid extraction.

Primer:	Primer Sequence:	Primer Length	*E. coli* Position
UnivBactF	5' GAG TTT GAT YMT GGC TC	17-mer	9 - 25
UnivBactR	5' GYT ACC TTG TTA CGA CTT	18-mer	1509 - 1492

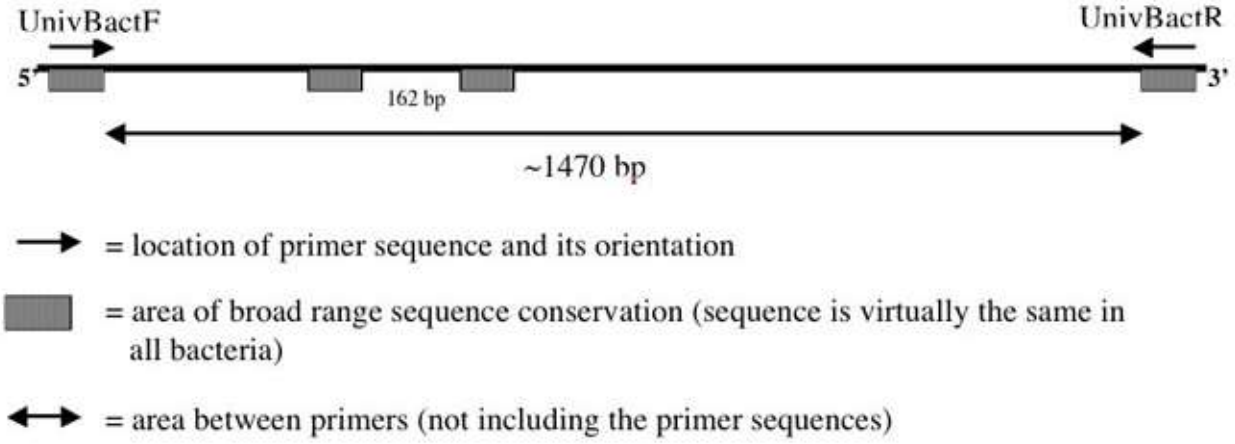


Figure 9. Areas of sequence conservation along the 16S rRNA gene allow for amplification of sequence containing highly variable regions which allow for phylotyping[13].

RATIONALE

Given the possible role of both Lipid 654 and Lipid 430 in the pathogenesis of periodontal disease as well as multiple sclerosis outlined above, further investigation into the mechanism of action of these lipids is clearly warranted. Most importantly, it is necessary to investigate whether the presence or absence of the R and S isoforms plays a role in the pathogenicity of these Lipids. Understanding the role these different isoforms play and which bacteria contain them will provide a better understanding of the disease pathogenesis. This information, in turn, will provide a better understanding of how to counteract their effects. Specifically, understanding which bacteria are more or less pathogenic in these diseases may allow for manipulation of the oral and gut microbiome to aide in disease prevention.

CHAPTER II: HYPOTHESIS AND AIMS

Hypothesis

We hypothesize that the relative levels of the R and S isoforms of Lipid 654 will vary among different Bacteroidetes present in the oral cavity, particularly those associated with chronic inflammatory periodontal disease.

Specific Aims

To determine the distribution of Lipid 654 R and S isoforms in Bacteroidetes known to produce Lipid 654. This aim is proposed because two isoforms have been identified in *P. gingivalis* lipid extracts and these isoforms vary in biological activity and enzyme susceptibility, both of which

may be important in the potential for specific Bacteroidetes to promote disease through this lipid class. We will determine the distribution of R and S isoforms of Lipid 654 produced by oral Bacteroidetes species and whether the isoforms of Lipid 654 recovered in dental tissues represent the two enantiomeric forms derived from *P. gingivalis* and perhaps other oral Bacteroidetes. Specifically, we will analyze oral isolates of *P. gingivalis* previously obtained from the Forsyth Institute and compare them with ATCC #33277. We will also analyze *P. gingivalis* isolates of 381, W83 and W50 strains obtained from ATCC as well as *Prevotella intermedia*, and *Tannerella forsythia*.

CHAPTER III: MATERIALS AND METHODS

Growth of Bacterial Cultures

Bacteria were inoculated into liquid basal medium (peptone, trypticase and yeast extract) supplemented with hemin, menadione (Sigma-Aldrich), and brain heart infusion (BHI). Bacteria were cultivated in liquid broth – being incubated for four days in an anaerobic chamber flushed with N_2 (80%), CO_2 (10%), and H_2 (10%) at 37ºC. Cultures were then harvested by centrifugation at 2000 x g for 2 hours. Bacterial pellets were then lyophilized to prepare for lipid extraction. Culture purity was verified by 16S rRNA gene amplification and sequencing as outlined below.

Verification of Culture Purity

Culture purity was verified using amplification and sequencing of the 16S rRNA gene from the bacterial pellets obtained. Amplification via touch PCR (polymerase chain reaction) was

performed using GoTaq Green Master Mix (Promega Corporation, Madison, WI). 50µl reaction mixtures were made as seen in Table 1.

GoTaq Green Mastermix	25µl
Upstream primer (10pmol/µl)	2µl
Downstream primer (10pmol/µl)	2µl
H_2O	18.5µl
$MgCl_2$ (50mM)	2.5µl

Table 1. PCR Reaction Setup

The upstream and downstream primers referred to in Table 1 are the universal forward and reverse 16S rRNA primers seen in Table 2. These primers have been shown to be universally effective in the amplification of the 16S rRNA gene from a broad spectrum of bacteria[15].

Primer Name	Sequence	Orientation	Comments
Universal Forward	GAGTTTGATYMTGGCTCAG	Forward	Amplification of 16S rRNA gene
Universal Reverse	GHTACCTTGTTACGACTT	Reverse	Amplification of 16S rRNA gene
AE50	TKACCGCGGCTGC	Reverse	Sequencing Primer at bp 521-533

Table 2. Primer Chart

Touch PCR was performed using sterilized glass pipettes to add template DNA from lyophilized bacterial pellets to each 50µl reaction mixture. PCR reactions were run in thin walled tubes using a PCR Sprint Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). A hot start protocol was utilized allowing the wells to reach 95ºC before the tubes were taken off of ice.

PCR cycling protocol was as follows: 95ºC for 10 minutes; 30 cycles of 95ºC for 45 seconds, 60ºC for 45 seconds, 72ºC for 2 minutes; 72ºC for 15 minutes.

Once PCR was completed, samples were shipped to GeneWiz (GeneWiz, South Plainfield, NJ) for PCR clean up and sequencing with reverse sequencing primer AE50 (see Table 2). Primer AE50 allowed sequencing of approximately the first 500 base pairs which were enough to phylotype the parent bacteria. Upon receipt of the sequencing reads from GeneWiz in the form of ab1 trace files, all files were downloaded. These files, which contained the chromatograms obtained from sequencing the first 500 base pairs of the amplicon produced in the steps above, were opened using Chromas sequencing software (Technelysium, South Brisbane, Australia). Chromatograms were inspected for double reads and trimmed as follows.

First, all chromatograms were inspected to see if single or multiple reads were present (see Figure 10). Single reads indicated that a single 16S rRNA gene had been amplified – a result consistent with a culture with only one bacterial species present. Chromatograms with 2 or more overlapping reads indicated that the culture had been contaminated by another species. Unfortunately, there was no way to untangle the multiple reads to determine which species had been cultured along with the intended bacterial species.

