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School of Science and Engineering

**PREDICTION AND COMPUTATIONAL ANALYSIS OF NOVEL CHROMOSOMAL  
TYPE II TOXIN ANTITOXIN SYSTEMS IN THE HUMAN ORAL MICROBIOME**

A Thesis Submitted to

The Biotechnology Master's Program

In partial fulfillment of the requirements for  
the degree of Master of Science in Biotechnology

By

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## DEDICATION

*I dedicate this work to Allah, my parents Mervat Bazan and Abdel-Raouf Bazan and to patients whom this project could affect their lives by any means.*

## ACKNOWLEDGEMENTS

### *Praise be to Allah*

I would like to acknowledge the helpful guidance and scientific assistance of my supervisors throughout the project. They have been more than a family believing in me and supporting me at the scientific and professional level as well as at the personal level.

I recognize the financial support of the American University in Cairo, in terms of the conference travel support, the research grant and the fellowships, which endorsed me for conducting such a project. I acknowledge the grant from the International Center for Genetic Engineering and Biotechnology (ICGEB), supporting this project. I appreciate the assistance of Zymo Research company that conducted much of the practical part of this project.

I would like to thank my mentors: Dr. Hamza El-Dorri, Dr. Asma Amleh, Dr. Ahmed Moustafa, Dr. Walid Fouad, Dr. Hassan El-Fawal, Dr. Hassan Azazy and Dr. Anwar Abdel-Nasser. I would like to thank Mr. Osama Said Ali and Mr. Ahmed El-Hossainy. I am principally grateful to Dr. Adham Ramadan. I would like to recognize Mrs. Aya Morsi, Mrs. Manar Zaki and Mr. John Sedky for their supportive provision during the study period at AUC.

I would like to express my gratitude to Dr. Asma's research group for their support and help throughout my journey. Special thanks to Amal Aboul-Fotouh, Mohamed Youssef and Diana George for their help in several occasions. My sincerest gratitude for Laila Ziko and Sara Sonbol who were role models in showing me how to truly work in dedication and good manners. The GSA community at AUC had a great impact on me during this journey, particularly, Chimson Chukwuemeka.

I am considerably grateful for the Microbiology and Immunology department at the Egyptian Russian University. My utmost appreciation goes to Dr. Fatma El-Zahraa, Dr. Ahmed Farouk and Dr. Tharwat El-Khamiessy along with the rest of the department for their plentiful support. The support I had from Dr. Mohamed Ehab Aboul-Fotouh, the dean of the pharmacy school, is unparalleled. Finally, I am deeply thankful for Mahmoud A. Shatta, Mohamed Al-Shehaby, Ahmed Safwat, Hussein Ghoneim and Sh. Saeed Abd-El-Mohsen.

## ABSTRACT

The American University in Cairo

### **Prediction and Computational Analysis of Novel Chromosomal Type II Toxin Antitoxin Systems in the Human Oral Microbiome**

By: Ashraf A. Bazan

Under supervision of: Dr. Ahmed Abdellatif, Dr. Tamer Salem and Dr. Heba Abostate

The importance of the human oral microbiome is progressively receiving considerable attention in recent research, serving as a model niche for studying microevolution. The impact of horizontal gene transfer by mobile genetic elements in such environment is the driving force for the mosaic nature of the oral microbiome. However, there is a missing link between the molecular systems interactions responsible for the plasticity of the genomes and the adaptations of the oral microbiome to physiological and pathological changes. The mobile chromosomal type II Toxin Antitoxin Systems (TASs). are known for their effective role in dynamic environment adaptation and stress response. In this study, we predicted and analyzed the genetic diversity and evolution of type II TAS in the oral microbiome of an Egyptian, presumably healthy, individual. 16S rRNA sequencing (submitted to GenBank). showed taxonomic analysis and microbial diversity and species abundance in three samples of supragingival plaque, subgingival plaque and buccal mucosa. Two hundred and seventy-eight type II TAS were identified from sequenced chromosomal genomes of the oral microbiome by means of exhaustive sequence and 3D structure homology, Hidden Markov Modelling and manual domain analysis. Gene family assignment were proposed since majority of the genes were previously annotated as hypothetical proteins. TAS network of the oral microbiome showed highly interconnected centralities which entails the extensive cross talk and intra-regulatory nature. Molecular ecology analysis of the type II TAS using diversity indexes confirms both diversity and relative abundance of these systems in the oral microbiome. Molecular evolutionary phylogenetic maximum likelihood analysis of the type II TAS, using modified Whelan And Goldman (WAG) as best fit evolution model, was performed for the predicted toxin antitoxin systems. Further analysis revealed evidence for the persistence of the toxin antitoxin systems throughout the oral microbiome. Molecular allometric analysis confirms uneven persistent distribution of the type II TAS. This comprehensive study of new chromosomal type II toxin antitoxin systems found in the oral microbiome provides insights on plasticity of the human oral microbiome and its adaptation to change in the host environment.

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## ABBREVIATIONS

ADP	Adenosine DiPhosphate
AMP	Adenosine MonoPhosphate
BLAST	Basic Local Alignment Search Tool
CAMP	Cyclic Adenosine MonoPhosphate
CDS	CoDing Sequence
CD-search	Conserved Domain Search
CFU/ml	Colony Forming Unit per milli liter
CPR	Candidate Phyla Radiation
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR associated
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDBJ	DNA Data Bank of Japan
DNA	DeoxyriboNucleic Acid
eHOMD	expanded Human Oral Microbiome Database
EMBL	European Molecular Biology Laboratory
GI	Genomic Island
GMO	Genetically Modified Organism
GO	Gene Ontology
HGT	Horizontal Gene Transfer
HMM	Hidden Markov Model
ICE	Integrative Conjugative Element
IRB	Institutional Review Board
IS	Insertion Sequence
MEGA	Molecular Evolutionary Genetic Analysis
MEME	Multiple Expression Motif for Motif Elicitation
MGE	Mobile Genetic Element
NCBI	National Center for Biotechnology Information
ORF	Open Reading Frame
PATRIC	PATHosystems Resource Integration Center



PHI Blast	Pattern Hit Initiated BLAST
PSI Blast	Position Specific Iterated BLAST
PSK	Post Segregation Killing
OTU	Operational Taxonomic Unit
QS	Quorum Sensing
RAST	Rapid Annotation using Subcellular Technology
RM	Restriction Modification
RNA	RiboNucleic Acid
rRNA	ribosomal RNA
SI	Super Integron
sRNAs	small RNA
TA	Toxin Antitoxin
TADB	Toxin Antitoxin DataBase
TALEN	Transcription Activator-Like Effector Nuclease
TAS	Toxin Antitoxin System
TCCRAS	Toxin Counter-electable Cassette Regulated by an Antitoxin Switch
tRNA	Transfer RNA

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# 1. LITERATURE REVIEW

## 1.1. Human Oral Microbiome

### 1.1.1. Introduction on Human Oral Microbiome

Since its inception, microbiology has focused on the human oral “Animalcules” as described by the very first scientist Antonie van Leeuwenhoek to discover microbes (Gest, 2004; Leeuwenhoek Antoni Van, 1677) Since then, it has been of great interest to study the human oral microbiome for reasons of easy accessibility, understanding diseases pathophysiology and studying host – microbe ecology to name a few (Dewhirst et al., 2010) Before the advent of the culture-independent analysis methods, most the studies were focusing on culturable microbes; however, recently, the focus has shifted toward a holistic view of the oral microbiome (Verma, Garg, & Dubey, 2018)

The complex system biology of the oral microbiome includes strikingly important cues for both the diversity and richness of the Operational Taxonomic Units (OTUs) of the regional ecosystem, where its evolutionary dynamics changes promptly to ecological inter and intra-species interactions including that of the host physiology and/or pathology (Krishnan, Chen, & Paster, 2017) Recognizing the extensive form and the non-zero-sum nature of the oral microbiome, the research has been reformed to study the elements of the system differently. Studying the interactions of the microbes alongside the molecular systems of these microbes holds informative and divergent ingenuity to understand short-comings of reductionist approach in understanding clinical diseases etiology and molecular biology (Cross et al., 2018; Edlund et al., 2013, 2015; Pál & Papp, 2017) A prime example for this is the dental caries, where it was believed for centuries that *Streptococcus mutans* is the sole cause of the condition. Currently, it is evident that the instability of the composition of the dental biofilm is what aggravate the periodontitis and dental caries (Bowen, Burne, Wu, & Koo, 2018; McLean, 2014) Another solid example for the holistic approach used in the oral microbiome research is the discovery of the mechanism of action and the evolution patterns of the miropin, a serpin-type suicidal endopeptidase inhibitor, found in the metagenome of the human oral microbiome (Goulas et al., 2017)

This approach has revolutionized the research where our fundamental understanding of the ecology, evolution and clinical diseases is more rationally restructured, yet, incompletely resolved and leave us with questions more than answers.

### **1.1.2. Ecology of the Human Oral Microbiome**

The human oral microbiome is the second largest microbial community right after the gut microbiome. The expanded Human Oral Microbiome Database (eHOMD) contains, as of April 2019, seven hundred and seventy-two different species (Chen et al., 2010; Human Microbiome Project Consortium, 2012; Verma et al., 2018) Although the salivary microbiome is considered broadly stable throughout different states, it is just a collection of planktonic microbes shredded from different sites in the buccal cavity (Belstrøm et al., 2016) The abundance and diversity varies in the oral microbiome of the buccal mucosa, hard palate, masticator mucosa, soft palate, different teeth enamels, supragingival tract, subgingival tract, tongue dorsum, keratinized gingiva and palatine tonsils (Moon & Lee, 2016)

The human oral microbiome has nearly third of the taxa unculturable. The culturable taxa can be broadly categorized into six phyla, namely, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria* and *Spirochetes* (Palmer, 2014; Verma et al., 2018) According to the updated eHOMD, the most abundant phylum is the *Firmicutes*, followed by *Bacteroides* (Dewhirst et al., 2010) However, false positives of taxonomic assignments are present due to Operational Taxonomic Units (OTU) similarity bias to the overrepresented *Firmicutes* in the databases used in the analysis. On the genus level, the most common are the *Streptococcus* and *Veillonella*. The major genera found are those of the *Actinomyces*, *Corynebacterium*, *Rothia*, *Capnocytophaga*, *Prevotella*, *Haemophilus*, *Granulicatella* and *Neisseria*. All of these constitute what is called the core oral microbiome because of their abundance. On the other hand, the variable oral microbiome is formed by the fluctuations of several other genera depending on the niche conditions (Verma et al., 2018; Zaura, Keijser, Huse, & Crielaard, 2009)

There are different niches in the buccal cavity which can be categorized into saliva, dental plaque and buccal mucosa. As mentioned above, whatever shed from the biofilms of the mucosa or plaque would be in the saliva in the plankton form. It has been estimated that there are around 140 million CFU/ml of the saliva. The major constituents of the salivary microbiome are *Actinobacteria*, *Bacteroides*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Spirochetes*, and TM7 (J.

He, Li, Cao, Xue, & Zhou, 2015; Human Microbiome Project Consortium, 2012; Zaura et al., 2009) The microbiome of the dental plaque forms biofilm on the teeth surface (L. Yang et al., 2011) Although the microbial community may vary based on which tooth, the main variation in the dental plaque biofilms is between the supragingival plaque and the subgingival plaque with the supragingival microbiome being more rich and diverse (Filoche, Wong, & Sissons, 2010; Gross et al., 2010; J. He et al., 2015; Keijser et al., 2008) Conversely, the buccal mucosa microbiome has lower diversity profile. The tongue dorsum is being extensively studied for research on halitosis. The characterized microbes of the tongue that are abundant include *Streptococcus salivarius* and *Rothia mucilaginosa* (J. He et al., 2015; Kazor et al., 2003)

In addition to the traditional bacterial phyla, there is a group of bacteria lineages that are extremely small in shape and genome size compared to most bacterial lineages. They are termed Candidate Phyla Radiations (CPRs) (Hug et al., 2016) The CPRs have provocative interspecies interactions that largely shapes the ecology of the oral microbiome. This newly categorized group is huge, consisting of around 15% of the bacterial domain with around 70 different phyla. Interestingly, they have less functional diversity and minimal metabolome, thus leading to the inference of being some sort of parasitic organisms (Danczak et al., 2017) Although they harbor the archaeal enzyme RubisCO that is used in carbon dioxide fixation, they are well-known obligate symbiont dependent on other bacteria (Baker, Bor, Agnello, Shi, & He, 2017; Wrighton et al., 2016) One of the best studied CPRs is the TM7x (Kianoush et al., 2014; Segata et al., 2012; Soro et al., 2014; Zhou et al., 2013). Under different physiological and environmental conditions, it can significantly alter the whole oral habitat ecosystem through its multivariate interactions with other species especially those of the *Actinomyces* (X. He et al., 2015; McLean et al., 2016) The abundance of such organisms increases from around 1% to approximately 21% of all of the microbiome in oral diseases such as periodontitis (B. Liu et al., 2012; Rylev, Bek-Thomsen, Reinholdt, Ennibi, & Kilian, 2011) Besides, they can expressively modify host immune responses, such as, macrophages response, cytokines concentrations and TNF- $\alpha$  inhibition (X. He et al., 2015) Thus, the dynamic ecology of the buccal cavity is more than just the richness, but also, the interactions within and between species.

Indeed, The human oral microbiome is more than just the bacteriome. In fact, there are about more than 100 species of known fungi that resides in the oral cavity (Bandara, Panduwawala, & Samaranayake, 2019; Dupuy et al., 2014; Ghannoum et al., 2010; Peters, Wu, Hayes, & Ahn,

2017) The oral mycobiome has low abundance compared to the oral bacterial communities (Baker et al., 2017); however, the impact should not be underestimated. This is because of two reasons; firstly, Despite the fungal communities low abundance, the larger fungal cell sizes constitutes a larger biomass in the oral ecosystem (Cui, Morris, & Ghedin, 2013) Secondly, hyphens of the fungi formulates backbone for the formation of multi-species biofilm (Baker et al., 2017) The medical impact of the fungi on the host is, clearly, huge. This is evident as clinicians encounters several fungal infections from local candidiasis to lethal systemic infections (Cui et al., 2013; Samaranayake, 2018) The mycobiome has massive inter-kingdom communication that is regarded as keystone species. For example, *Candida albicans* virulence is orchestrated with a handful of bacterial species like *Streptococcus mutans*, *Streptococcus oralis* and *Fusobacterium nucleatum*. On the other hand, biofilm formation abilities of such bacteria are reformed when interacting with *Candida albicans* (Allison et al., 2016; Baker et al., 2017; Diaz, Strausbaugh, & Dongari-Bagtzoglou, 2014; Janus, Willems, & Krom, 2016) We can infer from the *Candida albicans* interactions the importance of the oral mycobiome ecology (Bandara et al., 2019)

The virome of the oral microbiome is unique for individual hosts (Abeles et al., 2014; Naidu, Robles-Sikisaka, Abeles, Boehm, & Pride, 2014; Pérez-Brocal & Moya, 2018; Pride et al., 2012) Most of the virome are bacteriophages, mostly, the Myoviridae, Podoviridae and Siphoviridae families (Pérez-Brocal & Moya, 2018; Pride et al., 2012) The most abundant eukaryotic virus in the oral cavity is human Herpesviridae followed by Papillomaviridae and Anelloviridae (Baker et al., 2017; Pérez-Brocal & Moya, 2018) Beguilingly to explore, the extensive co-evolution, complex adaptation and exaptation of mobile genetic element is believed to modulate the ecology of the oral microbiome, which results in what has recently been termed “Ecological Diseases.” (Hoare, Marsh, & Diaz, 2017)

### **1.1.3. Association of Human Oral Microbiome and Clinical Diseases**

The healthy oral ecosystem is personalized. Nevertheless, it is, also, considered allostatic in the normal physiology of the host. This happens when dynamic interactions among elements of the microbiome and the host attain a homeostasis that can actively adapt to changing conditions in the oral cavity (Zaura & Cate, 2015) Accordingly, dysbiosis of the human oral microbiome is defined as failure of the host or microbial communities to adapt to the changing physiology of the buccal cavity. Dysbiosis of the human oral microbiome is statistically associated with various



clinical diseases which raise questions, not only for the causality predicament, but also the fundamental etiology of the diseases and, thereby, possibilities for effective treatment. The diseases could be categorized into two categories: oral diseases and systemic diseases.