Chromatograms showing single reads were used to trim obtained sequence to remove bases that could not clearly be identified by the software. Generally, these trimmed bases included the first 30 base pairs read – which are usually of poor quality in Sanger sequencing – as well as the end of the chromatogram extending beyond the first base of the amplicon being sequenced. Figure 11 shows an example of base pairs selected to be removed from the first 30 base pairs of a sample read.

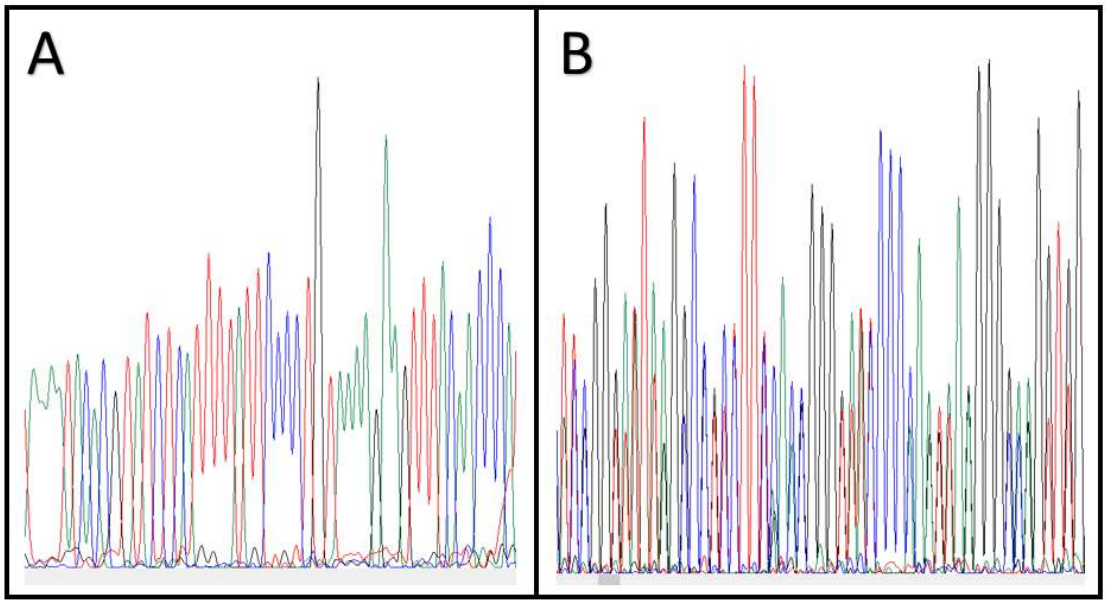


Figure 10. Box A depicts a chromatogram with one read. This chromatogram is indicative of a single amplicon having been sequenced – consistent with the presence of one bacterial species in the original culture. Box B depicts a chromatogram with 2 distinct reads. Note the double peaks present. This chromatogram is consistent with the presence of multiple amplicons, indicating that multiple bacterial species were present in the original culture.

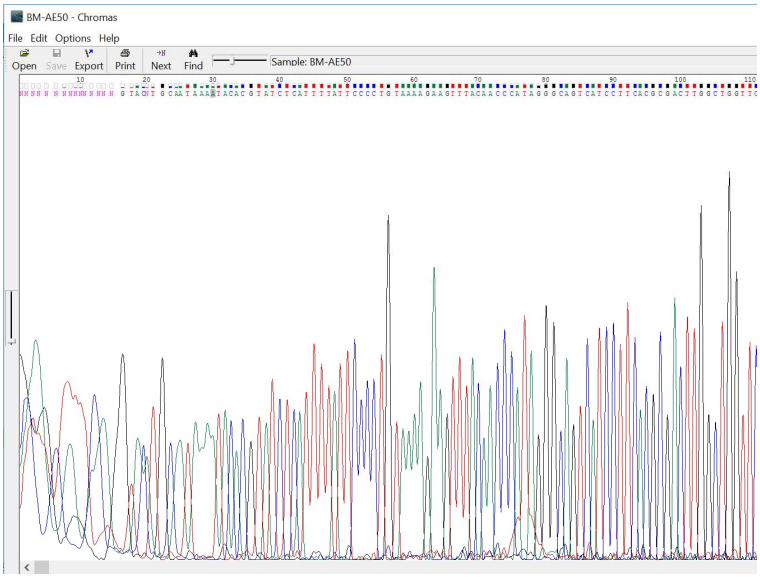


Figure 11. Base pairs 1-30 are trimmed from a sample .ab1 file due to poor reads. As can be seen, the software is unable to make a clear call on which base pair is present.

Once the sequence reads had been trimmed, the remaining 400-500 base pairs of quality sequence were used in a BLAST analysis to find significant alignments between the sequence obtained and the 16S rRNA gene library of the Greengenes database at greengenes.lbl.gov[16,17]. Alignments between the obtained sequence and a database match were displayed as seen in Figure 12. Alignments of greater than 99% were considered to confer identification of the parent bacteria.

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified	☐							
otu_4443	☐							
699032	☐		435	100.00	435	AB547660.1	Porphyromonas gingivalis str. JCM 12257	48898
k__Bacteria	☐							
p__Bacteroidetes	☐							
c__Bacteroidia	☐							
o__Bacteroidales	☐							
f__Porphyromonadaceae	☐							
g__Porphyromonas	☐							
s__Porphyromonas gingivalis	☐							
otu_1029	☐							
292895	☑		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292896	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292897	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292898	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
294927	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294928	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294929	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294930	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
35745	☐		435	100.00	435	AB035455.1	Porphyromonas gingivalis str. FDC 381	19162

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Figure 12. Table from BLAST search against Greengenes 16S rRNA gene database. Matches of greater than 99% were assumed to confer identity of the parent bacteria.

Lipid Extraction and Semipreparative HPLC Fractionation

To begin the extraction of bacterial lipids from the lyophilized bacterial pellets, the bacterial samples were first weighed. Each 2g of bacterial pellet was then suspended in 16ml of chloroform:methanol:water in a ratio of 1.33:2.67:1 (v/v/v). This mixture was vortexed every 15

minutes for 2 hours and then supplemented with 6ml of chloroform and 6ml of 2N of KCl + 0.5N K_2HPO_4. The mixture was once again vortexed and centrifuged at 2000 x g for 4 hours at 20ºC. At this time, the lower organic phase was collected, dried under nitrogen, and reconstituted into 24ml of the following HPLC solvent: hexane:isopropanol:water in a ratio of 6:8:0.75 (v/v/v). This mixture was then centrifuged at 2500 x g for 10 minutes and the supernatant was collected from HPLC analysis. Semipreparative HPLC fractionation was then performed using a Shimadzu 10ADvp HPLC system. Lipid fractionation was performed using normal phase isocratic separation (Ascentis Si, 25cm x 10mm, 5µm, Sigma-Aldrich) with a flow rate of 1.8ml/min. Fractions were collected in 1 minute increments and effluent was monitored at 205nm as shown in Figure 13. After pooling replicate fractions, they were dried under nitrogen and reconstituted in HPLC solvent (hexane:isopropanol:water mixture as mentioned above) for mass spectrometric analysis. This analysis is described below. Fractions which were determined to contain Lipid 654 by ESI MRM analysis were pooled and dried before further acidic HPLC fractionation to separate serine lipids from phospholipids of similar structure.