Oral disease includes caries, periodontal diseases, mucosal diseases and oral cancer. Systemic diseases include a plethora of gastrointestinal diseases, such as, irritable bowel syndrome, neurological diseases like Alzheimer's disease, endocrine diseases, such as, obesity, immunological diseases, such as, Rheumatoid Arthritis and cardiovascular diseases, such as, atherosclerosis. All of which have significant skew in the microbial communities in the polymicrobial biofilms on different buccal sites suggesting strong association and, surprisingly, causation of molecular etiology. These diseases are reviewed elsewhere as being out of the thesis's scope (Bowen et al., 2018; Cong & Zhang, 2018; L. Gao et al., 2018; Healy & Moran, 2019; Verma et al., 2018; Yangheng Zhang et al., 2018)

#### **1.1.4. Mobile Genetic Elements as Key Members of the Human Oral Microbiome**

Vital members of the human oral microbiome, yet understudied in this context, are the Mobile Genetic Elements (MGEs). The human oral microbiome has remarkable interplay among its elements at both the inter-genome and intra-genome levels. The biology of the mobile genetic elements is a key player in understanding the ecology, evolution and host response to the oral microbiome (Koonin Eugene V., 2016) The mobile genetic elements include integrative conjugative elements (ICE), transposons, group I and II introns, phages, prophages, plasmids, Insertion Sequence (IS) elements, Genomic Islands (GI), pathogenicity islands and selfish elements (Frost, Leplae, Summers, & Toussaint, 2005; Koonin Eugene V., 2016)

Most of the virome are bacteriophages living through most phyla of the oral bacteria (Pérez-Brocal & Moya, 2018; Pride et al., 2012) Both lysogenic prophages and lytic phages develop both adaptive and antagonized co-evolutionary red queen dynamics of the host bacteria (Baker et al., 2017; Fernández, Rodríguez, & García, 2018; Wahida, Ritter, & Horz, 2016; Yutin et al., 2018) Recently, there have been evidence of microbiome bacteriophages interacting with human mammalian cells albeit *in vitro* (Kaźmierczak & Dąbrowska, 2018) Beyond phages, the extensive horizontal gene transfer in the oral niche is predominantly central as the oral microbiome is primarily polymicrobial biofilm that facilitates exogenous flow of DNA (Bowen et al., 2018; Pinilla-Redondo, Cyriaque, Jacquiod, Sørensen, & Riber, 2018)

Horizontal Gene Transfer (HGT) provides augmented phenotype diversification upon co-evolution of the oral microbiome. This diversification outnumbers gene duplication leading to extensive co-speciation via elevated fitness cost of independent evolution (Shropshire & Bordenstein, 2016; Treangen & Rocha, 2011) The mosaic plastic nature of the bacterial communities enables release, passage and uptake of DNA – and more recently RNA – within and between species and kingdoms (Smillie et al., 2011) This communication of genotype occurs through different vehicles from secretion systems and integrative conjugative elements to, the newly discovered, bacterial nanotubes, viral-like gene transfer agents and membrane vesicles (Brimacombe, Ding, Johnson, & Beatty, 2015; Broaders, Gahan, & Marchesi, 2013; Dorward, Garon, & Judd, 1989; Dubey & Ben-Yehuda, 2011; Klieve et al., 2005; Koonin, 2016; Marrs, 1974; Wagner et al., 2017) Astonishingly, these transfers occur by unexpected rate and extent that reach estimates of 20% of the rate of point mutations (Vos, Hesselman, Te Beek, van Passel, & Eyre-Walker, 2015) It was calculated that on average there is 43.9 HGT event occur per one microbe in the human microbiome, at the time of the study. Thus, the whole human holobiont is considered a hotspot for HGT (L. Liu et al., 2012; Rosenberg & Zilber-Rosenberg, 2018) A study was conducted on human gut microbiome to analyze the HGT extent. By the compositional methods of calculating signature GC content, they found that up to 6.5% of all Open Reading Frames (ORFs) of all of the microbiome occurred by HGT (Sitaraman, 2018; Tamames & Moya, 2008)

Diving into the effector mechanisms for the host modulation of the HGT, the Cationic Anti-Microbial Peptides (CAMPs) of the innate immune system delivers a constant stress pressure on the microbial communities that collaterally empowers HGT events. This empowers evolutionary pathways required for adaptation (Andersson, Hughes, & Kubicek-Sutherland, 2016; Cullen et al., 2015; Wimley, 2010) Thus, HGT promotes resilience against host perturbations viciously allowing for numerous cascades like persistence until fitness adaptation, virulence to the host and resistance to host xenobiotics with quorum sensing orchestrating such endeavors among the microbiome population (Ferreiro, Crook, Gasparri, & Dantas, 2018) This gave rise to what is known as microbiome-derived resistome (Adu-Oppong, Gasparri, & Dantas, 2017, p.; Crofts, Gasparri, & Dantas, 2017) Different physiology and conditions of the host affects the HGT-mediated evolution of the microbiome such as host diet, heavy metal exposure and antibiotic chemotherapy

exposure (Ferreiro et al., 2018) This is a classic example of how elements of ecosystem evolve to escape local fitness optima in the ecological stability landscape. (Pál & Papp, 2017)

On another front, pitching on the HGT among the microbiome, it is noticeable the extensive evolutionary conflicts within individual microbe genome, among members of the same species, between different species, genera and, even, kingdoms (Ferreiro et al., 2018; Pinilla-Redondo et al., 2018) Certain communities of the oral microbiome produce bacteriocin that poses stress on other communities nearby (Burton et al., 2013; Hasannejad Bibalan, Eshaghi, Rohani, Pourshafie, & Talebi, 2017) The dental biofilm members *Streptococcus mutans*, *Streptococcus gordonii* and *Streptococcus salivarius* produce bacteriocins that negatively impact other species but itself. This is due to acquisition of several Integrative Conjugative Element (ICE) that codes for bacteriocin and its immune protein. Co-cultures of the two species mentioned above are capable of communicating and distributing the bacteriocin ICE. However, the other species in the community are susceptible to bacteriocins (Dahmane et al., 2017; Kreth, Merritt, Shi, & Qi, 2005; Mignolet et al., 2018) Another example is the bacteriolytic elements produced by the Type VI Secretion Systems in the ICE harbored by some species of the oral microbiome. This lysis occurs mainly for exogenous genetic uptake essential for competence in adaptive evolution as well as nutrition (Borgeaud, Metzger, Scignari, & Blokesch, 2015; Coyne, Roelofs, & Comstock, 2016; Finkel & Kolter, 2001)

In the arm race between the microbiome, there are several systems that interplay in the evolutionary conflicts driving adaptation (McLaughlin & Malik, 2017) They are protection mechanisms for the genome if required. Model examples are the Restriction Modification (RM) systems and the CRISPR-Cas systems. A supporting example is the CRISPR-Cas system in periodontal biofilm bacteria *Aggregatibacter actinomycetemcomitans* that increases the transformability and cell competence in the oral microbiome thereby driving diversity (Jorth & Whiteley, 2012) Although both RM and CRISPR-Cas systems are being horizontally transferred, they operate as checkpoints for mobile genetic elements disseminating harmful horizontal gene transfer events to attain genome stability (Furuta, Abe, & Kobayashi, 2010; Kobayashi, 2001) Both are key players in HGT of the human oral microbiome.(Sitaraman, 2018)

The study of mobilome of the oral microbiome is still in its infancy (Martínez, 2018; Sitaraman, 2018) Although there have been several studies on the horizontal gene transfer events in the oral microbiome, several questions remained unanswered, such as, how these mobile genetic

elements shape and “quorum-sense” communities as biofilms collaborating virulence and antibiotic resistance genes on the system level (Ferreiro et al., 2018) There are investigations on how phages and exogenous genes are invited or fought against depending on the need or the fitness cost. Studies attempted to explore mobile genetic elements that alleviate conflict molecular systems are insufficient to draw conclusive answers (Fernández et al., 2018) As being understudied in the human oral microbiome, it came clear that one molecular system holds a common role as described in the above questions; it suggests the chromosomal type II toxin antitoxin systems.

## **1.2. Toxin Antitoxin Systems**

### **1.2.1. Introduction on Toxin Antitoxin Systems**

The Toxin Antitoxin (TA) system appeared first in the literature in 1997 by Martin Holčák and V. M. Iyer (Holčák & Iyer, 1997) They categorized the post segregational killing effect of two components gene operons and labeled them as being a Toxin Antitoxin Systems (TAS). The first described TAS operon was in 1985 by Kenn Gerdes and colleagues (K Gerdes, Rasmussen, & Molin, 1986) They described a novel mechanism of plasmid maintenance in the population of *Escherichia coli*. At that time, it was known that the only plasmid maintenance mechanism is the partitioning system, which was less efficient with plasmids of low copy number as the bacterial replication could overwhelm the low copy numbered plasmids leading to limited partitioning. Additionally, several plasmids lack the partitioning system genes (Guynet & de la Cruz, 2011) Although the “Pilot-Fish” – like mechanism could drive the plasmid maintenance in such Partitioning – lacked plasmids, a negative selection hypothesis were believed to take place (Ogura & Hiraga, 1983) Gerdes group discovered the hok/sok system in the R1 plasmid that could apply a conditional negative selection pressure on plasmid-free cells; this is to say, the hok/sok system is killing of the plasmid free segregated cells (K Gerdes, Bech, et al., 1986)

Since then, the Post Segregation Killing (PSK) effect of several genes has been described in different plasmids which lead to their annotation by Holčák group as Toxin Antitoxin System. This is due to their neat molecular mechanism that involves two components: a stable toxic component that is rescued by a continuously expressed cognate antidote (Figure 1-1). Thus, in the progeny of mother cells, the labile antitoxin of lower half-life time than the toxin should be continuously expressed on the plasmid segregated cells, while the stable toxin is inherited through the cytoplasmic partitioning during replication. If the daughter cells are deficient of the plasmid,

the antitoxin will be depleted at a higher rate than the toxin leading to killing of the plasmid-less daughter cells.

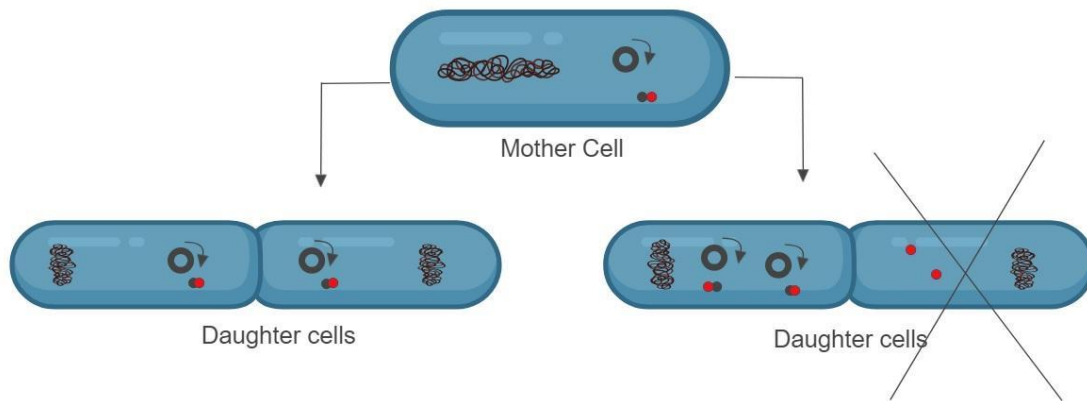


Figure 1-1: A cartoon diagram representing the basic idea of post segregation killing (PSK). of plasmid-less bacterial cells. The toxin (denoted as red dots) is expressed from the plasmid and inherited from the mother to daughters as they have high half-life time. On the contrary, the antitoxin (denoted as black dots) requires continuous expression from the plasmid; that is, the plasmid must be inherited for the daughter cell to survive the inherited toxin.

### 1.2.2. Classification of the Toxin Antitoxin Systems.

The Toxin Antitoxin Systems could be classified structurally and mechanistically. The common classification of the TAS is that according to the mechanisms of the neutralization of the antitoxin to the toxin. In that manner, the TAS has been recently categorized into seven types (Harms, Brodersen, Mitarai, & Gerdes, 2018a; Walling & Butler, 2019) On the other hand, the TAS families under each type are further classified based on the structural differences. This classification is sensible; as the mechanisms of action of the TAS has prominent association with their evolutionary patterns and abundance throughout the three life domains. Besides, structural homology is minimal between different mechanistic types of the TAS. Although there is a quite debate on the functions of the TAS, certain functions are allocated to certain types of the TAS which makes it more meaningful to classify the TAS according to the mechanisms of neutralization. Holistically, the antitoxins are proteins in case of types II, IV, V, VI and VII. In types I and III, the antitoxin are small RNAs (Goeders & Van Melderen, 2014; Page & Peti, 2016; Song & Wood, 2018a)

### **1.2.2.1. Type I Toxin Antitoxin Systems.**

This type of the TAS has the active toxin in a protein form. However, the antitoxin is a small antisense RNA that binds to the toxin's mRNA and hinder its translation (Fozo, Hemm, & Storz, 2008; Kenn Gerdes & Wagner, 2007) The toxin protein is a small hydrophobic protein with a mechanism that is not-fully understood; however, it is believed to increase the cellular membrane potential through depolarization (K Gerdes, Bech, et al., 1986; Ono, Akimoto, Ono, & Ohnishi, 1986; Weaver et al., 2003) The Type I Toxins have transmembrane domains much like several antimicrobial peptides albeit here the toxic effect is self-destructive (Arnion et al., 2017; Henriques, Melo, & Castanho, 2006; Unoson & Wagner, 2008) As for the antitoxin, the regulation of the toxins is through direct binding to the ribosomal binding site by simple base-pairing. Although this is not the case for the most studied case of Type I Toxin Antitoxin System, the Hok/Sok system, some antitoxins bind to the mRNA of the toxins in other sites blocking standby ribosomes (Fozo et al., 2008; Reif, Löser, & Brantl, 2018) It is evident that the type I Toxin Antitoxin Systems are usually in tandem repeat. The chromosomal TAS in this type are known to be laterally transferred. This could limit its breadth of taxonomic distribution; nonetheless, it is merely apparent to be increasingly abundant (Coray, Wheeler, Heinemann, & Gardner, 2017; Fozo et al., 2010)

### **1.2.2.2. Type II Toxin Antitoxin Systems.**

The Type II Toxin Antitoxin Systems are the most studied of the Toxin Antitoxin Systems. Both the toxin and the antitoxin are protein in nature. The type II toxins are small in size and have a large variety of target molecules. On the other hand, the type II antitoxins neutralize the toxin proteins. They, mainly, possess two domains; (1) DNA Binding Domain and (2) Protein Interacting Domain. The type II antitoxin proteins neutralize the toxin proteins by two main mechanisms; (1) They have direct interaction to the toxin protein through the C – terminal toxin binding domain and (2) the toxin-antitoxin complex exposes the N – terminus of the antitoxin and binds to the promoter of the Toxin Antitoxin Operon repressing the expression of the toxin. The DNA Binding Domain at the N – terminus is also responsible for the vast regulatory roles of the type II antitoxins that will be discussed below. The structure of the antitoxin is readily distorted exposing itself for proteolysis (Harms et al., 2018a; Makarova, Wolf, & Koonin, 2009a; Xia, Bao, Zhang, Linhardt, & Liang, 2019)

The Type II Toxin Antitoxin Systems can be *sensu lato* classified further into families. There are two systems for classification of Type II Toxin Antitoxin Systems. One that is based on toxin protein sequence similarity and the other is based on the domains of the toxin antitoxin systems independent from the proteins they are homologous to (Kenn Gerdes, Christensen, & Løbner-Olesen, 2005; Van Melderen & Saavedra De Bast, 2009)

The classification based on the toxin amino acid similarity is presented in the (Table 1-1) which embodies the 14 Toxin Antitoxin Systems super families. Eleven of which are of two components, which are the toxin and the antitoxin. They are the super families: ccd, HicBA, HipBA, mazEF(chpA), parD (PemKI), parDE, phd-doc, relBE, vapBC (vag), mosAT and yeeUV. Three of the TAS super families are three component systems, having a regulator beside the toxin and antitoxin genes as will be discussed below in the regulation section. These super families are Omega Epsilon Zeta ( $\omega$ - $\epsilon$ - $\zeta$ ), pasABC and paaR-paaA-parE.

It was suggested to categorize the type II toxins super families and Type II antitoxins super families separately because of the extensive cross talk between the members of each super families in “mix and match” phenomenon (Leplae et al., 2011)

Table 1-1: The Type II Toxin Antitoxin Systems Classification according to toxin protein sequence similarity.

<b>TAS SuperFamily</b>	<b>TAS SubFamily</b>	<b>Toxin Component</b>	<b>Antitoxin Component</b>	<b>Reference*</b>
<b>Ccd</b>	-		COG5302 CcdA	(De Jonge et al., 2010; Wilbaux, Mine, Guérout, Mazel, & Van Melderren, 2007)
		pfam01845		
		CcdB	pfam07362	
			CcdA	
			COG3609	
<b>HicBA</b>	-		COG4226 HicB	(Jørgensen, Pandey, Jaskolska, & Gerdes, 2009; Makarova, Grishin, & Koonin, 2006)
			pfam05534 HicB	
			COG1598	
			COG1724	
<b>HipBA</b>	-		COG1396 HipB	(Black, Irwin, & Moyed, 1994; Moyed & Bertrand, 1983; Rotem et al., 2010)
		HipA		
<b>mazEF (chpA).</b>	-		COG2002 AbrB	(Aizenman, Engelberg-Kulka, & Glaser, 1996; Engelberg-Kulka et al., 1998; Syed et al., 2011)
			COG2336 MazE	
			COG2337 MazF	
			pfam02452	
			PemK	
			pfam04014	
			AbrB	
<b>parD (PemKI).</b>	-	Kid (PemK).		(Kamphuis et al., 2007; Monti et al., 2007)
			Kis (PemI).	
<b>parDE</b>	-		COG3668 ParE	(Easter, Sobecky, & Helinski, 1997;
			COG3609	



<b>TAS SuperFamily</b>	<b>TAS SubFamily</b>	<b>Toxin Component</b>	<b>Antitoxin Component</b>	<b>Reference*</b>
				Yuan, Yamaichi, & Waldor, 2011)
<b>phd-doc</b>	-	COG3654 Doc	COG4118 Phd	(Garcia-Pino, Sterckx,
		pfam05012 Doc		Vandenbussche, & Loris, 2010; Gazit & Sauer, 1999; Lehnherr, Maguin, Jafri, & Yarmolinsky, 1993)
<b>relBE</b>	higBA		cd00093	(Arbing et al., 2010;
			HTH_XRE	Tian, Ohnishi,
		COG3549 HigB		Tabuchi, &
		COG3550 HipA		Terawaki, 1996)
			COG4691 StbC	
			smart00530 Xre	
		pfam05015		
			pfam01381	
			HTH_3	
	relBE	COG2026 RelE		(Grønlund & Gerdes,
			COG2161 StbD	1999; Han et al.,
			pfam04221 RelB	2011)
			COG3609	
		pfam05016		
	yefM-yoeB	YoeB		(Christensen et al.,
			YefM	2004; Yonglong Zhang & Inouye, 2009)

<b>TAS SuperFamily</b>	<b>TAS SubFamily</b>	<b>Toxin Component</b>	<b>Antitoxin Component</b>	<b>Reference*</b>
	ygiTU (mqsAR).	MqsR (YgiU).	MqsA (YgiT).	(Kasari, Kurg, Margus, Tenson, & Kaldalu, 2010; Kim et al., 2010)
	prlF-yhaV	YhaV	PrIF	(Schmidt et al., 2007)
<b>VapBC (vag).</b>	-	VapC	cd00093 HTH_XRE COG2002 AbrB COG3093 VapI COG4456 VagC pfam01850 PIN pfam04014 AbrB smart00530 Xre COG1848 COG3609 COG4113 COG4423 pfam01381 HTH_3	(Arcus, McKenzie, Robson, & Cook, 2011; Bloomfield, Whittle, McDonagh, Katz, & Cheetham, 1997; Cooper, Daugherty, Tachdjian, Blum, & Kelly, 2009)
<b>mosAT</b>	-	c100973 DUF1814	pfam11459 DUF2893	(Wozniak & Waldor, 2009)
<b>yeeUV</b>	-	yeeV	yeeU	(Brown & Shaw, 2003)

<b>TAS SuperFamily</b>	<b>TAS SubFamily</b>	<b>Toxin Component</b>	<b>Antitoxin Component</b>	<b>Reference*</b>
<b>ω-ε-ζ (PezAT).</b>	-	ζ zeta		(Mutschler, Reinstein, & Meinhart, 2010; Zielenkiewicz & Ceglowski, 2005)
			ε epsilon	
<b>pasABC</b>	-	PasB		(Matcher & Rawlings, 2009; Smith & Rawlings, 1998)
			PasA	
<b>paaR-paaA-parE</b>	-	ParE		(Hallez et al., 2010)
			PaaA	

\*Representative bibliography is only mentioned in this table.

The other classification system for type II Toxin Antitoxin Systems is according to the TAS domains. This domain-based classification is suggested by Makarova et al. (Makarova et al., 2009a) The reason behind this classification is that it is flexible to accommodate novel Toxin Antitoxin Systems that are predicted computationally, since there are a lot of interplay and cross talk between domains of different TAS super families of the previous classification (Goeders & Van Melderren, 2014) The list of Toxin Antitoxin domains is presented in (Table 1-2) and it has been concatenated by developers of the Toxin Antitoxin DataBase (TADB) (Xie et al., 2018a)

Table 1-2: Domain-based Classification of the Toxin Antitoxin Systems

<b>TA pair</b>	<b>T-domain</b>	<b>AT-domain</b>
<b>Xre-HipA</b>	HipA	Xre
<b>Xre-COG5654</b>	COG5654	Xre
<b>Xre-COG2856</b>	COG2856	Xre
<b>Xre-Bro</b>	Bro	Xre
<b>Xre-YgiU</b>	YgiU	Xre
<b>Xre-DUF397</b>	DUF397	Xre
<b>Xre-Fic</b>	Fic	Xre
<b>Xre-PIN</b>	PIN	Xre
<b>Xre-MazF</b>	MazF	Xre
<b>Xre-GNAT</b>	GNAT	Xre
<b>Xre-RelE</b>	RelE	Xre
<b>COG5606-RelE</b>	RelE	COG5606
<b>RHH-RelE</b>	RelE	RHH
<b>AbrB-RelE</b>	RelE	AbrB
<b>PHD-RelE</b>	RelE	PHD
<b>RHH-COG2929</b>	COG2929	RHH
<b>RHH-GNAT</b>	GNAT	RHH
<b>RHH-Fic</b>	Fic	RHH
<b>RHH-PIN</b>	PIN	RHH
<b>RHH-MazF</b>	MazF	RHH
<b>XF1863-MazF</b>	MazF	XF1863
<b>PHD-MazF</b>	MazF	PHD
<b>AbrB-MazF</b>	MazF	AbrB
<b>AbrB-Fic</b>	Fic	AbrB
<b>AbrB-PIN</b>	PIN	AbrB
<b>MerR-PIN</b>	PIN	MerR
<b>COG2442-PIN</b>	PIN	COG2442
<b>COG2886-PIN</b>	PIN	COG2886

<b>TA pair</b>	<b>T-domain</b>	<b>AT-domain</b>
<b>PHD-PIN</b>	PIN	PHD
<b>PHD-Fic</b>	Fic	PHD
<b>HicB-HicA</b>	HicA	HicB
<b>HEPN-MNT</b>	MNT	HEPN
<b>ArsR-COG3832</b>	COG3832	ArsR
<b>YhfG-Fic</b>	Fic	YhfG
<b>Xre-COG3832</b>	COG3832	Xre
<b>COG2886-RelE</b>	RelE	COG2886
<b>Xre-COG2929</b>	COG2929	Xre
<b>COG5304-COG2929</b>	COG2929	COG5304
<b>PHD-MazFn</b>	MazFn	PHD
<b>COG1753-PIN</b>	PIN	COG1753
<b>COG2880-PIN</b>	PIN	COG2880
<b>PHD-SMa0917</b>	SMa0917	PHD
<b>COG5606-COG4679</b>	COG4679	COG5606
<b>COG5642-COG5654</b>	COG5654	COG5642

### **1.2.2.3. Type III Toxin Antitoxin Systems.**

The third type of the Toxin Antitoxin systems has the antitoxin in form of sRNA that interact with the proteinaceous toxin. What distinct this type of the TAS is their unusual stoichiometry of the RNA-Protein interaction (Goeders, Chai, Chen, Day, & Salmond, 2016) The Type III TAS cassettes are bicistronic operon with the antitoxin composed of several repeats of short nucleotide sequences. The toxin protein processes this sRNA into monomers of these repeats forming dynamic complexes of the toxin interacting with one or more of the antitoxin monomers (Short et al., 2013) Although the half life time of the antitoxin is relatively higher than the other TAS types, the antitoxin can neutralize several toxins via the monomers. Also, the genetic organization of the type III operon contains intra-genetic Rho independent terminator which favours the antitoxin expression over the toxin protein (Rao et al., 2015) Under stress condition, the antitoxin half-life is shorter than the toxin protein; therefore, it survives and out paces the antitoxin interactions in a fashion not fully understood (Goeders et al., 2016)

The most common families of the type III TAS are the TenpIN, CptIN and ToxIN (Blower et al., 2012) In spite of some sequence homology with the type II TAS, the families of type III do not cross talk with each other or with other types of TAS. This could be due to the distinct pseudoknots the sRNA of the antitoxins form that differ in each family of the type III TAS (Short et al., 2013) Besides its function as addiction module, the characteristic role of the type III TAS is the antiviral abortive infection systems. Unlike the CRISPR and the Restriction Modification systems that solely protects the bacterium cell, the abortive infection systems perform “altruistic suicide” to protect the population of the colony (Short, Akusobi, Broadhurst, & Salmond, 2018)

#### **1.2.2.4. Type IV Toxin Antitoxin Systems.**

Both the toxin and the antitoxin of this poorly studies type of the TAS are protein in nature. In this type, the antitoxin interacts with the target of the toxin protecting it from the poisonous activity of the toxin. Thus, there is no direct interaction between the toxin and the antitoxin; instead, each independently acts on the target (Masuda, Tan, Awano, Wu, & Inouye, 2012; Zhongling Wen, Pengxia Wang, Chenglong Sun, Yunxue Guo, & Xiaoxue Wang, 2017) The vastly studied example is the cbeA/cbtA system (formerly known as YeeUV system), in which the target is the MreB/FtsZ proteins of the bacterial cytoskeleton. The toxin cbtA stalls the polymerization leading to fatal wobbly cell wall; on the other hand, the antitoxin promotes polymer bundling (Brown & Shaw, 2003)

#### **1.2.2.5. Type V Toxin Antitoxin Systems.**

The type V Toxin Antitoxin Systems are discovered in 2012 as Page and Wood group analyzed the GhoST system. It is established as a novel type of Toxin Antitoxin System as the antitoxin mechanism of neutralization is unique. The type V antitoxins are ribonucleases that specifically targets the mRNA of the toxin (Wang et al., 2012)

#### **1.2.2.6. Type VI Toxin Antitoxin Systems.**

The ClpXP proteases are potent proteolytic systems that can act on the type VI toxin proteins after being activated by the type VI antitoxin proteins. The antitoxins are, thus, adaptors that affects the toxin proteins indirectly through Clp-facilitated pathway. This indirect inhibition of the antitoxin is the reason to categorize these systems as an atypical type of TAS, namely, type VI toxin antitoxin systems (Aakre, Phung, Huang, & Laub, 2013)

### **1.2.2.7. Type VII Toxin Antitoxin Systems.**

The newly categorized Type VII Toxin Antitoxin Systems have the antitoxin gene codes for an enzyme that spontaneously deactivates the toxin by oxidizing the cysteine amino acid into sulphonic, sulphenic or sulphinic acid derivative (Marimon et al., 2016) This oxidation reduction reaction destabilizes the toxin making its half-life significantly shorter. This constitutes a novel mechanism of toxin neutralization as there is no stable complex formed between the toxin and the antitoxin. That been said had made it recently declared as a novel class of Toxin Antitoxin Systems (Song & Wood, 2018a) Thus far, Marimon and their colleagues characterized the Hha/TomB TAS member of this class leaving it open for further research (Marimon et al., 2016)

### **1.2.3. Roles of the Chromosomal Toxin Antitoxin Systems**

The chromosomal Toxin Antitoxin Systems (TAS) have wide range of molecular functions that translates to few, yet critical, biological functions (Harms et al., 2018a) The driving force for the TASs are the functions of the toxins. The antitoxin role is regulation of the toxins' actions. Although several antitoxin have numerous other roles in the global transcription regulatory networks of the bacterial physiology, the toxins roles are much more diverse (Makarova et al., 2009a; Slayden, Dawson, & Cummings, 2018; Xia et al., 2019)

#### **1.2.3.1. Molecular Functions**

Toxins, by definition, are enzymes that compromise cellular normal physiology. They perform this at every step on molecular biology central dogma and, mostly, at very low relative protein concentration (Harms et al., 2018a, 2017; Klein & Klein, 2016)

#### **Nucleases Toxins**

The majority of such are nucleases. Under this class, toxins include ribosome-dependent mRNA endonucleases like that of the RelE Superfamily (Christensen & Gerdes, 2003; Pedersen et al., 2003) Also, ribosome-independent mRNA endonucleases such as the MazF and HicA families (Jørgensen et al., 2009; Masuda & Inouye, 2017; Sofos, Xu, Dedic, & Brodersen, 2015; Yonglong Zhang et al., 2003) Also, the tRNA nucleases and rRNA nucleases are common among TAS families, for instance, the PilT N-terminus PIN containing toxins (Winther & Gerdes, 2011) Finally for the nucleases class, there are DNAses present in, for example, RalRA TAS (Guo et al., 2014)

## **Transferases Toxins**

Aside from the nucleases, toxins could alter the post translation machinery of the bacteria; the toxins Death on curing (Doc) and the HipA are kinases that phosphorylates the elongation factor EF-Tu and glutamyl-tRNA synthetase GltX, respectively (Castro-Roa et al., 2013; Germain, Castro-Roa, Zenkin, & Gerdes, 2013) The tRNA targeting acetyl transferases are the main action of the Gcn5-related N-acetyltransferases(GNAT) (Cheverton et al., 2016; Salah Ud-Din, Tikhomirova, & Roujeinikova, 2016; Yeo, 2018) The FicT toxin family are AMP transferases that targets enzymes of DNA replication, namely, topoisomerase and gyrase (Harms et al., 2015) PezT toxins interferes with peptidoglycan biosynthesis through phosphorylation (Mutschler, Gebhardt, Shoeman, & Meinhart, 2011) Lastly, the toxin component of the DarAT TAS is a reversible ADP-ribosyl transferases that act on DNA single strand (Jankevicius, Ariza, Ahel, & Ahel, 2016)

## **Oxidoreductase Toxins**

Even though it is poorly understood, the Hok toxins are depolarizing agents that interrupts the ATP synthesis process at the plasmic membrane (K Gerdes, Bech, et al., 1986; Verstraeten et al., 2015)

## **Stoichiometric and steric hindrance**

Unlike the topoisomerase inhibitor FicT discussed above, the CcdB toxin inhibits the topoisomerase, but through a different mechanism. It poses a steric hinderance upon physical interaction with the FtsZ or the MreB terminating the polymerization step and halting the DNA replication process with a non-enzymatic approach (Bernard & Couturier, 1992; Masuda et al., 2012)

### **1.2.3.2. Biological Functions**

The topic of biological roles of the toxin antitoxin systems is heated subject in the scientific community. It can be classified into dynamic evolution and environment adaptation.

#### **Dynamic Evolution**

The Toxin Antitoxin Systems are known for their Post-Segregational Killing of plasmid-lost daughter cells as discussed above. As for the TAS in the chromosome, several studies had proven its addiction properties for the dynamic portions of the genome; i.e. Chromosomal Mobile



Genetic Elements. Examples for this include stabilization of the prophage CP4So of the *Shewanella oneidensis* (J. Yao et al., 2018), the IncA/C genomic island in *Salmonella* spp. (Huguet, Gonnet, Doublet, & Cloeckert, 2016), SsPI-1 pathogenicity island in *Streptococcus suis* (X. Yao et al., 2015), several prophages in *Bacillus subtilis* (Durand, Jahn, Condon, & Brantl, 2012), the integrative conjugative element ICEAfe1 is addicted to the genome of *Acidithiobacillus ferrooxidans* (Bustamante, Tello, & Orellana, 2014), the ICE SXT is maintained through the *Vibrio cholera* genome by a TAS (Wozniak & Waldor, 2009) and, finally, a Super Integron (SI). is the first one to be verified that it can be diminished upon loss of the TAS found in the SI set of genes, which means it is indeed addicted to the chromosome (Szekeres, Dauti, Wilde, Mazel, & Rowe-Magnus, 2007) In spite of this, certain discrepancies and concerns on the experimental design for the functional assays of such TAS role (Song & Wood, 2018a)

The addiction can be for the unit gene as a selfish element within the genome of the same bacteria. The TAS maintained elements can be observed along with housekeeping genes localized near the origin of replication. This means that in an intra-genomic conflict, as a gene, it has high selfishness character over other genes of the bacteria (Rankin Daniel J., Turner Leighton A., Heinemann Jack A., & Brown Sam P., 2012) The selfishness of the TAS should not be taken as being only pathogenic to the genome. In such evolution, the bacteria mostly kept the TAS associated with beneficial impact to the bacteria (Ramisetty & Santhosh, 2017)

The TAS have anti-addiction functions. This means that the genome will not accept mobile genetic elements, plasmids or phages much like an immune system for the bacteria like the Restriction Modification (RM) systems and the clustered regularly interspaced short palindromic repeats CRISPR-Cas systems. On the other hand, the mobile genetic element, plasmid or phage get to choose its host. This happens by the incompatibilities of different Toxin Antitoxin Systems families where the cross-talk regulation favors the expression of the toxin for the uninvited MGE (Saavedra De Bast, Mine, & Van Melderen, 2008)

The abortive infection system is an innate immune system for bacteria where the infected individual altruistically suicide for the benefit of the population. This happens through the activation of the toxin of the TAS when the bacteriophage enters the bacteria and before its replication. This makes the TAS, especially Type III and IV, potent abortive infection systems (Dy, Przybilski, Semeijn, Salmond, & Fineran, 2014; Dy, Richter, Salmond, & Fineran, 2014; Goeders et al., 2016)

## **Environment Adaptation**

Under stress conditions and extreme environment, such as therapeutic antibiotic use, the bacterial population can tolerate such environment by keeping certain subpopulation as dormant as possible leaving out any energy expenditure. This phenomenon is known as persistence. The Toxin Antitoxin Systems (TAS) are an integral part in this pathway. With certain concentrations of the toxin, the cell become dormant and quit all translational biosynthesis cellular processes (Ronneau & Helaine, 2019) When the environment conditions return favorable, the antitoxin is expressed again and regulate the toxin actions reversing back the dormant persisters to normal population. This phenomenon is of tremendous importance as this adaptation allows the bacteria to evolve resistance mechanisms; besides, clinically, bacterial eradication from the host is difficult (Harms, Maisonneuve, & Gerdes, 2016; Page & Peti, 2016) Moreover, the persisters population often become heterogenic. These bet hedging phenotypes signals the rest of the population for a quicker response to the stress condition (Veening, Smits, & Kuipers, 2008) As global regulators, the Toxin Antitoxin Systems are implicated with extensive regulations of quorum sensing and biofilm formation (Chan, Espinosa, & Yeo, 2016; Wang & Wood, 2011; Wen, Behiels, & Devreese, 2014) These roles put the Toxin Antitoxins Systems as a clinically interesting target for drug design of novel anti-infective therapies and key molecular system to study to better understand basic science of bacterial communities including that of biotechnological – industrial microbes.

### **1.2.4. Importance of the Toxin Antitoxin Systems**

In the human oral microbiome, the toxin antitoxin systems have a lot of potential in translational medicine. Understanding the biology of the TAS helps in properly understanding the etiologies of oral and systemic diseases. On the other side, understanding the toxin antitoxin systems dynamics on a population level with a rich arena of the oral ecosystem is principal for making use of the TAS in numerous applications from high-yield industry to synthetic biological circuits.

There are numerous applications for the Toxin Antitoxin Systems. Possibly, we can design novel antibacterial without emerged resistance to it, which is a topic, currently, under extensive research. However, we will focus on the biotechnological applications of the Toxin Antitoxin systems as there are several up-to-date reviews on the clinical or chemotherapeutic applications

elsewhere in the literature (Bassegoda, Ivanova, Ramon, & Tzanov, 2018; Harms, Brodersen, Mitarai, & Gerdes, 2018b; Khusro, Aarti, Barbabosa-Pliego, & Salem, 2018; van Geelen, Meier, Rehberg, & Kalscheuer, 2018; Q. E. Yang & Walsh, 2017)

### **A Tool in Cloning**

DNA cloning has the problem of low frequency of the DNA insert. In addition to the problem of plasmid loss after several generations in the transformed cells. Therefore, Toxin Antitoxin systems were used as a toolbox in designing kits that enhance the cloning frequency. StabyCloning™ system and Gateway™ system are examples for kits that used CcdAB TA module for stabilizing the cloned DNA fragment insert (Stieber, Gabant, & Szpirer, 2008; Unterholzner, Poppenberger, & Rozhon, 2013) There are different ways to positively select the insert cloned. The insert could contain a toxin gene that is disrupted upon the insertion of the DNA fragment to be cloned. If the vector has the insert, the toxin will not be activated. Thus, positively selecting for the transformed hosts that have the insert (Bukowski, Rojowska, & Władyka, 2011) Another strategy is to design the vector to have a fragment of the antitoxin making the antitoxin inactive. However, the insert is ligated with the remaining fragment of the antitoxin that, if inserted successfully in the correct orientation, will produce a complete active antitoxin that will rescue the host cells from an inducible toxin expression (Stieber et al., 2008) The above-mentioned Gateway™ system uses another approach. The recombination sites attB1 and attB2 are to be flanking around the insert to be cloned. The vector should include the recombination sites attP1 and attP2 besides the integrase enzyme and the integration host factor. In the vector the attP1 and attP2 sites flank the toxin component of the CcdAB system. Here, a powerful selection will occur to the vectors that underwent integrase-mediated homologous recombination; the toxin is replaced by the insert (Stieber et al., 2008)

### **A Tool for Expression Vectors for Protein Production**

There are several major challenges for the protein production and gene expression systems. The plasmid is not stable and/or maintained throughout several generations of the microorganism. This expression vector could be lost due to its fitness cost. Other plasmids that lack the expression of the protein are more robust in their growth and replication than the expression vector, which lead them to be selected over the expression plasmid. The use of antibiotics to place a selective pressure on the plasmids could be an answer. However, it has several drawbacks. The product is

at danger of contamination with antibiotics that could have side effects on the consumers. The release of the antibiotic or antibiotic resistance in nature is risky. In large scale production, there is a problem that resistant cells could deactivate the antibiotic in the bioreactor leading to higher chances for competitor plasmids to outgrow the expression plasmid. Accordingly, the Toxin Antitoxin system could be used to avoid these drawbacks and increase the yield of protein production. Similar to its natural role in Post Segregationally Killing (PSK), TA systems could offer marvelous solution for plasmid stability (Pecota, Kim, Wu, Gerdes, & Wood, 1997) Several strategies from this concept are out there. The toxin gene could be engineered in the genome and the antitoxin on the expression vector; that way the plasmid is addicted to the host. One of the most successful systems is the food grade expression system developed in *Bacillus subtilis* in 2016 (S. Yang, Kang, Cao, Du, & Chen, 2016)

### **Single Protein Production systems**

One of the systems to have higher yields out of an expression vector is the design of a Single Protein Production (SPP) system. In *Escherichia coli*, the toxin component of the MazEF TA system is an endoribonuclease enzyme that can act to interfere with the total mRNA of the cell. Specifically, the MazF toxin targets the degradation of the mRNA containing the sequence ACA. The cell growth is arrested; however, the translation machinery was still operating for four days after the induction of the toxin. The protein of interest to be produced is engineered to lack the sequence ACA (ACA-less mRNA). Upon inducing the toxin expression, all mRNA of the host is degraded except for the ACA-less mRNA of the protein of interest. All resources of the cell will be available for this single protein to be expressed in high yields. This system is proved to be superbly successful (Suzuki, Zhang, Liu, Woychik, & Inouye, 2005)

### **A Tool to Target Cell Ablation in Plants**

For developing model systems, a cell ablation tool was developed using the Toxin Antitoxin system. The antitoxin is derived by a promoter that is expressed in all plant tissue except the only one to be ablated. On the contrary, the toxin is downstream of a promoter that is specifically expressed in the tissue to be ablated. This system was proven to be successful (Baldacci-Cresp et al., 2016)

## **Stable Killer Switch**

A synthetic genetic circuit was developed using the Toxin Antitoxin systems as a switch to kill the bacteria *Escherichia coli* in response to a certain stimulus. This killing switch is designed to be evolutionary stable. This tool is extremely powerful; it can be used in different applications. The microbial growth in mammalian gut is controlled by the temperature as a killer switch (Stirling et al., 2017) Another application is a killer switch that is engineered to bacteria that escaped certain path or to control the bacteria for containment. The unwanted bacteria are switched to be killed by a certain stimulus, for example cold shock causing “Cryodeath” (Boettner, 2017) This phenomenon hold humongous potential as the killer switch can be used for bacteria that perform certain therapeutic and/or theranaustics role giving it a license to kill and, then, by the killer switch the bacteria itself is killed, meaning the killing machines are temporally and spatially controllable.