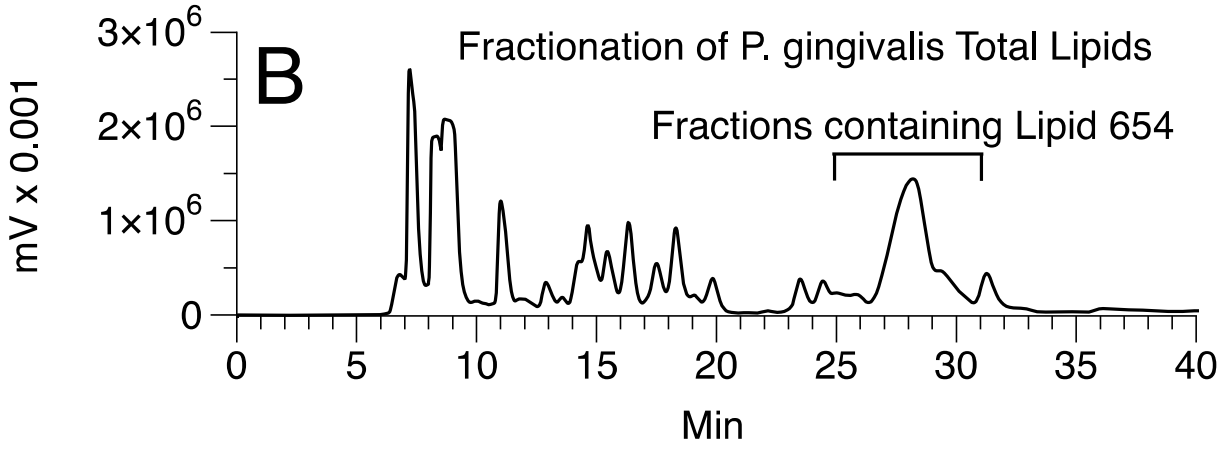

Figure 13. Total lipids of *P. gingivalis* fractionated by normal phase HPLC. The fractions containing Lipid 654 are shown.[18]

Mass Spectrometry

Mass spectrometry was performed by applying lipids from recovered HPLC fractions to a normal phase column (Ascentis Si, 3cm x 2.1mm, 5µm, Sigma Aldrich) connected to an Qtrap 4000 instrument (Sciex, Framingham, MA). Under isocratic conditions, neutral HPLC solvent was delivered at a flow rate of 100-120 µl/min using a Shimadzu LC-10ADvp pump. Using a mass range of 100 to 1600 amu, and MS/MS acquisition parameters optimized for specific lipid products, total ion chromatograms were obtained. Negative ion ESI was carried out at -4500 V with a focusing potential of -10 V, a declustering potential of -90V, and an entrance potential of -10 V. Collision energies for negative ion products ranged from -30 to -55 volts. The MRM negative ion transitions which were monitored for Lipid 654 were m/z 653.5/131.1, 653.5/306.2, and 653.5/381.4.

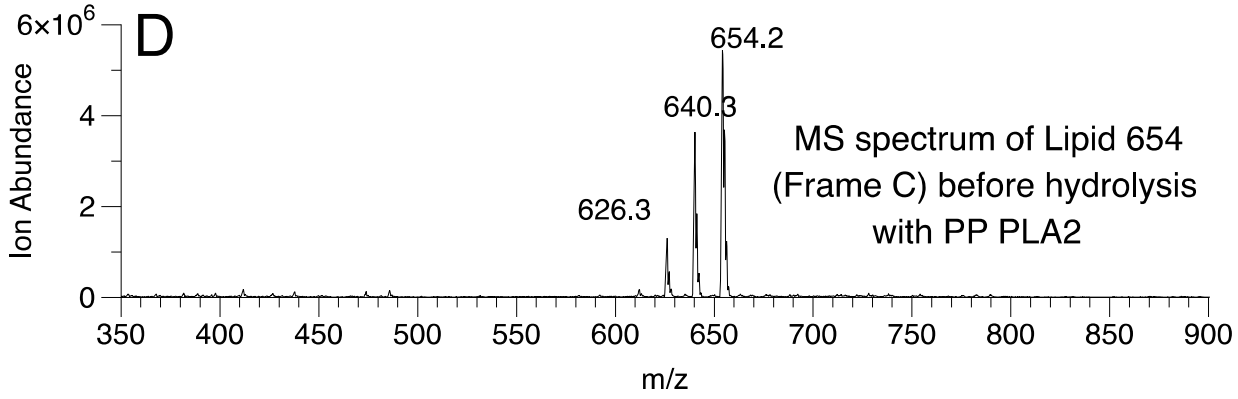

Figure 14. MS spectrum of Lipid 654.[18]

Acidic HPLC Fractionation

To further purify Lipid 654 from co-eluting lipids after initial fractionation, acidic HPLC fractionation was performed. Acidic HPLC allowed Lipid 654 to be separated from co-migrating phospholipids. To perform this separation, the factions from the previous semipreparative HPLC which contained Lipid 654 were pooled, dried under nitrogen, and reconstituted in the same HPLC solvent used above [hexane:isopropanol:water in a ratio of 6:8:0.75 (v/v/v)] with the addition of 0.1% acetic acid. This mixture was then fractionated once again using the same Shimadzu 10ADvp HPLC system along with the same normal phase HPLC column (Ascentis Si, 25cm x 10mm, 5µm, Sigma-Aldrich). The flow rate was 1.8ml/min and fractions were collected in 1 minute increments. Lipid 654 eluted in the 10-12 min fractions, ahead of other phospholipids which eluted at later time points. Fractions containing Lipid 654 were collected, pooled, and refractionated in order to produce a highly enriched Lipid 654 sample as illustrated in Figure 15. This enriched sample was finally divided into 250µg aliquots.

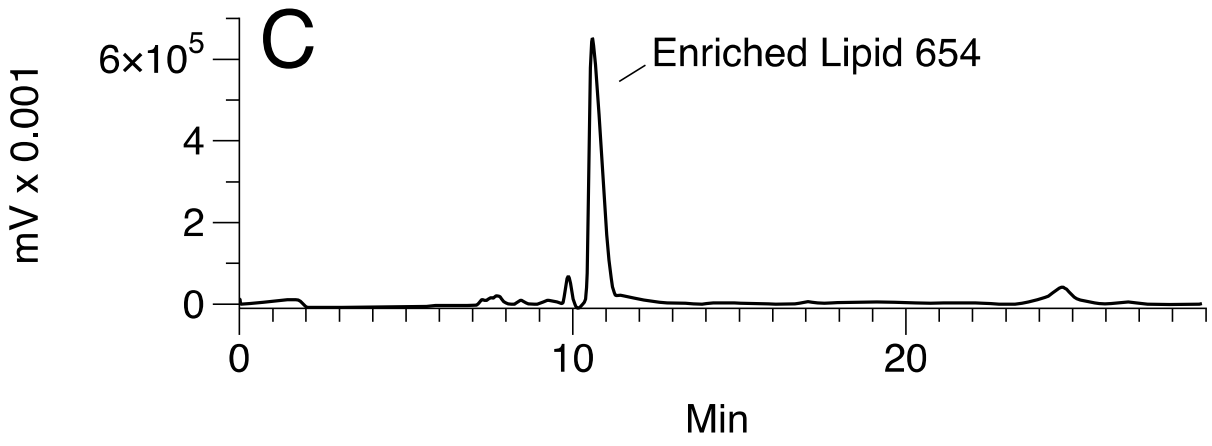

Figure 15. Enriched Lipid 654 sample obtained from acidic HPLC fractionation.[18]

Chiral HPLC Fractionation

Once an enriched Lipid 654 sample was obtained, the R and S isoforms were separated via chiral HPLC fractionation. The enriched Lipid 654 sample was dried and reconstituted in a solvent composted of hexane:isopropanol, 9:1 (v/v) and supplemented with 0.1% diethylamine. Fractionation was performed by eluting the enriched sample over a LUX-cellulose 4 (25 cm x 4.6 mm, 10µm, Phenomenex, Torrence, CA) at a flow rate of 0.6ml/ min. The effluent was monitored at 205nm. A racemic mixture of synthetic L-serine-(*R*)- and *(S*)-Lipid 654 was prepared as reported by Deitz *et al*[19] for use as a standard to demonstrate at which time points the R and S isoforms elute. This standard was run over the same column and chromatograms were compared to see relative levels of R and S isoforms recovered from the enriched samples.