## **Inducible Cell Lysis Systems in Bio-production**

One of the problems that bio-production faces is the release of the protein expressed outside of the cells. Mechanical, chemical or enzymatic cell lysis techniques are commonly used to release the protein for further purification. Here, the researchers could engineer a cell lysis system that is cost-efficient, controllable, reproducible and efficacious. The regulatory system involves the Toxin Antitoxin system that is conditionally inducible to lyse the cells after they complete their role in the bio-reactors (Y. Gao, Feng, Xian, Wang, & Zhao, 2013)

## **Engineering Resistance to Phage in Bioreactor Cells**

The bacteria *Geobacillus thermoglucosidasius* is used in biofuel production. However, the common problem to these bacteria is phage infection that drastically affect the fermentation process. The toxin *csaB* of the TA system is engineered to promote the resistance to the phage GVE3 that hinder the bacteria from biofuel production, thus, leading to higher viability in the bacterial population (van Zyl, Taylor, & Trindade, 2016)

## **A Reporter for Identification of Quorum Sensing**

The phenomenon of Quorum Sensing (QS) in prokaryotes changed our view of the microorganisms. They are sociable with several communication pathways and signals intraspecies and interspecies. However, not all microorganisms and/or all pathways are discovered. In fact, there is a deep need in detecting new signal pathways in both quorum sensing and quorum

quenching as both are potential novel chemotherapies. Identifying the biomolecules involved in these pathways are made easier using a reporter system. The reporter system, here, is *Escherichia coli* that is engineered with CcdAB TA system linked to promoters that can detect the QS families of signals in both Gram-positive and Gram-negative bacteria, namely, acyl homoserine lactone and autoinducer-2 families. The reporter system was validated and were able to detect the QS of 34 species (Weiland-Bräuer, Pinnow, & Schmitz, 2015)

### **TA Systems and Bio-photonic Imaging**

Imaging of bacteria during the disease in real-time manner is important to study pathophysiology and etiology of infectious diseases. This is possible through luminescence optical imaging. The bio-luminescence technology has many benefits to illuminate the sample for real-time optical imaging. The Toxin Antitoxin systems can play an important role in developing this technology. For example, the research group at University of Auckland developed a system using  $\omega$ - $\epsilon$ - $\zeta$  TA system; it stabilized the plasmid containing luciferase reporter genes in Group A *Streptococcus* (GAS) strains. The bioluminescence signals were quantifiable to be analyzed in culture and animal disease models (Loh & Proft, 2013)

### **TA in Genome Editing**

Genome editing revolutionized our approach in biotechnology. Now, no viral vectors or plasmids would remain in the host. The application arena is vast, starting from crop editing bypassing the GMO labelling regulation dilemma to advancing medical and therapeutic tools. With the appearance of tools like CRISPR and TALEN, genome editing became much easier process. However, the off-targeting remains a problematic drawback (Germini et al., 2018)

Although the system works on Gram-positive bacteria only, the genome editing tool toxin counter-selectable cassette regulated by an antitoxin switch (TCCRAS) holds a very promising versatile tool for genetic engineering and synthetic biology. Several approaches could be done with this tool including, large scale deletions, in-frame deletions, point mutations, large scale insertions and gene knock-ins. For example, the researchers successfully engineered Lycopene biosynthesis pathway in *Bacillus subtilis* using TCCRAS claiming minimal off targets. In the TCCRAS tool, the Toxin Antitoxin system RelBE has been adapted to a switch with inducible promoter and the whole system is harbored on plasmid that is integrated in the chromosome (Wu et al., 2018)

### **1.3. Objectives**

The Toxin Antitoxin Systems have huge potentials in both the industry and on the bed side. However, to fully understand the TAS, we should understand them collectively on the system level in bacterial populations, especially those related to the dynamic human host niche with rich HGT events like that of the human oral microbiome. This project aims to study the distribution, abundance and the evolutionary ecology of the chromosomal type II toxin antitoxin systems in the human oral microbiome which requires hitherto predictions of putative toxin antitoxin systems in such microbial community.

## **2. METHODOLOGY**

### **2.1. Ethical Statement**

All of the human sampling in this study has been approved by the Institutional Review Board (IRB) of the American University of Cairo which adequately address the participating subjects of this study in an ethical manner.

### **2.2. Microbiome Analysis**

Three samples were taken from a 26-years-old Egyptian, presumably, healthy individual claiming good oral hygiene. The location of the sampling was in Atlanta, United states of America (33.7590 N 84.3987 W). The samples were taken by gentle rubbing of a sterile cotton-headed tip to the left canine supragingival and subgingival dental plaque. The third sample was for the buccal mucosa neighboring the same canine tooth. The sample was sent to Zymo-Research® Company for targeted 16S rRNA sequencing.

The ZymoBIOMICS® 96 MagBead Kit (Zymo Research, Irvine, CA) was used for total DNA extraction of the three samples using the company's manual protocol. The Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA) was used to prepare the samples for targeted sequencing of the 16S ribosomal RNA gene variable regions of V3-V4 using customized primers. PCR reactions were conducted in real-time PCR machines to control cycles. qPCR fluorescence readings quantified the final PCR products and pooled them together on equal molarity basis. The Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA) cleaned up the final pooled library. Afterwards, the pooled library was quantified with TapeStation® and Qubit®. Illumina® MiSeq™ was used for sequencing the final library prepared. This was done with a v3 reagent kit (600 cycles) with >10% PhiX spike-in.

Two methods were applied for unique amplicon sequences identification and chimera errored sequences; one using vsearch version 1.41.3 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016; Schloss et al., 2009) The other is using the Dada2 pipeline. (Callahan et al., 2016) Then, Sequence data were deposited in NCBI Genbank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2005) with assigned accession number, BioProject number and BioSample number that are mentioned in the results section. Taxonomic assignments were done using three databases as



reference, an internal script with Zymo's own curated 16S database, GreenGene database (DeSantis et al., 2006) and SILVA database (Quast et al., 2013) These assignments were done using Uclust from Qiime version 1.9.1 (Bolyen et al., 2018) and Mothur version 1.35.1 (Schloss et al., 2009) Composition visualization of box plots and Bray-Curtis dissimilarity heatmaps, alpha-diversity and beta-diversity analyses were performed with Qiime v.1.9.1 (Bolyen et al., 2018) and plotly in R (<https://www.rstudio.com/>).

## **2.3. Toxin – Antitoxin Systems Prediction**

### **2.3.1. Retrieval of Genomic Data**

From the genera found in the oral microbiome, selected species were chosen for downstream analysis. The genomic data were retrieved from NCBI genome database and RefSeq that showed sequence similarity with 16S rRNA gene as assigned from the SILVA database. The fifty most abundant Operational Taxonomic Units (OTUs). that are more than 97% similarity with the afore-mentioned SILVA database were included in downstream analysis.

### **2.3.2. Pipeline for Prediction**

The database Toxin Antitoxin Database (TADB) is the principal database used in the primary detection of the type II toxin antitoxin systems (Xie et al., 2018b, p. 0) Using the tool TAFinder (<http://202.120.12.133/TAFinder/>), the genomic data was run through BlastP (McGinnis & Madden, 2004) with e-value threshold of 0.001 against TADB where the Coding Sequences (CDS) of each genome were blasted and results of the toxins and antitoxins were only considered if there are genomic interspace that ranges from being overlapped by 40 bases and up to interspace distance of 300 bases. Another search is done against TADB Hidden Markov Model profiles through HMMER (Finn, Clements, & Eddy, 2011) with e-value threshold of 0.01.

Afterwards, the output of such processes was comprehensively analyzed by Position Specific Iterated Blast (PSI Blast) of minimum six iterations or upon convergence. Additionally, it was concurrently used with Pattern Hit Initiated Blast (PHI Blast) and Reverse PSI Blast using MEME, InterproScan or CD-search to detect distant similarities with highly similar domain architecture (Altschul, Gish, Miller, Myers, & Lipman, 1990; Altschul et al., 1997; Bailey, Williams, Mischel, & Li, 2006; Jones et al., 2014; Marchler-Bauer et al., 2002; Mitchell et al., 2018; Z. Zhang et al., 1998)

Homology modelling of the 3D structure of selected potential TA loci was done using either of I-Tasser or SWISS Model based on the TAS in question (Benkert, Biasini, & Schwede, 2011; Bertoni, Kiefer, Biasini, Bordoli, & Schwede, 2017; Bienert et al., 2017; Guex, Peitsch, & Schwede, 2009; Schwede, Kopp, Guex, & Peitsch, 2003; Waterhouse et al., 2018; J. Yang & Zhang, 2015, 2015) Then, information was analyzed for the structural similarity with BtoxDB database of Toxin Antitoxin Systems structures from Protein Data Bank using flexible jFATCAT algorithm (Barbosa, Garrido, & Marchetto, 2015; Prlić et al., 2010; Ye & Godzik, 2003)

TA loci that directly inferred from above pipeline were manually curated for annotation. For novel TAS declarations, manual curation and literature-based study of each individual potential TA loci along with the assembly of motifs and domains architecture, families and super families were done to declare them or demote them as putative novel Toxin Antitoxin Systems. Toxin Antitoxin genes nomenclature was avoided and only membership to TAS super-families and families classification was employed in this study.

#### **2.4. Toxin Antitoxin Systems Abundance and Diversity**

The Toxin Antitoxin Systems (TAS) abundance was calculated in different contexts. Abundance of the TAS in the oral microbiome, relative molecular abundance of the TAS in the pool of genes of the oral microbiome and heatmap of specific abundance pair-wised for TAS in each OTU were visualized and graphed by GraphPad Prism, R studio, and heatmapper program (Babicki et al., 2016; Khan, 2013; Swift, 1997) Circos is used for tabular visualization of TAS domains abundance in OTUs of the oral microbiome (Krzywinski et al., 2009) The diversity of the Toxin Antitoxin Systems is calculated by different indices. These are Berger-Parker index, Margalef index, Simpson indices, Shannon index and Pielou index (Berger & Parker, 1970; E. K. Morris et al., 2014; Shannon, 1948a; Simpson, 1949)

#### **2.5. Toxin Antitoxin Systems Network**

Undirected protein – protein interaction network was built for the toxin antitoxin systems using both CytoScape version 3.7.1 and OmicsNet using internal scripts for calculation of necessary centralities of degree, betweenness, eigen vector, transitivity and closeness (Kofia, Isserlin, Buchan, & Bader, 2015; G. Zhou & Xia, 2018, 2019)

## **2.6. Toxin Antitoxin Systems Evolutionary and Phylogenetic Analysis**

Molecular Evolutionary Genetic Analysis (MEGA) version X is the principal tool used to compute phylogenetic analysis of the Toxin Antitoxin Systems predicted in the Oral Microbiome (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) The putative TAS loci were multiple-aligned using MUSCLE accessed from MEGA X. (Edgar, 2004) Then, a best fit model was calculated to be used to produce bootstrapped Maximum Likelihood phylogeny using MEGA X (Felsenstein, 1973; Henderson, 2005) Finally, test of molecular clocks was computed for the TAS phylogeny (Goldman & Whelan, 2000; Tajima, 1989; Whelan & Goldman, 2001) Molecular allometric analysis was done by comparing genome size with number of genes and/or number of TAS loci by linear regression analysis of Pearson's correlation represented on a scatter plot with linear regression line visualized through GraphPad Prism tool (Swift, 1997)

## **2.7. Statistics**

Detailed statistics are described in place as per the statistical tests' results inference. The statistical analysis employed in this study tests the null hypothesis and considers significance in rejection and/or failure of rejection by p-value of maximum value 0.05, unless, otherwise specifically stated elsewhere in the thesis document.

### 3. RESULTS AND DISCUSSION

This project aims to study the chromosomal type II Toxin Antitoxin Systems in the oral microbiome in terms of predictions, abundance, distribution, diversity, network and evolution. The reason for this study in the oral microbiome is because of the dynamics of this rich niche. Recently, studies on the association of such niche with oral and systemic pathologies are extensive and show insufficient understanding of the etiology (Healy & Moran, 2019; Krishnan et al., 2017) Thus, diving into the molecular systems might hold clear insights for the behavior of the microbiome related to these clinical diseases. As a primer to this approach, predictions and distribution of the toxin antitoxin systems in the oral microbiome is the core of this project.

Contrast to the whole systems approach, the chromosomal type II toxin antitoxin systems had been studied comprehensively in isolated species. As of January 2018, the toxin antitoxin systems have been studied in 613 species with a total of 5634 predicted TAS, of which 144 TAS have been validated experimentally (Xie et al., 2018). However, there are limited studies associates specifically with the oral species; example of these include *Aggregatibacter actinomycetemcomitans*, *Streptococcus mutans*, *Treponema denticola*, *Lactobacillus rhamnosus*, *Bacillus globigii*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (Ahn & Rice, 2016; Dufour et al., 2018; Krügel et al., 2015; Mitchell et al., 2010; Schneider, Weigel et al., 2018; Sijbrandij et al., 2014; Tikhomirova et al., 2018). Clearly, the toxin antitoxin systems are studied in isolated species leaving space for research in studying TAS roles in systems of multicellular organismal behavior of microbiomes.

In environmental microbiomes, there had been few attempts to study the TAS primarily to understand their roles in stress response, persisters formation, quorum sensing, disseminating antimicrobial resistance genes and virulence determinants (Hörak & Tamman, 2017). In the abiotic environment, studies on a polluted river called Zenne in Brussels, Belgium showed metagenomic clusters of TAS in proximity to beta-lactamase resistance gene and histidine biosynthesis operon (Vercammen et al., 2013). Another study was conducted on metagenome of the ground water where plasmidial TAS were found spanning multimetal resistance genes (Kothari et al., 2019). For wastewater environments, metagenomic studies on integrons revealed several hypothetical proteins that are implicated with toxin antitoxin systems among other stress related genes (Gatica et al., 2016). Most interestingly, a study was done on the microbiome of decaying wood and soil of a rainforest in Puerto Rico. They studied a special category of the microbial interactions in the

microbiome that involves the toxin antitoxin systems, secondary metabolites, fitness genes, quorum sensing and quorum quenching systems. These systems are main players of the complexity nature of the microbiome, for which they specifically termed these as the “sociomicrobiome.” The researchers promote studying other ecosystems based on the same concept (Santiago-Rodriguez, Toranzos, Bayman, Massey, & Cano, 2013).

Aside from the sociomicrobiome, there has been recent studies on the complexity of microbial interactions in biotic environments. A metagenomic study on the marine sponge showed highly developed organismal behavior such as specialization of metabolic pathways, but universal unity in defense interplayed by global regulatory network of toxin antitoxins systems and restriction modification systems (Slaby, Hackl, Horn, Bayer, & Hentschel, 2017). The human microbiome projects that involves TAS analysis are few. In 2019, Danilenko research group had comprehensively studied the Toxin Antitoxin Systems in the gut microbiome for developing software called TAGMA (Klimina et al., 2019). Finally, a comprehensive study on all metagenome of the human microbiome project that is deposited in the ftp website for NCBI before February 2014 was done for CRISPR -Cas systems prediction and analysis. This study collaterally predicted neighboring Toxin Antitoxin Systems in several body niche including the oral microbiome (Zhang, Doak, & Ye, 2014). As obvious, there is a lack in the research of the Toxin Antitoxin Systems in the Human Microbiome.

By searching the literature, from June 2017 till April 2019, by several keywords including logic combinations of “Toxin”, “Antitoxin”, “Oral”, “Microbiome” and “Metagenome” in public databases of “PubMed” (<https://www.ncbi.nlm.nih.gov/pubmed/>), there is no research journal article that discusses the analysis of the toxin antitoxin systems in the human oral microbiome. Therefore, the importance of this project, which analyze the human oral microbiome of a healthy individual, lies in understanding roles of molecular systems of the oral microbial interactions that influence pathogenesis.

### 3.1. Human Oral Microbiome Analysis

The analysis of the targeted sequencing of the 16S rRNA gene of three samples taken from the biofilms of the supragingival, subgingival plaques and buccal mucosa. The samples are termed oral 1, oral 2 and oral 3, respectively. They are presented in terms of taxonomy composition and abundance (From Figure 3-1 to Figure 3-3). This Targeted Locus Study project has been deposited at DDBJ/EMBL/GenBank under the accession KCXV000000000. The version described in this thesis is the first version, KCXV010000000. The BioProject accession number is PRJNA527703 and the BioSample accession number is SAMN11158134. The total OTUs are 89601 sequences. Figure 3-1 and Figure 3-2 are box plots that visualize the abundance percentage of the phyla, genera and species of the oral microbiome. Different colors represent different taxa. The legends for figure colors are omitted for redundancy and simplicity. The heat map represented in (Figure 3-3) denote the abundance of each unique Operational Taxonomic Unit (OTU) of those used in the downstream analysis for TAS predictions. The Microbiome abundance table S1 is in the appendix section.