Chapter IV: RESULTS

Cultures were inoculated, incubated, and tested for verification of purity as described above for eleven common oral bacteria. Results for culture purity are depicted below in Table 3. Final trimmed 16S rRNA gene sequence reads obtained with sequencing primer AE50 may be found in Appendix I. BLAST results against the Greengenes database may be found in Appendix II.

Sample Description	Greengenes BLAST result	% Match
Porphyromonas gingivalis ATC33277	*Porphyromonas gingivalis* ATC33277	100
*Porphyromonas gingivalis 0450**	*Porphyromonas catoniae*	94.53
Porphyromonas gingivalis 4087	*Porphyromonas gingivalis* W83	99.5
Porphyromonas gingivalis 0568	*Porphyromonas gingivalis* W83	99.56
Porphyromonas gingivalis 0569	*Porphyromonas gingivalis W83*	99.78
Porphyromonas gingivalis 381	*Porphyromonas gingivalis* FDC 381	99.57
Porphyromonas gingivalis W83	*Porphyromonas gingivalis* W83	100
Prevotella intermedia	Two reads present after bp 290	
Porphyromonas gingivalis 0570	*Porphyromonas gingivalis* W83	99.5
Porphyromonas gingivalis 0580	Multiple matches- none were *Porphyromonas gingivalis*	
Tannerella forsythia	*Tannerella forsythia*	100

Table 3.

*BLAST result against Greengenes database returned a match with less than the 99% homology required to confirm strain identity.

As can be seen above in Table 3, most strains returned BLAST results with the required 99% homology match required to confirm sequence identity. The two notable exceptions were the *P. gingivalis* strain 0450 and *P. intermedia* obtained from the late Ed Moore of VPI. In the first case, the trimmed sequence read obtained for *P. gingivalis* strain 0450 was used in a BLAST search against the Human Oral Microbiome Database.[11,15] This BLAST search returned a homology match of 98.7% against "*Porphyromonas* sp. Strain_F0450c," confirming that the culture was a pure culture of the intended species. In the case of the *P. intermedia* culture, the ab1 files analyzed by Chromas showed 2 reads after base pair 290. The 260 base pairs of quality read (the first 30 base pairs were removed due to poor quality as described above) returned a BLAST search result showing homology to *Prevotella nigrescens*. These BLAST results may also be found in Appendix II.

At the time of this writing, not all cultures had undergone lipid extraction, Lipid 654 enrichment and separation of R and S Lipid 654 isoforms as outlined above. The results for those samples which have been completed are shown below in Table 4.

Sample	R-isoform %	S-isoform %
*Porphyromonas gingivalis ATC33277**	100	0
*Porphyromonas gingivalis 0450**	100	0
Porphyromonas gingivalis 0569	100	0
*Porphyromonas gingivalis W83**	100	0
*Prevotella intermedia**	100	0
Porphyromonas gingivalis 0570	100	0
*Porphyromonas gingivalis 0580**	100	0

Table 4. Relative percentage of R and S isoform Lipid 654 present in multiple oral Bacteroidetes species. Results for the species above with an asterisk (*) present were taken from unpublished work by Reza Nemati.[20]

Much of the data presented in Table 4 came from unpublished work done by Reza Nemati.[20] Chiral HPLC chromatographs used to analyze lipid samples from the other bacterial species can be found in Appendix III.

CHAPTER V: DISCUSSION AND CONCLUSION

Relevance and Summary of Findings

Lipid 654 is a serine dipeptide lipid that has been shown to play a key role in inflammatory disease.[1,6] While originally thought to act through TLR4 receptors,[4,5] the mechanism of action for Lipid 654 has been demonstrated to work by engaging TLR2 receptors. Through TLR2 activation, Lipid 654 has been demonstrated to directly inhibit osteoblast differentiation,[21] further bolstering the idea that Lipid 654 from *P. gingivalis* plays a key role in periodontitis. Despite these findings, gingival tissue samples taken from both healthy and diseased tissues

show similar levels of Lipid 654 present. This finding in itself seems to refute Lipid 654 as a pathogen in periodontitis, until you consider the fact that two forms of the Lipid exist; enantiomers differing in the R and S configuration in the beta carbon of the 3-OH iso C17:0 fatty acid. A closer look at these enantiomers reveals that only one, the R isoform, can be cleaved to generate the de-esterified hydrolysis product Lipid 430. This hydrolysis reaction is catalyzed by Phospholipase A2 (PLA2), which is in itself a very significant finding as Lipid 654 is not a phospholipid and also does not contain a glycerol moiety.

Lipid 430, much like Lipid 654, has been shown to elicit inflammatory responses via TLR2 activation[21]. With regard to its potential role in periodontitis, Lipid 430 has also been shown to inhibit osteoblast differentiation and function. Unlike Lipid 654, Lipid 430 is present at extremely low levels in bacterial lipid extracts. While Lipid 430 levels are also extremely low in blood samples, they begin to show up in tissue samples. This evidence suggests that the hydrolysis of Lipid 654 to produce Lipid 430 occurs within tissues.

To sum up these findings, if Lipid 430 is the pathogen responsible for bone loss in periodontitis, and Lipid 430 is a hydrolysis of the R isoform, and only the R isoform, of Lipid 654, then it is the presence of this R isoform of Lipid 654 in bacterial lipids that is responsible for pathogenesis. This demonstrates the need to understand which oral bacteria contain which isoforms of Lipid 654.

Our results show that the seven species tested so far contain only the R isoform. This is in contrast to our hypothesis that the relative levels of both isoforms would vary among different species – but is an important finding nonetheless.

Purity of Tested Cultures

Each of the eleven cultures grown were tested for culture purity using PCR techniques outlined above. Of the eleven, eight cultures had one clear sequencing read with greater than 99% match in the Greengenes database to the bacterial species intending to be cultured. The single read indicated that only one 16S rRNA gene was amplified, thereby confirming the presence of only one bacterial species in the culture. There were three cultures for which culture purity could not be verified. These cultures are outlined below:

1) *P. gingivalis* 0450: Sequencing results with the AE50 primer (see Table 2) showed that one 16S rRNA gene was amplified from this culture – consistent with one species being present in the culture. However, when a BLAST search was performed with the sequence against the Greengenes database, no match was present with homology greater than the 99% cutoff. In this case, the trimmed read was then used in a BLAST search against the Human Oral Microbiome (HOMD) database. Against the HOMD database, the read produced a 98.7% match for "HOT_279 (Porphyromonas sp. Strain_F0450c)," thereby verifying that the intended species had been grown. BLAST results against the HOMD database may be found in Appendix II.
2) *P. intermedia:* Sequencing results with the AE50 primer showed two overlapping reads present after base pair 290. This is consistent with either a contaminating bacterial species in the culture or mispriming of either the sequencing or amplification primers. The single read present from the 260 base pairs of quality read was used in a BLAST search against the Greengenes database and returned a greater than 99% homology match against *P. nigrescens*. BLAST results may be found in Appendix II.

3) *P. gingivalis 0580:* Sequencing results with the AE50 primer were used in a BLAST search against the Greengenes database and returned matches with greater than 99% homology against multiple species – none of which were the intended species. These species included: *Bifidobacterium urinalis, Metascardovia creceti, and Alloscardovia omnicolens*. More detailed analysis of which species was present could be determined by fully sequencing the amplified 16S rRNA gene – but this was not deemed necessary. The culture was not of the intended species. BLAST results may be found in Appendix II.

Shortcomings in Verification of Culture Purity

The greatest shortcoming in the method chosen for verification of culture purity is that it completely depends on the ability of the universal forward and reverse primers used (see Table 2) for amplification of the 16S rRNA gene to anneal to and amplify this gene from any contaminating species. While the selected primers have been shown to amplify the 16S rRNA gene of a wide range of bacterial species, previous oral microbiome studies have shown a few rare phyla are not detected with these primers.[22] Despite this, PCR amplification of the 16S rRNA gene has been validated as a reliable method for detection of bacterial contamination.[23]

Future Directions

Following the work that has been completed thus far, the next step taken would be to extract lipids and determine the relative levels the R and S isoforms of Lipid 654 present in the remaining four cultures that have been grown and purified: *P. gingivalis* 0487, *P. gingivalis* 0568, *P. gingivalis* 381, and *T. forsythia*. After this, other bacterial species may be analyzed including *Prevotella intermedia*, *Tannerella forsythia*, *Capnocytophaga* species, and *Treponema denticola*.

The future goal of this research is to find species with varying levels of Lipid 654 R and S isoforms and see if these levels vary along with pathogenicity of the host bacteria.

If such a relationship can be found between the relative levels of the R and S isoforms of Lipid 654 and the pathogenicity of the host bacteria, the next logical step would be to investigate how this information could be used to mitigate or fully prevent diseases involving Lipid 654. The possibility of mitigating and preventing diseases such as periodontitis and multiple sclerosis surely warrant further investigation of these lipids.

Chapter VI: REFERENCES

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Chapter VII: APPENDICES

Appendix I

Trimmed sequence reads obtained from sequencing with primer AE50 as described above are listed below for each bacterial culture. Each read is listed under the bacteria which was intended to be cultured.

Porphyromonas gingivalis ATC33277

AAGAAGTTTACAATCCTTAGGACAGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCT
CTCGCCCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCT
CAGTACCAGTGTGGGGGATAAACCTCTCAGTTCCCCTACCCATCGTCGCCTTGGTGA
GCCGTTACCTCACCAACTAGCTAATGGGACGCATGCCTATCTTACAGCTATAAATAT
TTCCTTGTAATATCATGCAATAATACAAGTGTATGCGGTTTTAGTCCGTCTTTCAACG
GGTTATCCCCCTCTGTAAGGCAAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCG
CCATCAACCTTAGCAAGCTAAGATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCT
ATCGCTAGCGTTCATCCTGAGCCAGGATCAAACT

Porphyromonas gingivalis 0450

TCATCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACCAATATTCCTCAC
TGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGTTCAACCT
CTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAAT
GGGACGCATGCCTATCTCACAGCGAATTTCATTTCCTTTACGTTGGATGCCCACCAT
GAAGTGTACGCGGTATTAGTCCGTCTTTCGACGGGTTATCCCCCTCTGTGAGGCAAG
TTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCAGTCTTAGCAAGCTAAGAC
CATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTGAGCCAT
GATCAAACTCA

Porphyromonas gingivalis 4087

TTCATGCATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTAGG
ACTGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTATCGCCCATTGACCAATATTCC
TCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGATAA
ACCTCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACAAGC
TAATGGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCAAT
AATACAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAGGC

AAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCGACCTTAGCAAGCTAA
GATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTGAGC
CAGGATCAAACTC

Porphyromonas gingivalis 0568

GCAATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTAGGACT
GTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACCAATATTCCTCA
CTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGATAAACC
TCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACCAGCTAAT
GGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCAATAATA
CAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAGGCAAGT
TGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCGACCTTAGCAAGCTAAGATC
ATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTGAGC

Porphyromonas gingivalis 0569

GCAATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTAGGACT
GTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACCAATATTCCTCA
CTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGATAAACC
TCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACCAGCTAAT
GGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCAATAATA
CAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAGGCAAGT
TGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCAACCTTAGCAAGCTAAGATC
ATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTGAG

Porphyromonas gingivalis 381

TTCAATGCAATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTA
GGACAGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACCAATATT
CCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGAT
AAACCTCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTA
GCTAATGGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCA
ATAATACAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAG
GCAAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCAACCTTAGCAAGCT
AAGATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTG
AGCCAGG

Porphyromonas gingivalis W83

TTCAATGCAATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTA
GGACTGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACCAATATT
CCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGAT
AAACCTCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACAA
GCTAATGGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCA
ATAATACAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAG
GCAAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCAACCTTAGCAAGCT
AAGATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTG
AGC

Prevotella intermedia (First 260 base pairs with single read present)

GGCGCACACGTGCGCCAATTTATTCCCACATAAAAGCAGTTTACAACCCATAGGGCC
GTCATCCTGCACGCTACTTGGCTGGTTCAGACTTGCGTCCATTGACCAATATTCCTCA
CTGCTGCCTCCCGTAGGAGTTTGGACCGTGTCTCAGTTCCAATGTGGGGGACCTTCC
TCTCAGAACCCCTACTGATCGTCGCCTTGGTGGGCCGTTGCCCCGCCAACTAGCTAA
TCAGACGCATCCCCATCCCTTACCGGAAAAA

Porphyromonas gingivalis 0570

ACGGTACATTCAATGCAATACTCGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTA
CAATCCTTAGGACTGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTG
ACCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGT
GTGGGGGATAAACCTCTCAGTTCCCCTACCCATCGTCGCCTTGGTGAGCCGTTACCT
CACCAACCAGCTAATGGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAA
TATCATGCAATAATACAAGTGTATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCC
CTCTGTAAGGCAAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCGACCT
TAGCAAGCTAAGATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCG
TTCATCCTGAGC

Porphyromonas gingivalis 0580

ACTCACTCACGCTTGCTCCCCAATAAAAGCGGTTTACAACCCGAAGGCCGTCATCCC
GCACGCGGCGTCGCTGCATCAGGGTTCCCCCCATTGTGCAATATTCCCCACTGCTGC
CTCCCGTAGGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGG
CCGGCTACCCGTCGAAGCCTTGGTAGGCCACTACCCCACCAACAAGCTGATAGGAC
GCGATCCCATCGCATAGCACTAAAACGTTTTCCCACACACCCATGCGAGCATGTGGA
ACATTCGGCATTACCACCCGTTTCCAGGAGCTATTCCAAACTACACGGCAGGTTAAT
CACGCGTTACTCACCCGTTCGCCACTCTCACCCCCCTAGCAAGCTAGAAAGGATCCC
GTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCTGAGCCA

Tannerella forsythia

TACATGCAATAAAATACACGTATCTCATTTTATTCCCCTGTAAAAGAAGTTTACAAC
CCATAGGGCAGTCATCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGACC
AATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTG
GGGGACCTTCCTCTCAGAACCCCTAACCATCGATGGCTTGGTGAGCCGTTACCTCAC
CAACTACCTAATGGTACGCATGCCCATCCGCAACCAATAAATCTTTAACAAATAGCC
CCATGCGGAACCCCTGTTTTATGAGGTATTAGTCCGACTTTCGCCGGGTTATCCCTCT
GTTGCGGGCAGGTTACATACGCGTTACTCACCCGTGCGCCGGTCGCCGACAGGTATT
GCTACCATCGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTG
AG

<u>Appendix II</u>

BLAST results against the Greengenes database are shown for each read above. Each result is shown under the bacteria which was intended to be cultured. The BLAST results against both the Greengenes and Human Oral Microbiome Database are shown for *Porphyromonas gingivalis* 0450.