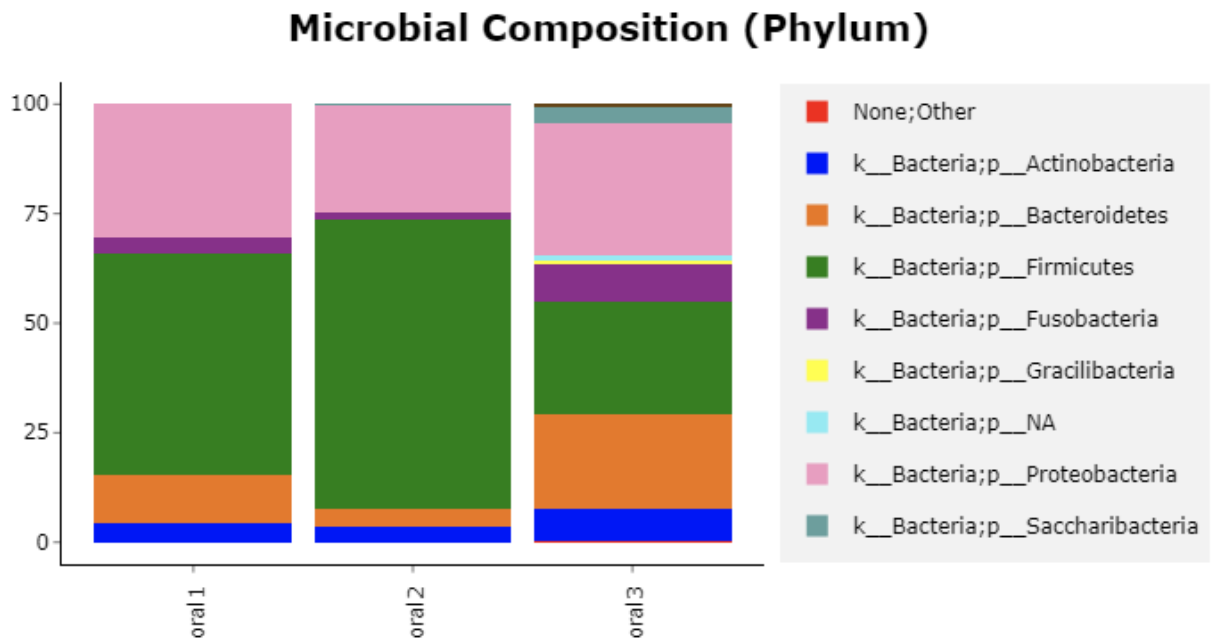
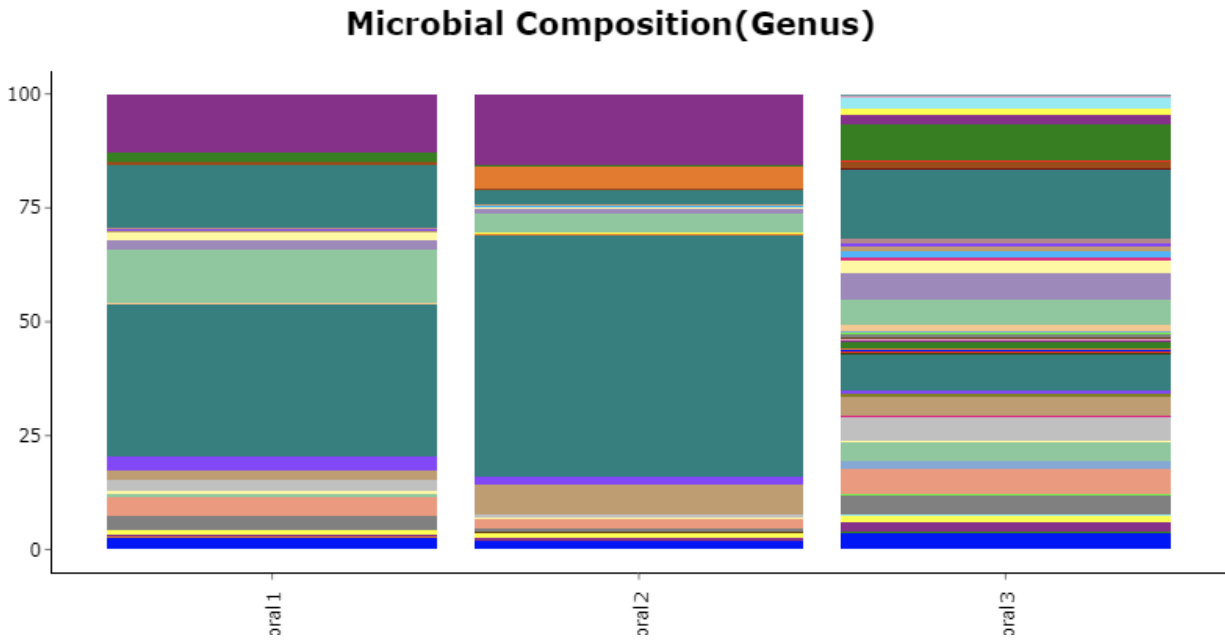


Figure 3-1: The composition of the microbial community in the oral microbiome from three different samples from supragingival, subgingival plaque and buccal mucosa respectively as oral 1, oral 2 and oral 3. The composition on the level of phyla is represented by different colors as represented.

(A)



(B)

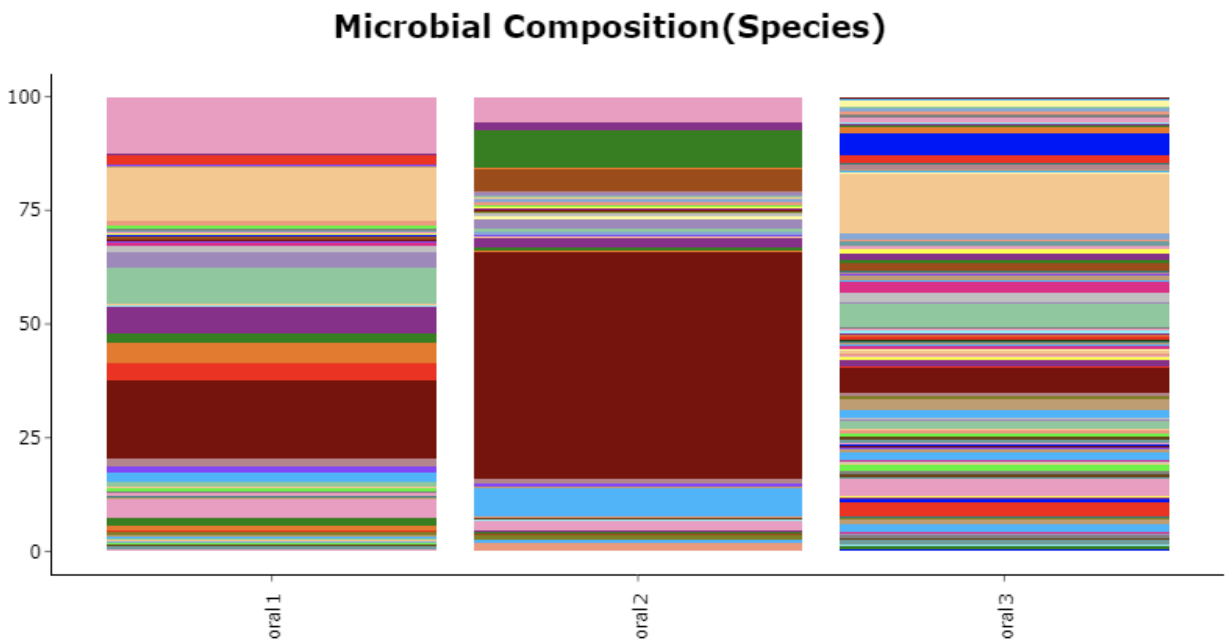


Figure 3-2: The composition of the microbial communities in the oral microbiome from three different samples from supragingival, subgingival plaque and buccal mucosa respectively as oral 1, oral 2 and oral 3. Figure (A) shows the composition on the genus level and figure (B) on the species level with different colors representing abundance. Refer to text for details of the taxonomy.

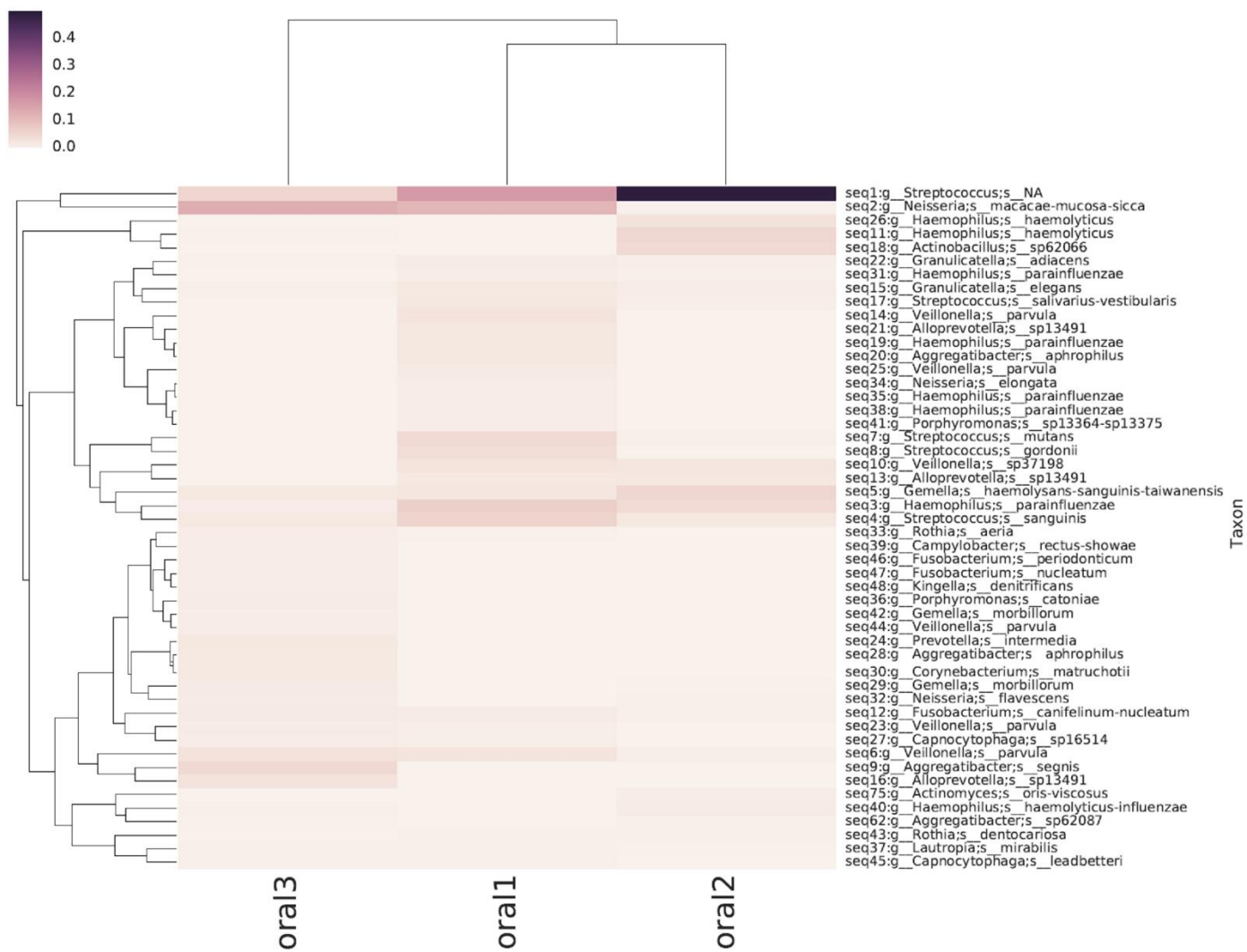


Figure 3-3: Heatmap for Unique Operational Taxonomic Units in three samples from the Oral Microbiome.



Three samples of the oral microbiome were taken from different sites in the oral microbiome. Although these sampling sites were not meant to be studied vis a vis comparing each to another, the analysis is done separately for each sample. These sampling sites are specifically chosen because they are of the thickest and rich biofilms in the oral microbiome compared to other biofilms of the hard palate, soft palate, ...etc. (Bowen et al., 2018; Mark Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016) However, the reason of taking the samples from the canine tooth is just for ease of access.

Much like the literature, the most abundant phyla here in this study for the subgingival and supragingival plaque is the Firmicutes (Escapa et al., 2018; Mark Welch et al., 2016) In the third sample of the buccal mucosa, it showed higher abundance in the Bacteroides in more or less the same as Firmicutes. This coincides with the more abundant phyla of the gut mucosal biofilms which is the Bacteroides (Barko, McMichael, Swanson, & Williams, 2018; Sweeney & Morton, 2013) The most abundant genus in the supragingival and subgingival plaque is the Streptococcus, while that of the buccal mucosa is the Neisseria. It is evident from (Figure 3-2) that the buccal mucosa has higher richness of taxa over the other two samples. In fact, heatmap in (Figure 3-3) shows clustering of the gingival plaques samples together and out-grouping the buccal mucosa.

The diversity of the three samples are observable and quantifiable by different calculations. The diversity within the samples is called alpha diversity. The calculations for this diversity are best visualized by rarefaction curves. The brilliance of these methods lies on the fact that the diversity potential of the samples is assessed accurately. For example, the diversity index for the number of assigned species is measured for a very low number of sequences. Then, a plot for gradually increasing number of sequences is done with measuring the diversity for each point. This increase in diversity, that is due to increase in the observed sequence, is a better measure than just assessing the richness of the assigned taxa for the total sequence because the diversity could increase as a function for increasing observed sequence and not for the innate diversification of the sample. Here, in the rarefaction curves, the innate diversification is observed after attaining plateau of the curve (Gotelli & Colwell, 2001) Figure 3-4 shows the rarefaction curve for the observed assigned taxa for the samples oral 1, oral 2 and oral 3. It shows the oral 3 (buccal mucosa) as the most diverse.

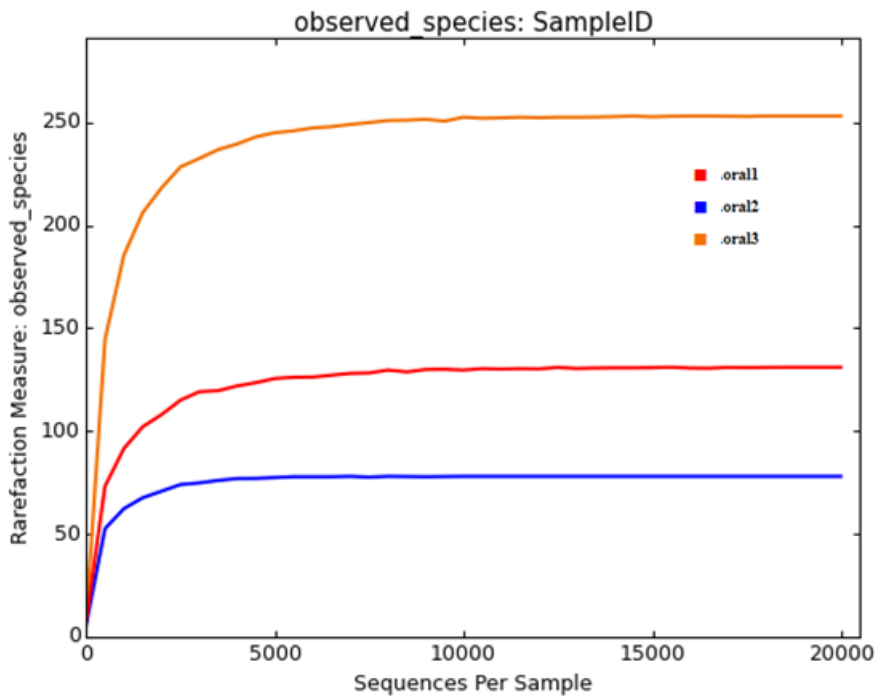


Figure 3-4: represents rarefaction curve based on the observed species.

Other diversity indexes, such as the Shannon (Shannon, 1948b), accurately measures the alpha diversity as observed in (Figure 3-5) (E. K. Morris et al., 2014) This is because they combine the measure of evenness with that of the richness (number of OTUs). The buccal mucosa has the highest diversity (within sample) index compared to the subgingival and supragingival plaque. This is aligning with the literature for the case of healthy patients with good oral hygiene (Moon & Lee, 2016) For the subgingival and supragingival plaque the alpha diversity is lower. Nonetheless, the supragingival plaque (oral 1) has higher within sample diversity compared to the subgingival plaque (oral 2); however, this observation is slightly contradictory to several oral microbiome studies. Aside from the small sample size variations, this could be justified as each individual has slightly different microbiome fingerprint and it been not, at least yet, indicative to any major change in the host physiology or dysbiosis. This might be considered inter-personal microbiome normal dynamics (Hall et al., 2017)

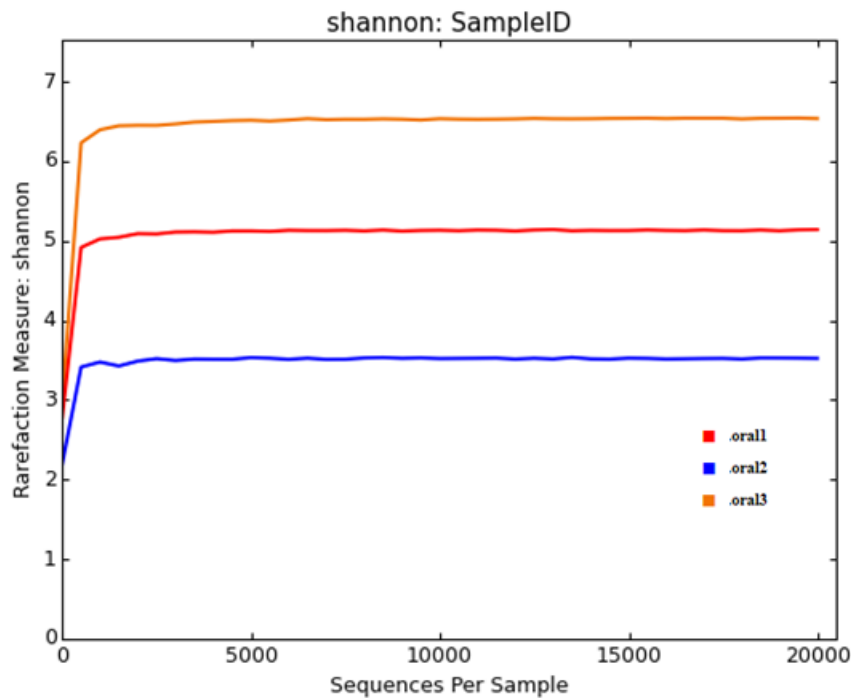


Figure 3-5: Rarefaction curve representing Shanon Index. Upper and lower confidence intervals are not included in this graph.

The beta diversity between the three samples are not of interest of this study as we are not comparing the samples to each other. However, Figure 3-6 is a Principle Component Analysis PCoA based on the ecological pairwise distance between the taxa compositions of the samples calculated by Bray Curtis dissimilarity matrix. The graph is mocked by other points to help visualize the profiles of the microbial community between samples. It is obvious from the figure that the three oral sample are clustered near each other in comparison with the mock points. This analysis is predictable; thus, differential details among the samples are out of the scope for this project.

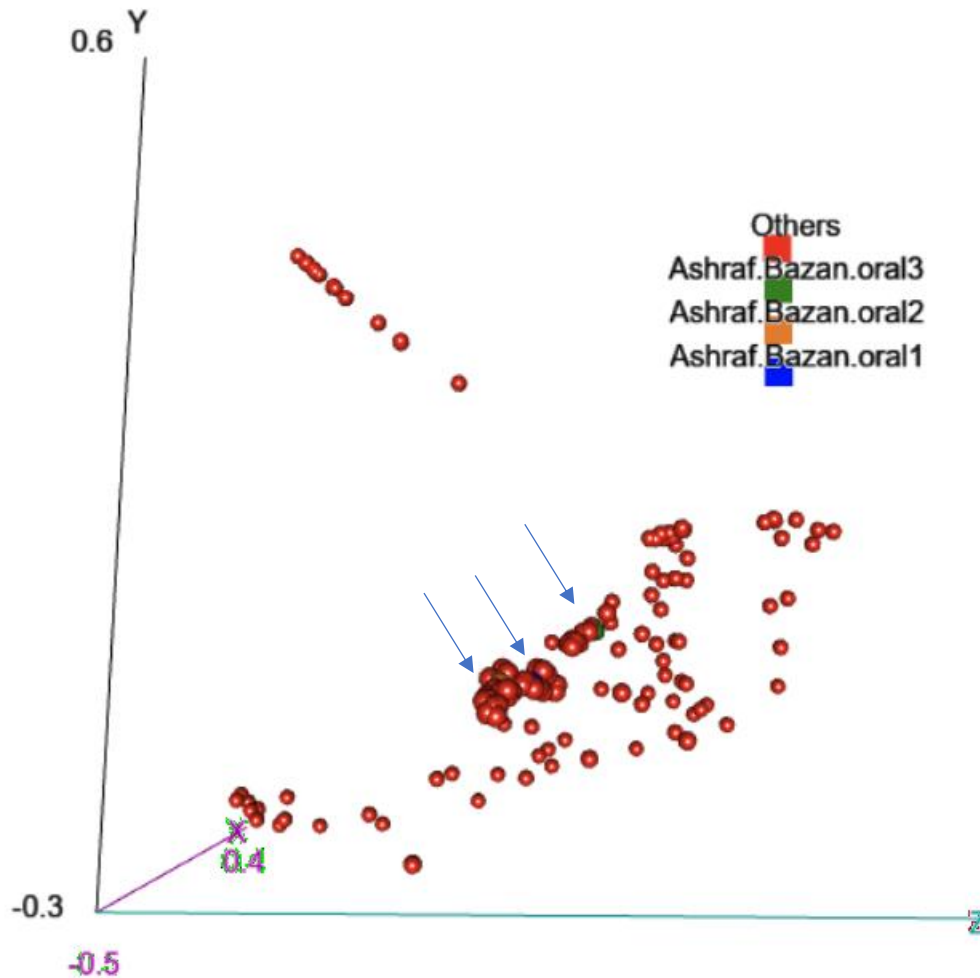


Figure 3-6: Principle Component Analysis to visualize the beta diversity between the three oral samples taken from the oral microbiome. The three arrows points to the three samples (Orange, Green and Blue) among the mock red points that are termed “others”.

From a system point of view, we are going to treat the oral microbiome as averaging the abundance and diversity from the three samples for the downstream analysis of the chromosomal type II toxin antitoxin systems analysis.

### 3.2. Chromosomal Type II Toxin Antitoxin Systems Predictions

The chromosomal toxin antitoxin systems are of a great interest to this study. This is because their exact roles are quite debatable as mentioned in the literature review section of the thesis. On the other hand, the plasmidial toxin antitoxin systems are well established as post segregational killing that addicts the plasmids to the population (Hayes, 2003) They are almost ubiquitous in all plasmids (Schuster & Bertram, 2013) The chromosomal abundance and diversity of the TAS are not properly addressed; studying their distribution patterns could give us insights

about their role especially in stress response and adaptation to changing environments in the buccal ecosystem (Shidore, Zeng, & Triplett, 2019)

The type II toxin antitoxin systems are the most studied type of the toxin antitoxin systems; yet, there are a lot of ambiguity in detecting them in the chromosome. This ambiguity in detecting TAS genes are because of the small size of the gene. Another reason is the unusual GC content and codon usage of these genes in comparison with the rest of the context genome. (Makarova, Wolf, & Koonin, 2009b; Song & Wood, 2018b) For example, *ab initio* gene prediction tools, generic homology-based tools, phylogenetic and network functional enrichment tools are all lacking the sensitivity and specificity for detection of the TAS gene sequence pairs due to the miniature size and overlapping nature of the two protein-coding sequences of the TAS in addition to the different codon usage and GC content. Even tools that incorporate metabolic pathways predictions, subsystems technology, Hidden Markov Models and structural homology to the annotation pipelines, with default Open Reading Frame (ORF) calculations, misses most of the TAS identifications. Examples include DAVID, NCBI prokaryotic genome annotation pipeline and PATRIC/RAST annotation server (Aziz et al., 2008; Brettin et al., 2015; Gillespie et al., 2011; Haft et al., 2018; Huang, Sherman, & Lempicki, 2009a, 2009b; Tatusova et al., 2016) These specific examples for annotation tools expressively announced the exclusion of such genes. However, such tools could identify uncomprehensive minority annotations, especially plasmidial TAS (Makarova, Wolf, & Koonin, 2019) Conclusive discussions for such problems occur in nearly most of the research that aims at identifying genes that codes for bacterial immune response like, CRISPR and RM systems (Koonin & Makarova, 2013; Makarova et al., 2011; Makarova, Wolf, & Koonin, 2013; Mruk & Kobayashi, 2014) Clearly, there is a need for specific tools and pipelines for chromosomal TAS detections.

There are specialized tools that are tailored to address the problem of missing ORFs. One tool, that is now obsolete, is called RASTA. This stands for Rapid Automated Scan for Toxins and Antitoxins (Sevin & Barloy-Hubler, 2007) Another tool, that “replaced” this one is called TAFinder. This one is currently supported and inhouse the most comprehensive database for the Toxin Antitoxin Systems (Xie et al., 2018a, p. 2) This tool uses specific default parameters for BlastP and HMMer pipelines that are tailored for detecting the TAS and they use the comprehensive database of TADB which minimizes the distance similarities with other genes. Therefore, there is a problem which is detecting novel TAS genes that are not yet in the TADB,

still display domain architecture and structure homology with that of the toxin and antitoxins in the TADB. These novel TAS are vastly derived from the existing knowledgebase of the domains and related proteins to the TAS genes that waits to be mined into (Makarova et al., 2009b) Thus, an exhaustive survey that is manually bio-curated is required to expand the putative TAS database and unleash trends in their distribution in the human oral microbiome.

The genomic data retrieved for the analysis are not the full metagenome in the oral microbiome. OTUs that assigned to species that share 97% similarity with the 16S rRNA are only included to decrease inaccurate data as the genomic sequence is obtained from NCBI RefSeq database and not from the direct genomes of the samples (Haft et al., 2018) Whenever there is unassembled genomic sequence, the downstream analysis is applied on each contig of the project. To avoid duplicate detections of TAS from the contigs that are possibly overlapping, manually screened TAS genes of exact sequences are removed from contigs of the same taxa. As there are 89601 OTUs, there are some that are extremely scarce and minimally abundant in the oral microbiome; they are omitted from TAS analysis for the unlikelihood of changing the abundance of TAS genes. The OTUs used in the analysis are presented in (Table 3-1) and their abundance is visualized in (Figure 3-3) as a clustered heatmap.

Table 3-1 The taxa that are involved in the down-stream analysis of the Toxin Antitoxin Systems

<b>UTO</b>	<b>Accession or Assembly Number</b>
<i>Capnocytophaga leadbetteri</i>	CP022384.1
<i>Lautropia mirabilis</i>	LR134378.1
<i>Rothia dentocariosa</i>	CP002280.1
<i>Haemophilus influenzae</i>	LS483480.1
<i>Actinomyces oris</i>	CP014232.1
<i>Aggregatibacter segnis</i>	LS483443.1
<i>Capnocytophaga ochracea</i>	CP001632.1
<i>Fusobacterium canifelinum</i>	NZ_RQYY00000000.1
<i>Neisseria flavescens</i>	LAEI00000000.1
<i>Corynebacterium matruchotii</i>	ACEB00000000.1
<i>Prevotella intermedia</i>	CP030094.1
<i>Gemella morbillorum</i>	LS483440.1
<i>Kingella denitrificans</i>	AEWV00000000.1
<i>Fusobacterium nucleatum</i>	AE009951.2
<i>Fusobacterium periodonticum</i>	CP028108.1
<i>Campylobacter showae</i>	UWOJ00000000.1
<i>Rothia aeria</i>	AP017895.1
<i>Streptococcus sanguinis</i>	LS483346.1
<i>Gemella sanguinis</i>	PNGT00000000.1
<i>Veillonella atypica</i>	CP020566.1
<i>Streptococcus gordonii</i>	LS483375.1
<i>Streptococcus mutans</i>	LS483349.1
<i>Porphyromonas catoniae</i>	AMEQ00000000.1
<i>Neisseria elongata</i>	CP031255.1
<i>Aggregatibacter aphrophilus</i>	LS483485.1
<i>Alloprevotella rava</i>	ACZK00000000.1
<i>Veillonella parvula</i>	CP019721.1
<i>Streptococcus salivarius</i>	CP013216.1

UTO	Accession or Assembly Number
<i>Granulicatella elegans</i>	NZ_KI391971.1
<i>Haemophilus parainfluenzae</i>	FQ312002.1
<i>Granulicatella adiacens</i>	NZ_ACKZ00000000.1
<i>Actinobacillus minor</i>	ACQL01
<i>Haemophilus haemolyticus</i>	LS483458.1
<i>Neisseria mucosa</i>	CP028150.1
<i>Streptococcus mitis</i>	NC_013853.1

The chromosomal type II Toxin Antitoxin Systems predicted are classified according to the Type II Toxin Antitoxin Systems super-families and families classification described and referenced before in (Table 1-1). If the putative novel TAS do not fall into the classification in (Table 1-1), the domain-based classification is used as described in (Table 1-2). Although commonly in the literature, the putative novel TAS are assigned to be named, the nomenclature of the novel TAS in this project are avoided due to the recency of the detections and the common nebulous nature of the nomenclature as proposed by experts in the biology of the toxin antitoxin systems (Song & Wood, 2018b) In general, the putative predictions of the toxin antitoxin systems are considered novel in the scientific community; however, the computational detections of the TAS are not *bona fide* due to the lack of experimental validation and *in vivo* functional assay.

The chromosomal type II toxin antitoxin systems in the oral microbiome are 278 gene sequences (i.e. 139 pair of TAS) that are predicted by the pipeline described in the methods. They are denoted in (Table 3-2). This includes manual review of the InterPro domains of each sequence, Gene Ontologies GO predictions, PSI Blast hits after convergence and of minimum 6 iterations, structural homology in abstruse cases. The family classification serves as gene-like style of declaring novel genes. For example, all predicted genes that have the RelB annotation are declared as novel RelB-like gene. In case the classification deems a domain, the declaration of the gene will be as domain-containing gene. For example, all predicted genes with HTH annotation are declared as novel HTH-domain-containing gene. Genes that are annotated in the RefSeq genome differently, yet showed homology throughout all stages of the pipeline, are kept as TAS genes with the original domain declaration. Some of the taxa has no TAS predicted; they are not tabulated in (Table 3-2).



Table 3-2: The predicted chromosomal type II toxin antitoxin systems in the oral microbiome.

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
<i>Capnocytophaga leadbetteri</i>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	CGC53_RS02185	470181..470486	MNT
	0	AT	CGC53_RS02180	469856..470191	HEPN
	TA_2	T	CGC53_RS03910	910662..911189	START
	0	AT	CGC53_RS03905	909922..910602	HTH
	TA_3	T	CGC53_RS04105	952696..952992	YoeB
	0	AT	CGC53_RS04100	952451..952708	RHH
	TA_4	T	CGC53_RS10070	2256679..2257134	VapC
0	AT	CGC53_RS10065	2256471..2256698	RelB	
<i>Rothia dentocariosa</i>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	HMPREF0733_10180	201492..201863	VapC
	0	AT	HMPREF0733_10181	201860..202057	VapB
	TA_2	T	HMPREF0733_10355	377568..378707	GNAT
	0	AT	HMPREF0733_10354	376739..377443	HTH
	TA_3	T	HMPREF0733_10590	644678..645097	VapC
	0	AT	HMPREF0733_10591	645101..645406	Phd
	TA_4	T	HMPREF0733_10761	816485..816787	MNT
	0	AT	HMPREF0733_10760	816135..816488	HEPN
	TA_5	T	HMPREF0733_11193	1280854..1282620	GNAT
	0	AT	HMPREF0733_11192	1280138..1280851	AcrR
	TA_6	T	HMPREF0733_11254	1364766..1365056	MNT
	0	AT	HMPREF0733_11255	1365056..1365391	HEPN
	TA_7	T	HMPREF0733_11511	1660539..1661009	HTH
	0	AT	HMPREF0733_11512	1661006..1661326	HTH
	TA_8	T	HMPREF0733_11698	1882625..1882885	YoeB

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	HMPREF0733_11697	1882368..1882622	Phd
<b><i>Haemophilus influenzae</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC13377_00047	48268..48666	VapC
	0	AT	NCTC13377_00046	48035..48268	MazE
	TA_2	T	NCTC13377_00076	76885..77175	RelE
	0	AT	NCTC13377_00075	76641..76895	Phd
	TA_3	T	NCTC13377_00298	300005..300313	RelE
	0	AT	NCTC13377_00299	300313..300609	RelB
	TA_4	T	NCTC13377_00347	349919..350239	HipA
	0	AT	NCTC13377_00346	349623..349931	HTH
	TA_5	T	NCTC13377_00352	358344..358703	RelE
	0	AT	NCTC13377_00353	358696..358992	HTH
	TA_6	T	NCTC13377_01012	997694..998038	MNT
	0	AT	NCTC13377_01011	997261..997692	HEPN
	TA_7	T	NCTC13377_01294	1293131..1293838	HipA
	0	AT	NCTC13377_01295	1293938..1294603	HTH
	TA_8	T	NCTC13377_01416	1396539..1397246	HipA
	0	AT	NCTC13377_01417	1397346..1398011	HTH
	TA_9	T	NCTC13377_01430	1403404..1403709	HigB
	0	AT	NCTC13377_01429	1403114..1403407	HigA
	TA_10	T	NCTC13377_01437	1406854..1407105	RelE
	0	AT	NCTC13377_01436	1406523..1406870	HTH
	TA_11	T	NCTC13377_01632	1614045..1614350	RelE
	0	AT	NCTC13377_01631	1613711..1614034	HTH
<b><i>Actinomyces oris</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	AXE84_01550	344964..345341	Fido

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	AXE84_01545	344750..344953	RelB
	TA_2	T	AXE84_02440	545051..545683	GNAT
	0	AT	AXE84_02445	545810..546946	LsrR
	TA_3	T	AXE84_02825	658137..658391	YoeB
	0	AT	AXE84_02830	658391..658642	Phd
	TA_4	T	AXE84_02845	661089..662333	HipA
	0	AT	AXE84_02850	662330..662617	HTH
	TA_5	T	AXE84_03195	747284..747661	VapC
	0	AT	AXE84_03190	747090..747287	VapB
	TA_6	T	AXE84_03905	913152..913553	VapC
	0	AT	AXE84_03900	912928..913155	CcdA
	TA_7	T	AXE84_03930	917777..918169	VapC
	0	AT	AXE84_03935	918166..918387	VapB
	TA_8	T	AXE84_04220	991997..992584	VapC
	0	AT	AXE84_04225	992588..993073	MerR
	TA_9	T	AXE84_04425	1037940..1038410	MNT
	0	AT	AXE84_04420	1037554..1037943	HEPN
	TA_10	T	AXE84_06645	1623445..1623798	RelE
	0	AT	AXE84_06640	1623125..1623448	HTH
	TA_11	T	AXE84_06935	1712733..1713014	RelE
	0	AT	AXE84_06940	1713001..1713312	HTH
	TA_12	T	AXE84_06970	1718075..1719739	GNAT
	0	AT	AXE84_06965	1717239..1717979	AcrR
	TA_13	T	AXE84_08665	2160245..2160514	YoeB
	0	AT	AXE84_08660	2160009..2160251	Phd
	TA_14	T	AXE84_09955	2478807..2479232	START

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	AXE84_09960	2479229..2479789	MarR
	TA_15	T	AXE84_11560	2864573..2866099	GNAT
	0	AT	AXE84_11555	2864007..2864576	AcrR
	TA_16	T	AXE84_11985	2963479..2963826	MazF
	0	AT	AXE84_11990	2963813..2964052	RelB
<b><i>Aggregatibacter segnis</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10977_00366	368843..369205	RelE
	0	AT	NCTC10977_00367	369202..369579	HTH
	TA_2	T	NCTC10977_00853	824328..825641	MNT
	0	AT	NCTC10977_00852	823368..824225	ParD
	TA_3	T	NCTC10977_01792	1838998..1840278	HipA
	0	AT	NCTC10977_01791	1838720..1838998	HTH
<b><i>Capnocytophaga ochracea</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	Coch_0735	889703..889999	HigB
	0	AT	Coch_0736	890004..890237	HTH
	TA_2	T	Coch_0780	939775..940101	Fido
	0	AT	Coch_0779	939063..939773	Phd
	TA_3	T	Coch_0815	978517..978822	RelE
	0	AT	Coch_0816	978825..979049	RelB
	TA_4	T	Coch_0841	1003635..1003955	RelE
	0	AT	Coch_0840	1003367..1003642	RHH
	TA_5	T	Coch_0997	1170527..1170850	RelE
	0	AT	Coch_0998	1170843..1171100	RelB
	TA_6	T	Coch_1196	1403828..1404136	RelE
	0	AT	Coch_1197	1404140..1404382	RelB
	TA_7	T	Coch_1229	1435589..1436116	START

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	Coch_1228	1434881..1435531	HxlR
<b><i>Gemella morbillorum</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11323_00623	628213..628653	ImmA/IrrE
	0	AT	NCTC11323_00624	628643..629044	HTH
<b><i>Fusobacterium nucleatum</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	FN1664	162255..162590	RelE
	0	AT	FN1665	162655..162954	HTH
	TA_2	T	FN1998	500269..500451	HipA
	0	AT	FN1997	499898..500218	HTH
	TA_3	T	FN2046	551001..551450	GNAT
	0	AT	FN2045	550464..550892	PerR
	TA_4	T	FN2066	576417..576833	ImmA/IrrE
	0	AT	FN2065	575990..576457	HTH
	TA_5	T	FN0056	692832..693311	GNAT
	0	AT	FN0055	692298..692804	GNAT
	TA_6	T	FN0211	837364..837630	RelE
	0	AT	FN0210	837138..837356	RelB
	TA_7	T	FN0497	1141273..1141545	RelE
	0	AT	FN0496	1141050..1141271	RelB
	TA_8	T	FN1100	1744317..1744583	RelE
	0	AT	FN1099	1744105..1744332	RelB
	TA_9	T	FN1294	1952607..1953158	GNAT
	0	AT	FN1295	1953248..1953655	GNAT
<b><i>Fusobacterium periodonticum</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	C4N17_08000	1625268..1625546	YoeB
	0	AT	C4N17_08005	1625540..1625779	Phd

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	TA_2	T	C4N17_09110	1824443..1824727	RelE
	0	AT	C4N17_09115	1824714..1824953	RelB
	TA_3	T	C4N17_09580	1921236..1921721	GNAT
	0	AT	C4N17_09585	1921750..1922253	GNAT
	TA_4	T	C4N17_09585	1921750..1922253	GNAT
	0	AT	C4N17_09595	1922516..1922770	YefM
	TA_5	T	C4N17_09590	1922263..1922523	YoeB
	0	AT	C4N17_09595	1922516..1922770	Phd
	TA_6	T	C4N17_10050	2023458..2023649	HicA
	0	AT	C4N17_10045	2022996..2023421	HicB
	TA_7	T	C4N17_10615	2118410..2119150	ImmA/IrrE
	0	AT	C4N17_10610	2118037..2118423	HTH
	TA_8	T	C4N17_10875	2153007..2153279	RelE
	0	AT	C4N17_10880	2153281..2153544	RelB
	TA_9	T	C4N17_11255	2235387..2236526	HipA
	0	AT	C4N17_11250	2234505..2235152	AcrR
	TA_10	T	C4N17_11255	2235387..2236526	HipA
	0	AT	C4N17_11260	2236513..2236833	HTH
	TA_11	T	C4N17_12205	2431810..2431992	HicA
	0	AT	C4N17_12210	2432030..2432428	HicB
	TA_12	T	C4N17_12260	2440858..2441268	ImmA/IrrE
	0	AT	C4N17_12265	2441228..2441695	HTH
<b><i>Rothia aeria</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	RA11412_0178	154222..154647	VapC
	0	AT	RA11412_0179	154651..154956	Phd
	TA_2	T	RA11412_0821	741232..742998	GNAT