Porphyromonas gingivalis ATC33277

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified	☐							
otu_4443	☐							
699032	☐		435	100.00	435	AB547660.1	Porphyromonas gingivalis str. JCM 12257	48898
k__Bacteria	☐							
p__Bacteroidetes	☐							
c__Bacteroidia	☐							
o__Bacteroidales	☐							
f__Porphyromonadaceae	☐							
g__Porphyromonas	☐							
s__Porphyromonas gingivalis	☐							
otu_1029	☐							
292895	☑		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292896	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292897	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292898	☐		435	100.00	435	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
294927	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294928	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294929	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294930	☐		435	100.00	435	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
35745	☐		435	100.00	435	AB035455.1	Porphyromonas gingivalis str. FDC 381	19162

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 0450

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
605636			342	94.53	384	AB547656.1	Porphyromonas catoniae str. JCM 13863	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
Unclassified								
otu_1026								
1931			327	92.95	383	X82823.1	Porphyromonas catoniae str. ATCC 51270	15323
254464			304	89.58	384	EU012310.1	Porphyromonas sp. str. UQD 309	43505
255301			304	89.58	384	EU012308.1	Porphyromonas sp. str. UQD 448	43505
255703			304	89.58	384	EU012305.1	Porphyromonas sp. str. UQD 302	43505
256564			304	89.58	384	EU012309.1	Porphyromonas sp. str. UQD 450	43505
256819			304	89.58	384	EU012330.1	Porphyromonas sp. str. UQD 313	43505
257509			304	89.58	384	EU012306.1	Porphyromonas sp. str. UQD 449	43505
257673			304	89.58	384	EU012317.1	Porphyromonas sp. str. UQD 300	43505
567158			327	92.95	383	NR_026230.1	Porphyromonas catoniae str. ATCC 51270	15323

Make changes to My Interest List | Reset check-boxes

All sequences within a checked node (taxa) will be included in My Interest List. To select individual sequences within a node, make sure the node itself i

Search results: 1 query sequence(s) searched against **HOMD 16S rRNA RefSeq Version 14.51 (Starts at position 28)**

Minimal identity for color highlight 98.5 % Change

1	Query[2]	Query length(nt)	Links[3]	Hit HOMD files[4]	HOMD clone name	Identities(%)[5]	Mismatch[5]	Identities(%)[6]	Mismatch[6]	Score (bits)	Query start[7]	Sbjct start[7]	Query End[7]	Sbjct End[7]
☑	Seq1	375		279F450c	Porphyromonas pasteri | HOT_279 | Strain_F0450c | GB_tbd | Named | G | 6	98.7	5/374	98.7	5/374	652	1	374	374	1
				279F450d	Porphyromonas pasteri | HOT_279 | Strain_F0450d | GB_tbd | Named | G | 6	98.4	6/374	98.4	6/374	648	1	374	374	1
				279CW034	Porphyromonas pasteri | HOT_279 | Clone_CW034 | GB_AY008310 | Named | G | 6	98.4	6/374	98.4	6/374	648	1	374	374	1
				283_2823	Porphyromonas catoniae | HOT_283 | Strain_ATCC 51270 | GB_X82823 | Named | G | 5	97.9	8/374	97.9	8/374	639	1	374	374	1

Search ID: **7mvs7akjpbc4irurrohunh20n4** Run Time: **7** seconds Date/Time: **March 12, 2017, 3:07 pm**

1 Check individual or all results to be downloaded.
2 Click to toggle the sort order of the results by query ID
3 Access individual query sequence or original blast result.
4 The table will show the top four hit results for each query sequence.
5 Total mis-match nt / total match nt
6 Total mis-match nt / total match nt (excluding gaps and non-AGCTU).
7 Start or end positions of query or subject in the alignment.
* Percent identities >= 98.5% are highlighted in red

Porphyromonas gingivalis 4087

BLAST summary by My Taxonomy shown below. Complete BLAST output can be viewed.

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
129018			418	99.53	422	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129019			418	99.53	422	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129021			418	99.53	422	NC_002950.2	Porphyromonas gingivalis str. W83	22072
147692			418	99.53	422	AE015924.1	Porphyromonas gingivalis str. W83	22072
147713			418	99.53	422	AE015924.1	Porphyromonas gingivalis str. W83	22072
147733			418	99.53	422	AE015924.1	Porphyromonas gingivalis str. W83	22072
147743			418	99.53	422	AE015924.1	Porphyromonas gingivalis str. W83	22072
46900			416	99.29	422	AB035458.1	Porphyromonas gingivalis str. A7A1-28	19162
49110			418	99.53	422	AB035456.1	Porphyromonas gingivalis str. W83	19162
93513			418	99.53	422	NC_002950.2	Porphyromonas gingivalis str. W83	22072

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 0568

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
621567			457	99.57	461	AB547661.1	Porphyromonas gingivalis str. JCM 8525	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
129018			457	99.57	461	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129019			457	99.57	461	NC_002950.2	Porphyromonas gingivalis str. W83	22072
147692			457	99.57	461	AE015924.1	Porphyromonas gingivalis str. W83	22072
147713			457	99.57	461	AE015924.1	Porphyromonas gingivalis str. W83	22072
147733			457	99.57	461	AE015924.1	Porphyromonas gingivalis str. W83	22072
147743			457	99.57	461	AE015924.1	Porphyromonas gingivalis str. W83	22072
46900			459	99.78	461	AB035458.1	Porphyromonas gingivalis str. A7A1-28	19162
49110			457	99.57	461	AB035456.1	Porphyromonas gingivalis str. W83	19162
93513			457	99.57	461	NC_002950.2	Porphyromonas gingivalis str. W83	22072

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 0569

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
621567			452	99.78	454	AB547661.1	Porphyromonas gingivalis str. JCM 8525	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
129018			452	99.78	454	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129019			452	99.78	454	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129021			452	99.78	454	NC_002950.2	Porphyromonas gingivalis str. W83	22072
147692			452	99.78	454	AE015924.1	Porphyromonas gingivalis str. W83	22072
147713			452	99.78	454	AE015924.1	Porphyromonas gingivalis str. W83	22072
147733			452	99.78	454	AE015924.1	Porphyromonas gingivalis str. W83	22072
147743			452	99.78	454	AE015924.1	Porphyromonas gingivalis str. W83	22072
49110			452	99.78	454	AB035456.1	Porphyromonas gingivalis str. W83	19162
93513			452	99.78	454	NC_002950.2	Porphyromonas gingivalis str. W83	22072

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 381

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
699032			466	100.00	466	AB547660.1	Porphyromonas gingivalis str. JCM 12257	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
292895			466	100.00	466	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292896			466	100.00	466	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292897			466	100.00	466	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
292898			466	100.00	466	NC_010729.1	Porphyromonas gingivalis str. ATCC 33277	35168
294927			466	100.00	466	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294928			466	100.00	466	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294929			466	100.00	466	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
294930			466	100.00	466	AP009380.1	Porphyromonas gingivalis str. ATCC 33277	35168
35745			466	100.00	466	AB035455.1	Porphyromonas gingivalis str. FDC 381	19162