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	RA11412_0820	740558..741229	AcrR
	TA_3	T	RA11412_1191	1091799..1092269	HTH
	0	AT	RA11412_1192	1092266..1092721	HTH
	TA_4	T	RA11412_2432	2261884..2262381	GNAT
	0	AT	RA11412_2431	2261591..2261884	RHH
	TA_5	T	RA11412_2432	2261884..2262381	GNAT
	0	AT	RA11412_2433	2262592..2263131	HTH
	TA_6	T	RA11412_2436	2264486..2264653	VapC
	0	AT	RA11412_2437	2264851..2265048	VapB
<b><i>Streptococcus sanguinis</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11085_00324	309578..309856	RelE
	0	AT	NCTC11085_00323	309322..309585	RelB
	TA_2	T	NCTC11085_00917	948027..948443	GNAT
	0	AT	NCTC11085_00918	948617..949228	AcrR
	TA_3	T	NCTC11085_01051	1097626..1098483	GNAT
	0	AT	NCTC11085_01052	1098603..1099505	LysR
	TA_4	T	NCTC11085_01635	1689503..1690072	GNAT
	0	AT	NCTC11085_01633	1688763..1689299	HTH
	TA_5	T	NCTC11085_02225	2284014..2284886	ImmA/IrrE
	0	AT	NCTC11085_02226	2284886..2285248	HTH
<b><i>Veillonella atypica</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	B7L28_00880	227561..227965	VapC
	0	AT	B7L28_00875	227301..227564	Phd
	TA_2	T	B7L28_00985	253813..255006	GNAT
	0	AT	B7L28_00990	255064..255951	LysR
	TA_3	T	B7L28_01445	340243..340506	RelE

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	B7L28_01440	340015..340239	RelB
	TA_4	T	B7L28_02060	465243..465509	RelE
	0	AT	B7L28_02055	465032..465253	RelB
	TA_5	T	B7L28_09065	1992530..1992907	HicA
	0	AT	B7L28_09060	1992342..1992518	HicB
	TA_6	T	B7L28_09085	1997335..1997610	RelE
	0	AT	B7L28_09080	1997064..1997345	RelB
	TA_7	T	B7L28_09285	2039161..2040165	Fido
	0	AT	B7L28_09290	2040218..2041081	HTH
<b><i>Streptococcus gordonii</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC3165_00371	383729..384496	PezT
	0	AT	NCTC3165_00370	383253..383729	HTH
	TA_2	T	NCTC3165_00373	385018..385389	Fido
	0	AT	NCTC3165_00372	384764..385021	Phd
	TA_3	T	NCTC3165_01298	1313648..1314064	GNAT
	0	AT	NCTC3165_01297	1312863..1313474	AcrR
	TA_4	T	NCTC3165_01704	1732532..1733392	ImmA/IrrE
	0	AT	NCTC3165_01703	1731839..1732522	HTH
<b><i>Streptococcus mutans</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10449_00169	173400..173732	MazF
	0	AT	NCTC10449_00170	173719..174024	RelB
	TA_2	T	NCTC10449_00182	183970..184224	YoeB
	0	AT	NCTC10449_00183	184217..184483	YefM
	TA_3	T	NCTC10449_00189	187863..188072	RelE
	0	AT	NCTC10449_00190	188142..188414	RelB
	TA_4	T	NCTC10449_00193	190470..191264	ImmA/IrrE



<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	0	AT	NCTC10449_00192	190044..190406	HTH
	TA_5	T	NCTC10449_00202	200685..201017	MazF
	0	AT	NCTC10449_00201	200446..200691	MazE
	TA_6	T	NCTC10449_00413	401966..402157	VapC
	0	AT	NCTC10449_00412	401677..401982	MazE
	TA_7	T	NCTC10449_00760	741130..741321	HicA
	0	AT	NCTC10449_00761	741339..741716	HicB
<b><i>Neisseria elongata</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	DV445_RS02635	524666..525214	GNAT
	0	AT	DV445_RS02640	525321..525806	HTH
	TA_2	T	DV445_RS05995	1188449..1189306	Bro
	0	AT	DV445_RS06000	1189372..1190073	HTH
	TA_3	T	DV445_RS12620	2480707..2481126	VapC
	0	AT	DV445_RS12615	2480471..2480710	RHH
	TA_4	T	DV445_RS12655	2491470..2491742	RelE
	0	AT	DV445_RS12660	2491732..2491923	RHH
<b><i>Aggregatibacter aphrophilus</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11096_01165	1141294..1142574	HipA
	0	AT	NCTC11096_01166	1142574..1142852	HTH
	TA_2	T	NCTC11096_02169	2251436..2252749	MNT
	0	AT	NCTC11096_02170	2252853..2253710	ParD
<b><i>Streptococcus salivarius</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	HSISS4_00658	705771..706136	HigB
	0	AT	HSISS4_00659	706126..706419	HigA
	TA_2	T	HSISS4_00840	910234..911448	FmhB
	0	AT	HSISS4_00841	911450..912259	HTH

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	TA_3	T	HSISS4_01146	1242750..1243268	GNAT
	0	AT	HSISS4_01147	1243285..1244328	HTH
	TA_4	T	HSISS4_01298	1438024..1439409	GNAT
	0	AT	HSISS4_01299	1439435..1439845	MarR
	TA_5	T	HSISS4_01524	1722127..1722381	YoeB
	0	AT	HSISS4_01525	1722385..1722639	Phd
<b><i>Granulicatella elegans</i></b>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	ORF1_138	164191..165234	Fido
	0	AT	ORF1_139	165451..166302	HTH
	TA_2	T	ORF1_182	211296..211862	GNAT
	0	AT	ORF1_183	211872..212714	AraC
	TA_3	T	ORF1_377	431367..431636	GNAT
	0	AT	ORF1_376	430909..431334	Rrf2
	TA_4	T	ORF1_630	693298..693477	HicA
	0	AT	ORF1_631	693514..693969	HicB
	TA_5	T	ORF1_835	907257..907529	RelE
	0	AT	ORF1_834	906979..907254	RelB
	TA_6	T	ORF1_854	919944..920765	Fido
	0	AT	ORF1_853	919487..919753	HTH
	TA_7	T	ORF1_928	998474..998653	HicA
	0	AT	ORF1_927	997982..998437	HicB
	TA_8	T	ORF1_1375	1460943..1461380	GNAT
	0	AT	ORF1_1374	1460493..1460750	Fido
	TA_9	T	ORF1_1491	1589708..1589899	HicA
	0	AT	ORF1_1490	1589299..1589676	HicB
	TA_no.	T/A	Locus_tag	Location	

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
<i>Haemophilus parainfluenzae</i>	TA_1	T	PARA_11550	1159230..1159496	RelE
	0	AT	PARA_11560	1159480..1159824	RelB
<i>Actinobacillus minor</i>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	AM305_00230	39922..40356	VapC
	0	AT	AM305_00225	39686..39925	MazE
<i>Haemophilus haemolyticus</i>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10839_00505	499226..499486	HipA
	0	AT	NCTC10839_00504	498930..499238	HTH
	TA_2	T	NCTC10839_01017	1007834..1008127	MNT
	0	AT	NCTC10839_01018	1008111..1008530	HEPN
	TA_3	T	NCTC10839_01455	1463601..1463855	HicA
	0	AT	NCTC10839_01454	1463105..1463587	HicB
	TA_4	T	NCTC10839_01455	1463601..1463855	HicA
	0	AT	NCTC10839_01456	1463929..1464594	HTH
	TA_5	T	NCTC10839_01587	1597372..1598652	HipA
	0	AT	NCTC10839_01586	1597094..1597372	HTH
	TA_6	T	NCTC10839_01662	1690384..1690746	MazF
	0	AT	NCTC10839_01661	1690121..1690390	MazE
	<i>Neisseria mucosa</i>	TA_no.	T/A	Locus_tag	Location
TA_1		T	NM96_RS06400	1295082..1295564	START
0		AT	NM96_RS06405	1295628..1296032	HTH
TA_2		T	NM96_RS10690	2119236..2119961	Aat
0		AT	NM96_RS10695	2120154..2120651	Fur
<i>Streptococcus mitis</i>	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	smi_0100	106784..107131	RelE
	0	AT	smi_0099	106507..106794	RelB

<b>Organism</b>	<b>TA no.</b>	<b>T/A</b>	<b>Locus Tag</b>	<b>Location</b>	<b>Manually Curated Gene Class</b>
	TA_2	T	smi_0438	414401..414754	MazF
	0	AT	smi_0439	414738..414953	MazE
	TA_3	T	smi_0611	553127..553477	RelE
	0	AT	smi_0610	552850..553137	RelB
	TA_4	T	smi_0698	647243..647530	RelE
	0	AT	smi_0697	646896..647201	RelB
	TA_5	T	smi_1101	1089095..1089865	PezT
	0	AT	smi_1102	1089865..1090341	HTH
	TA_6	T	smi_1200	1192110..1192673	GNAT
	0	AT	smi_1199	1191656..1192144	GNAT
	TA_7	T	smi_1262	1265315..1265629	MNT
	0	AT	smi_1261	1264960..1265334	HEPN
	TA_8	T	smi_1262	1265315..1265629	MNT
	0	AT	smi_1263	1265676..1265936	RelB
	TA_9	T	smi_1273	1270051..1270410	RelE
	0	AT	smi_1272	1269690..1270061	RelB

The distribution of the type II toxin antitoxin systems on the chromosomes of the genome of the oral microbiome is segregated and shows non-uniform distribution as shown from Figure 3-7 till Figure 3-29. This distribution could imply that the TAS genes are displaying a specific function and not randomly occurred. One reason could be that several TAS shows proximity to the Origin of Replication which accentuates its selfishness character in the intra-genome (Melderer & Bast, 2009; Ramisetty & Santhosh, 2017) The intragenomic existence of the Toxin Antitoxin module is not necessary a conflict. The inclusiveness of the gene does not contradict fitness of the bacterial host (Gardner & Úbeda, 2017; McLaughlin & Malik, 2017; Rankin Daniel J. et al., 2012) The TA gene promotes maintenance of fitness factors, virulence and resistance for xenobiotics and phages. Thus, the TAS evolution is not best described as having Red Queen evolutionary dynamics where the co-evolution of selfish entities is only attributed to attack or defend the host in an arm-race manner (McLaughlin & Malik, 2017) It is thought that TAS are skewing to the Black Queen evolutionary dynamics as they provide fitness characters with reduced fitness cost as they persist near the origin of replication of the bacterial chromosome. Although this require further investigations, this proximity to origin of replication ensures the quality expression of the fitness genes in demand that could be released to the surrounding population. On the other hand, the bacterium can be dependent on costly fitness genes present in the ecosystem but not invited by the antiaddiction role of the TAS modules. This leakiness and adaptive dependency are the main pillars of the Black Queen Hypothesis (Cairns et al., 2018; Goormaghtigh et al., 2018; Kang, Kim, Jin, & Lee, 2018; J. J. Morris, 2015; J. J. Morris, Lenski, & Zinser, 2012)

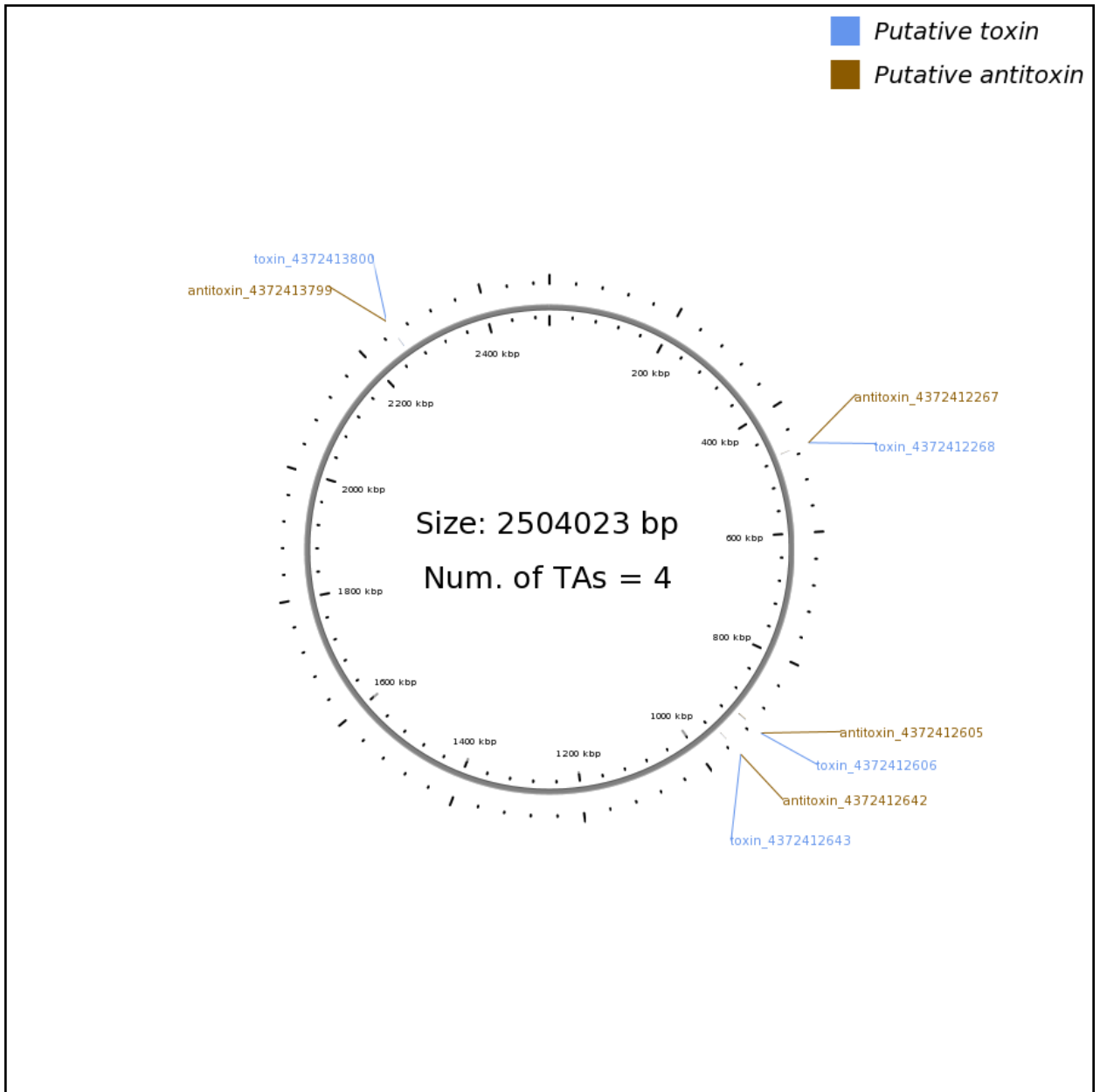


Figure 3-7: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Capnocytophaga leadbetteri* bacteria in the oral microbiome.

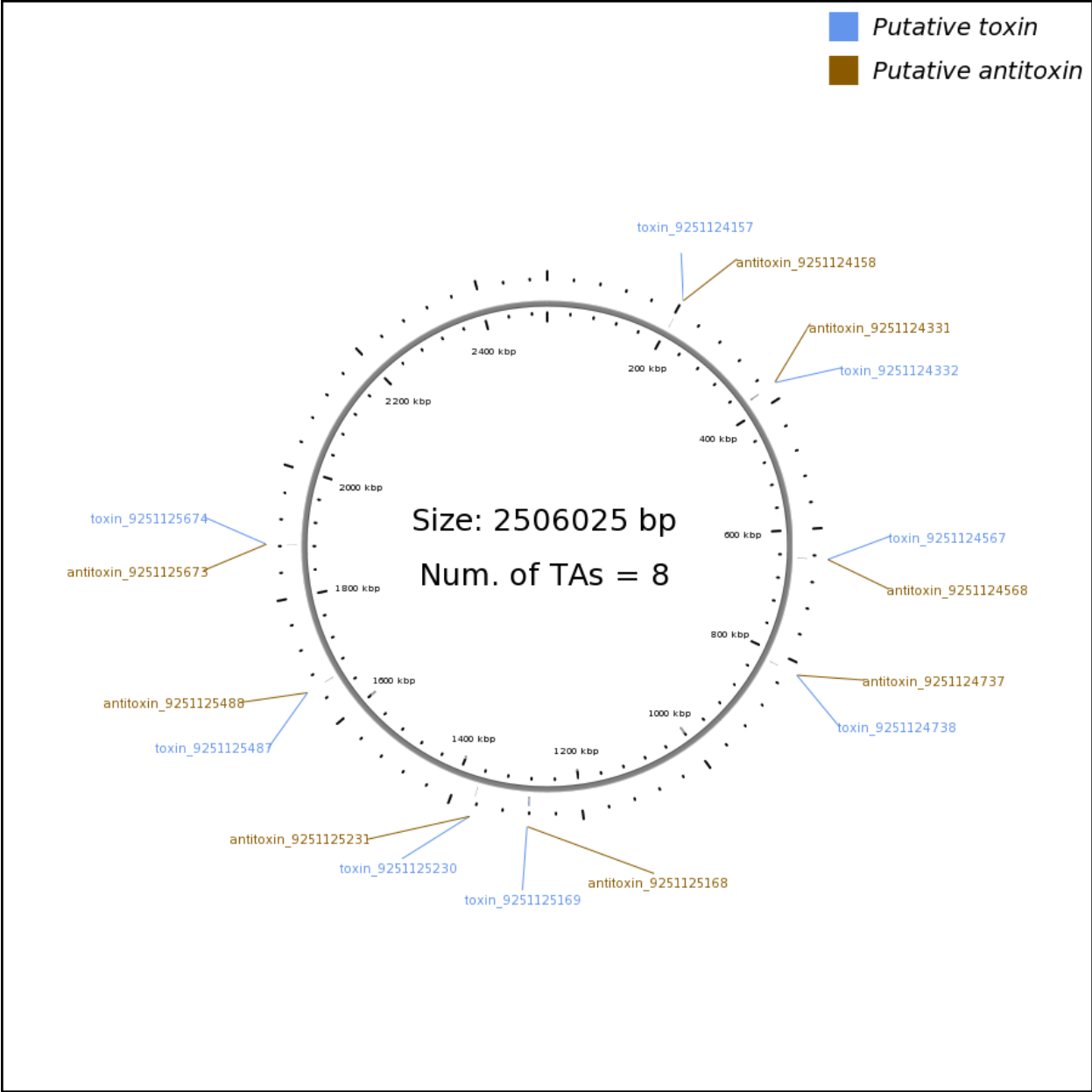


Figure 3-8: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Rothia dentocariosa* bacteria in the oral microbiome.