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis W83

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
621567			462	100.00	462	AB547661.1	Porphyromonas gingivalis str. JCM 8525	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
129018			462	100.00	462	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129019			462	100.00	462	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129021			462	100.00	462	NC_002950.2	Porphyromonas gingivalis str. W83	22072
147692			462	100.00	462	AE015924.1	Porphyromonas gingivalis str. W83	22072
147713			462	100.00	462	AE015924.1	Porphyromonas gingivalis str. W83	22072
147733			462	100.00	462	AE015924.1	Porphyromonas gingivalis str. W83	22072
147743			462	100.00	462	AE015924.1	Porphyromonas gingivalis str. W83	22072
49110			462	100.00	462	AB035456.1	Porphyromonas gingivalis str. W83	19162
93513			462	100.00	462	NC_002950.2	Porphyromonas gingivalis str. W83	22072

Make changes to My Interest List | Reset check-boxes

Prevotella intermedia

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
667369			257	99.61	259	AB547696.1	Prevotella nigrescens str. JCM 12250	48898
698429			257	99.61	259	GU561338.1	Prevotella nigrescens str. SG15	49080
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Prevotellaceae								
g__Prevotella								
s__Prevotella nigrescens								
otu_1044								
107329			259	100.00	259	AY689227.1	Prevotella nigrescens str. ChDC KB5	13761
35662			257	99.61	259	AF414834.1	Prevotella nigrescens str. 7-PNIG	17973
70986			256	98.85	260	AF414839.1	Prevotella nigrescens str. 11-PNIG	17973
71190			258	99.62	260	AF414837.1	Prevotella nigrescens str. 31-PNIG	17973
71240			256	99.23	259	AF414836.1	Prevotella nigrescens str. 9-PNIG	17973
71569			257	99.23	260	AF414840.1	Prevotella nigrescens str. 13-PNIG	17973
72329			258	99.61	259	AF414835.1	Prevotella nigrescens str. 8-PNIG	17973
73473			256	98.84	259	AF414833.1	Prevotella nigrescens str. ATCC 33563	17973

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 0570

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
621567			466	99.57	470	AB547661.1	Porphyromonas gingivalis str. JCM 8525	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Porphyromonas								
s__Porphyromonas gingivalis								
otu_1029								
129018			466	99.57	470	NC_002950.2	Porphyromonas gingivalis str. W83	22072
129019			466	99.57	470	NC_002950.2	Porphyromonas gingivalis str. W83	22072
147692			466	99.57	470	AE015924.1	Porphyromonas gingivalis str. W83	22072
147713			466	99.57	470	AE015924.1	Porphyromonas gingivalis str. W83	22072
147733			466	99.57	470	AE015924.1	Porphyromonas gingivalis str. W83	22072
147743			466	99.57	470	AE015924.1	Porphyromonas gingivalis str. W83	22072
46900			468	99.79	470	AB035458.1	Porphyromonas gingivalis str. A7A1-28	19162
49110			466	99.57	470	AB035456.1	Porphyromonas gingivalis str. W83	19162
93513			466	99.57	470	NC_002950.2	Porphyromonas gingivalis str. W83	22072

Make changes to My Interest List | Reset check-boxes

Porphyromonas gingivalis 0580

BLAST summary by My Taxonomy shown below. Complete BLAST output can be viewed.

greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
k__Bacteria								
p__Actinobacteria								
c__Actinobacteria (class)								
o__Bifidobacteriales								
f__Bifidobacteriaceae								
g__Alloscardovia								
s__Alloscardovia omnicolens								
otu_895								
111587			341	98.35	363	AY880049.1	Bifidobacterium sp. S18-11	43551
111606			341	100.00	341	AY880048.1	Bifidobacterium sp. S13-05	43551
143171			343	94.04	403	AB241105.1	Metascardovia criceti str. OMB105	45079
177852			402	99.75	404	AM419459.1	Alloscardovia omnicolens str. CCUG 18650	29290
179433			389	98.77	405	AM419458.1	Alloscardovia omnicolens str. CCUG 44766	29290
186424			404	100.00	404	AM419461.1	Alloscardovia omnicolens str. LMG 23791	29290
190137			404	100.00	404	AM419460.1	Alloscardovia omnicolens str. CCUG 31649	29290
63869			410	99.76	412	AJ278695.1	Bifidobacterium urinalis	20544
63870			405	99.76	413	AJ278694.1	Bifidobacterium urinalis str. CCUG 26938	20544
72858			398	99.75	400	AF385524.1	Bifidobacterium sp. oral strain H6-M4	18944

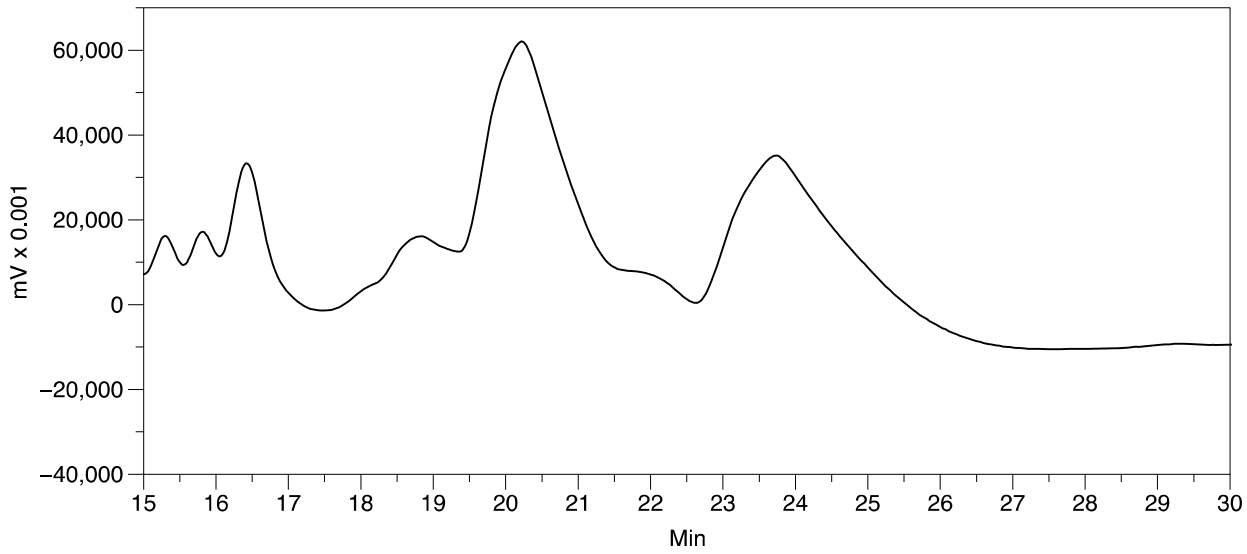
Make changes to My Interest List | Reset check-boxes

Tannerella forsythia

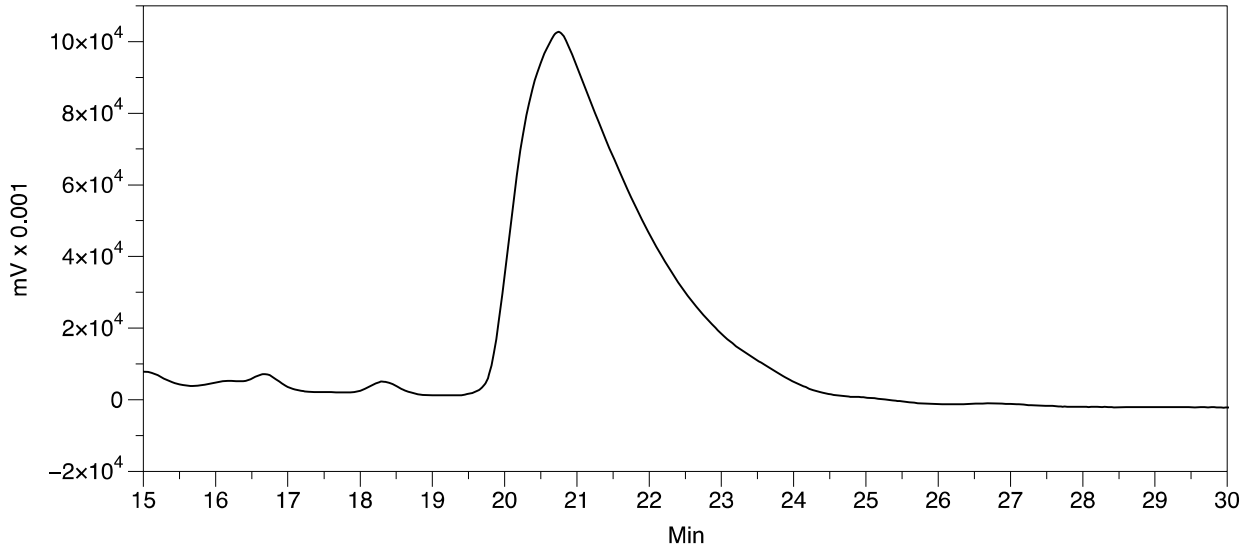
greengenes Taxonomy	MIL	Bel	Score	Identity	Match Length	Accession	prokMSAname	Study
Unclassified								
otu_4443								
739431			445	100.00	445	AB547708.1	Tannerella forsythia str. JCM 10827	48898
k__Bacteria								
p__Bacteroidetes								
c__Bacteroidia								
o__Bacteroidales								
f__Porphyromonadaceae								
g__Tannerella								
s__Tannerella forsythia								
otu_1032								
22635			439	99.33	445	AB053946.1	Tannerella forsythensis str. Sai5	43579
34893			441	99.55	445	AB053942.1	Tannerella forsythensis str. KM3	43579
36831			441	99.55	445	AB053938.1	Tannerella forsythensis str. FJ1	43579
38366			441	99.55	445	AB053941.1	Tannerella forsythensis str. HG3	43579
40709			441	99.55	445	AB053939.1	Tannerella forsythensis str. G9	43579
40924			441	99.55	445	AB053944.1	Tannerella forsythensis str. KS16	43579
46214			441	99.55	445	AB053945.1	Tannerella forsythensis str. L7	43579
46574			441	99.55	445	AB053947.1	Tannerella forsythensis str. TR6	43579
49167			443	99.78	445	AB053940.1	Tannerella forsythensis str. HA3	43579

Appendix III

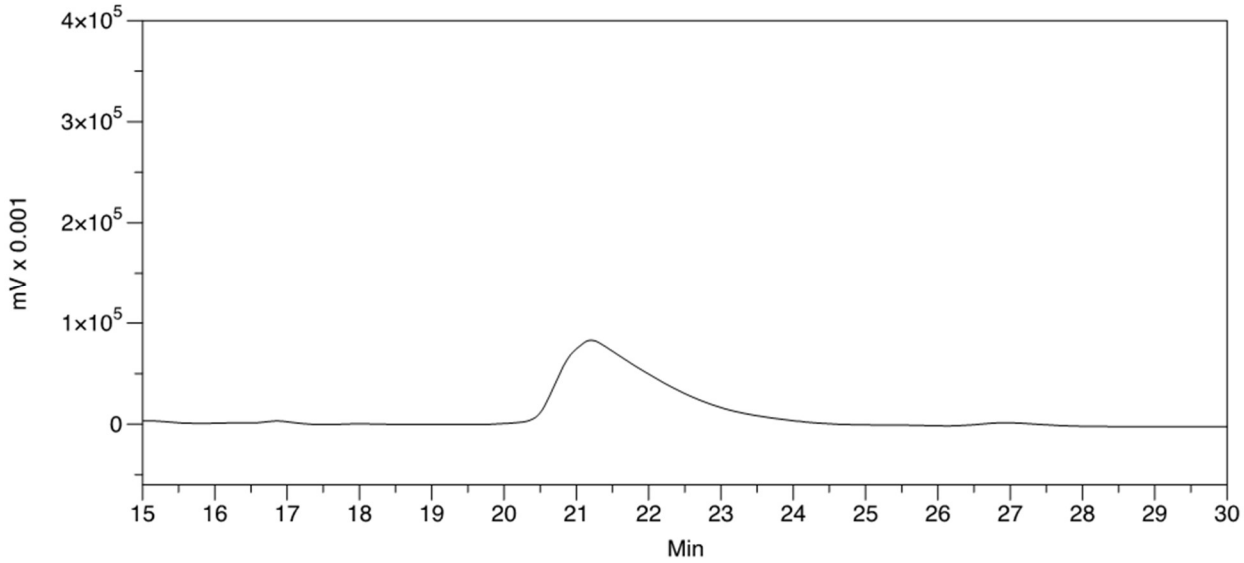
Chromatographs obtained from chiral HPLC as described above.



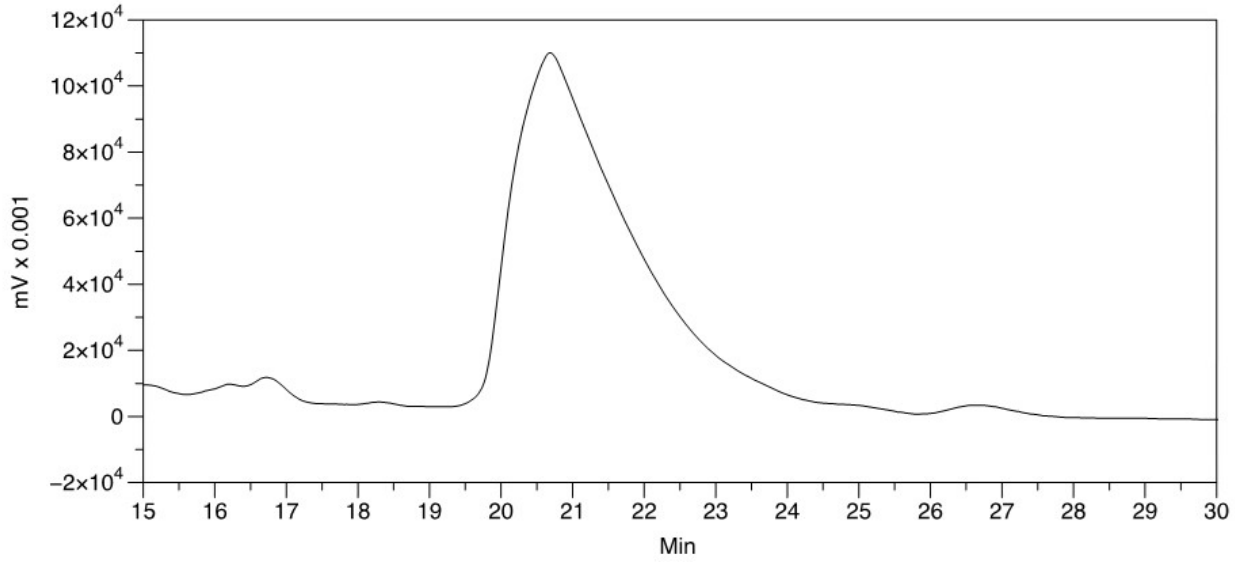
Chromatograph obtained from racemic mixture of synthetic R and S isoform Lipid 654



Chromatograph obtained from *P. gingivalis* 0450 Lipid 654 enrichment



Chromatograph obtained from *P. gingivalis* 0569 Lipid 654 enrichment



Chromatograph obtained from *P. gingivalis* 0570 Lipid 654 enrichment