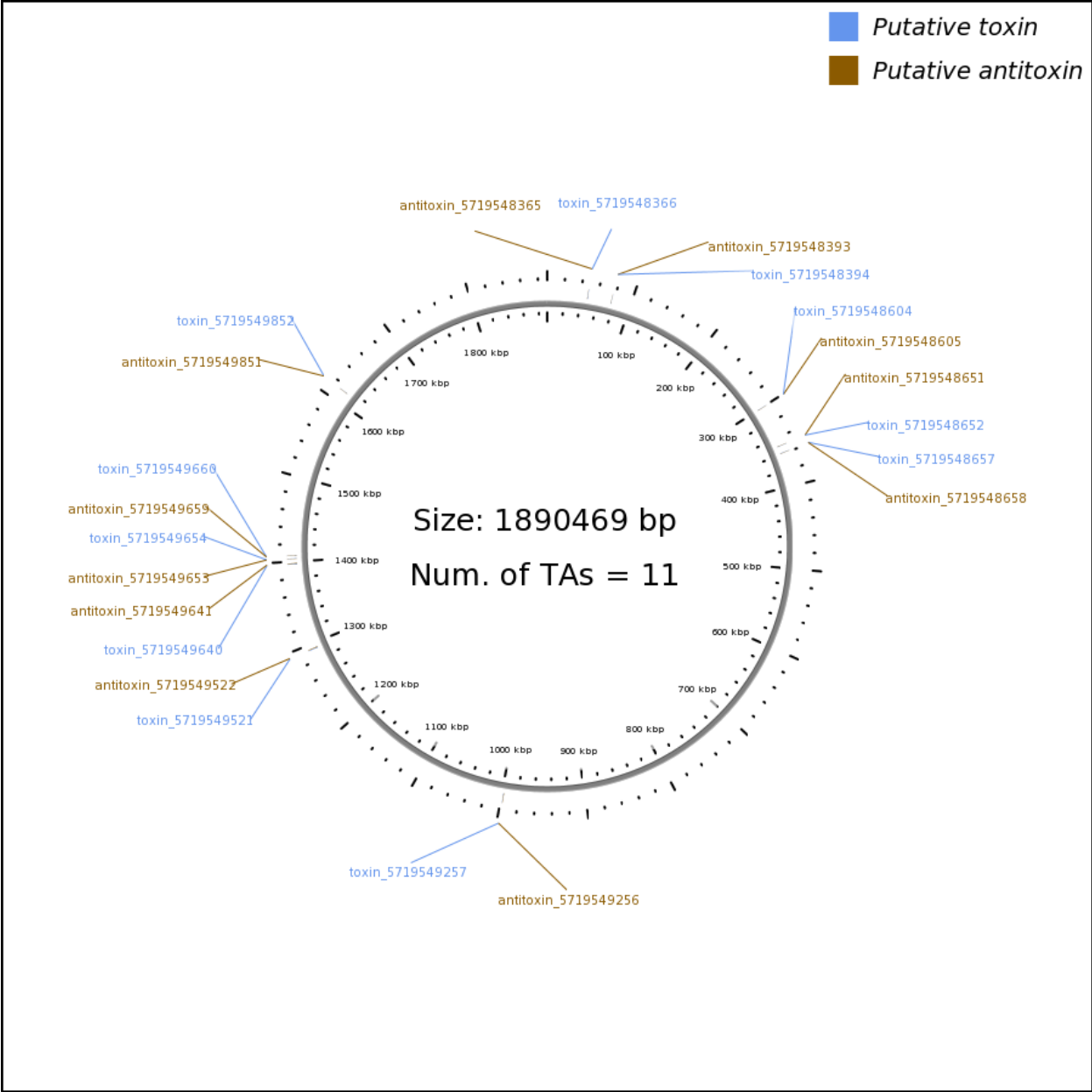


Figure 3-9: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus influenzae* bacteria in the oral microbiome.



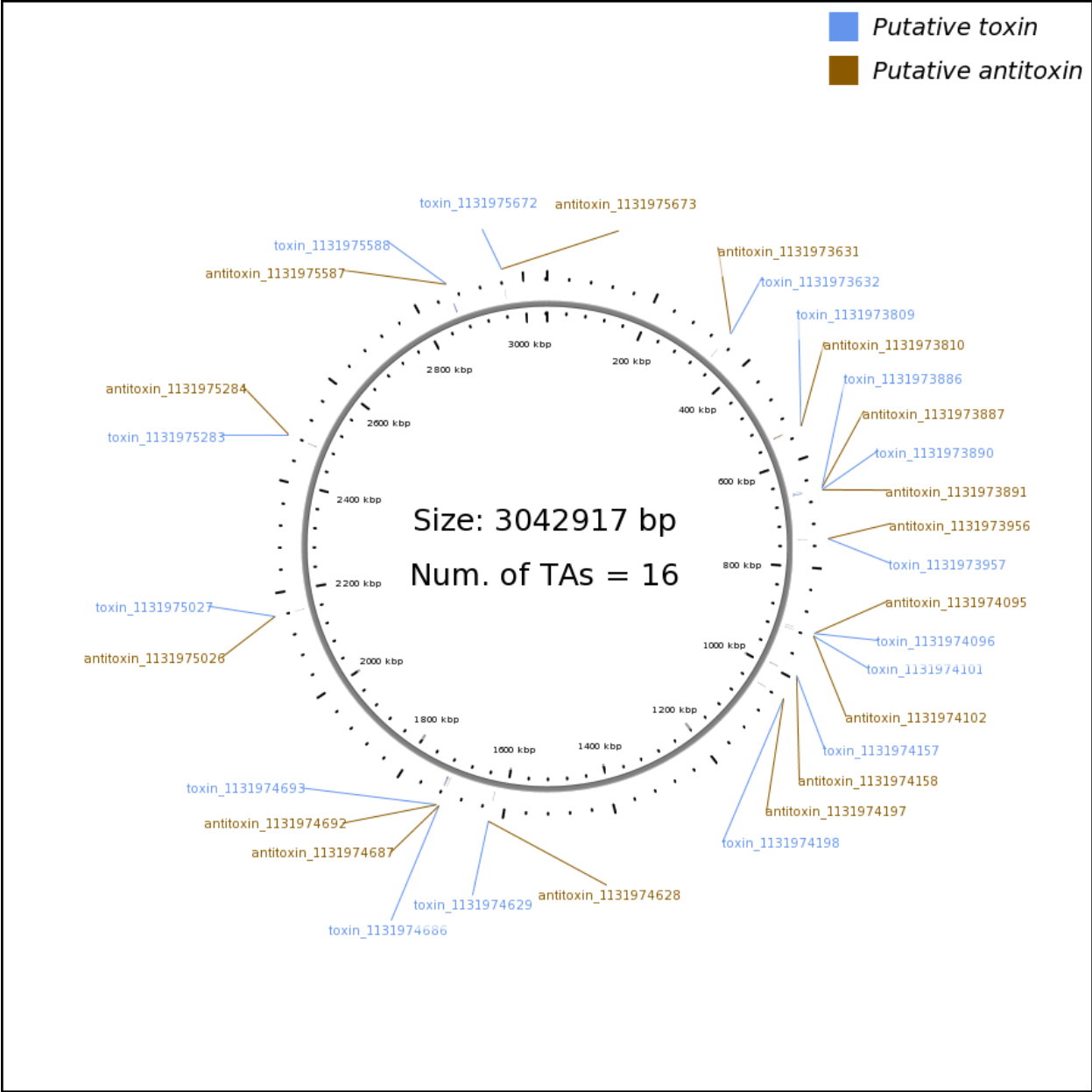


Figure 3-10: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Actinomyces oris* bacteria in the oral microbiome.

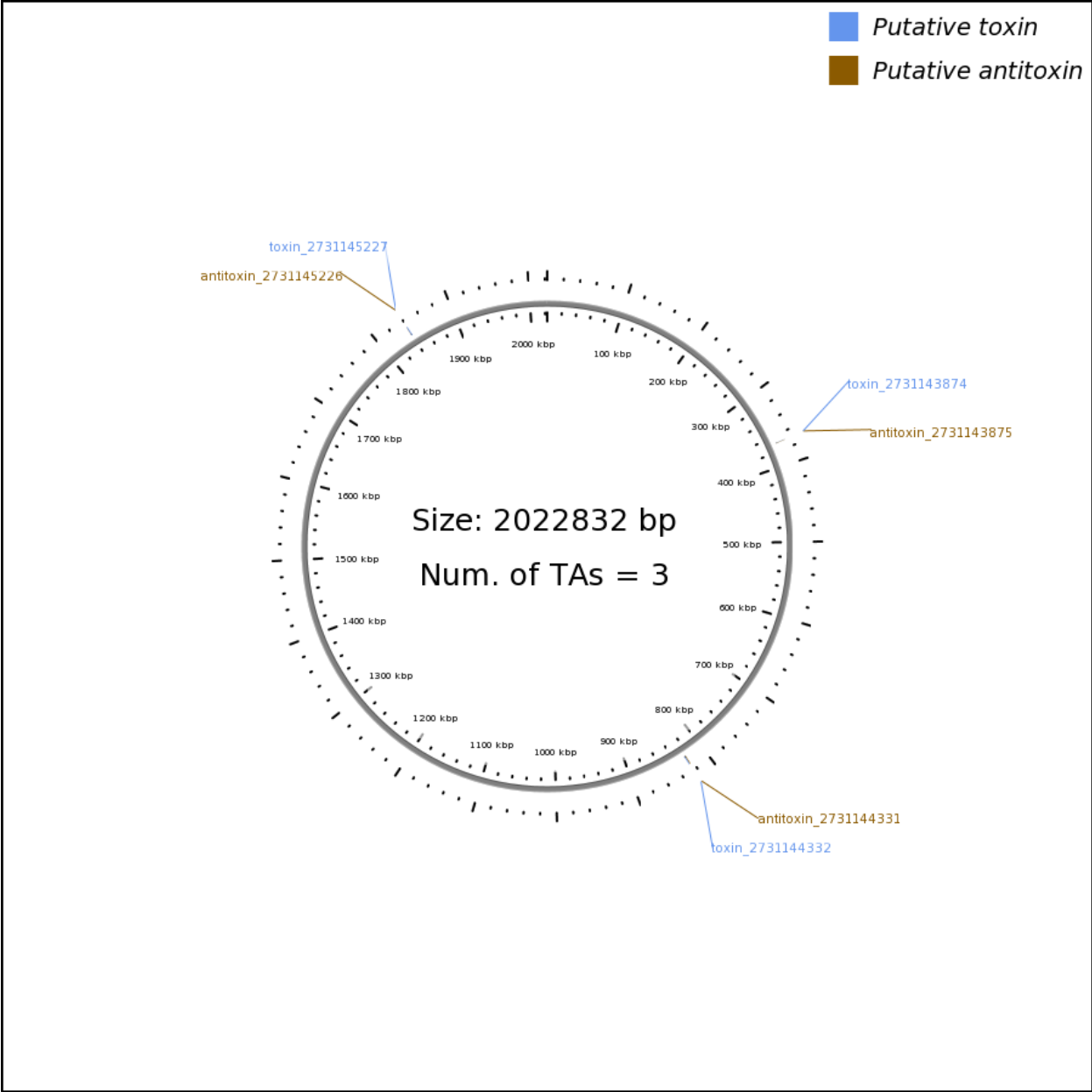


Figure 3-11 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Aggregatibacter segnis* bacteria in the oral microbiome.

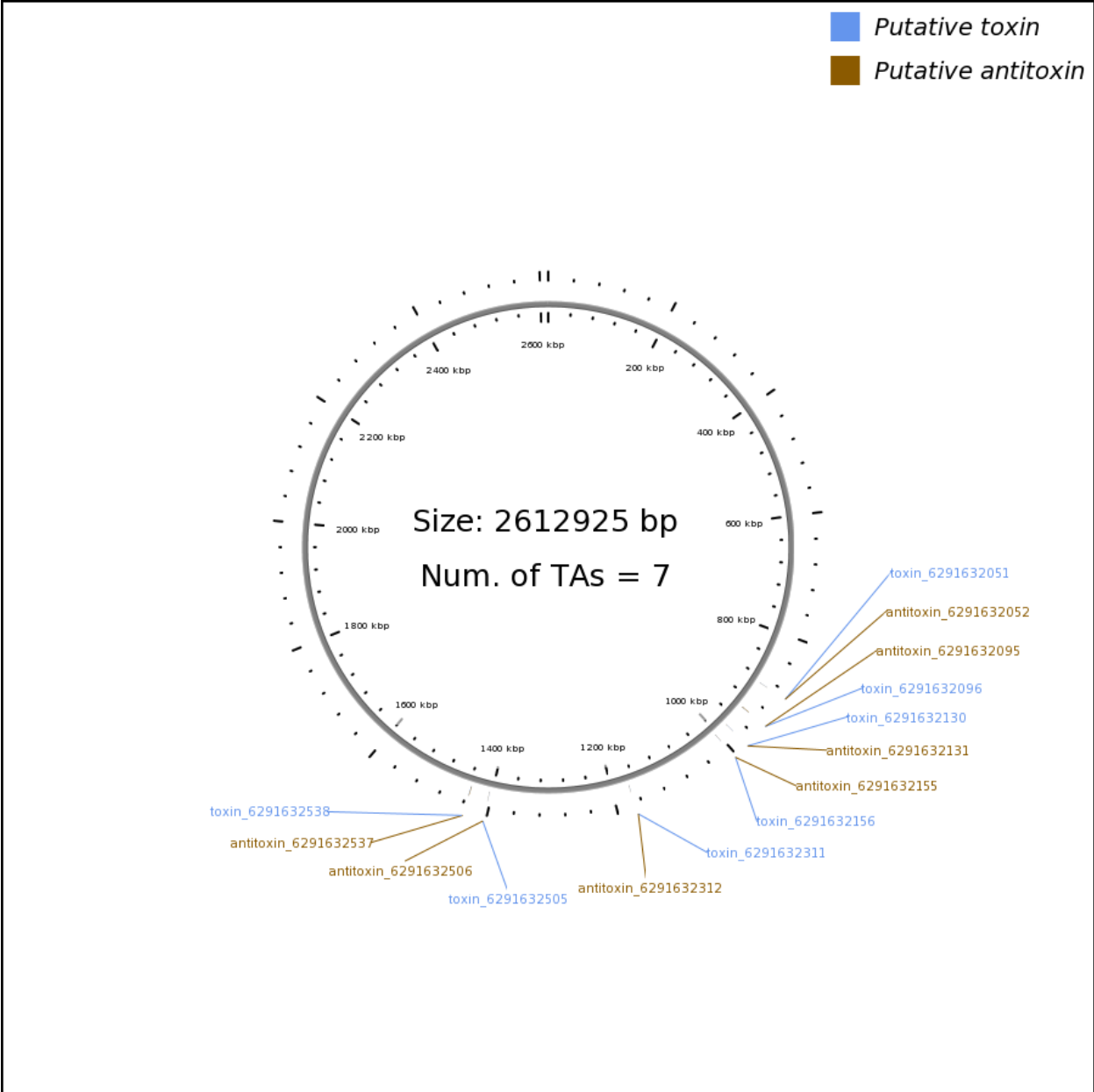


Figure 3-12 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Capnocytophaga ochracea* bacteria in the oral microbiome.

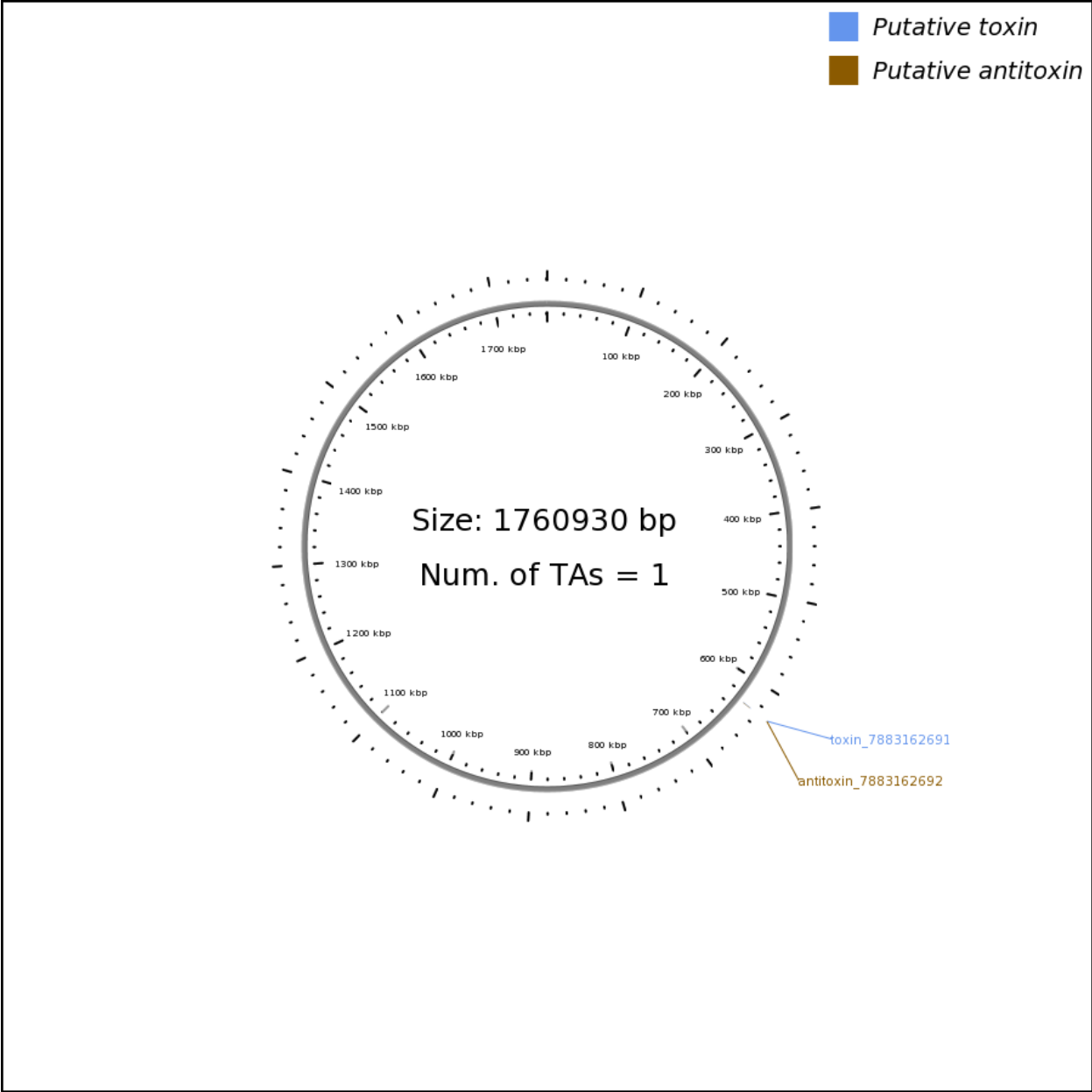


Figure 3-13: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Gemella morbillorum* bacteria in the oral microbiome.

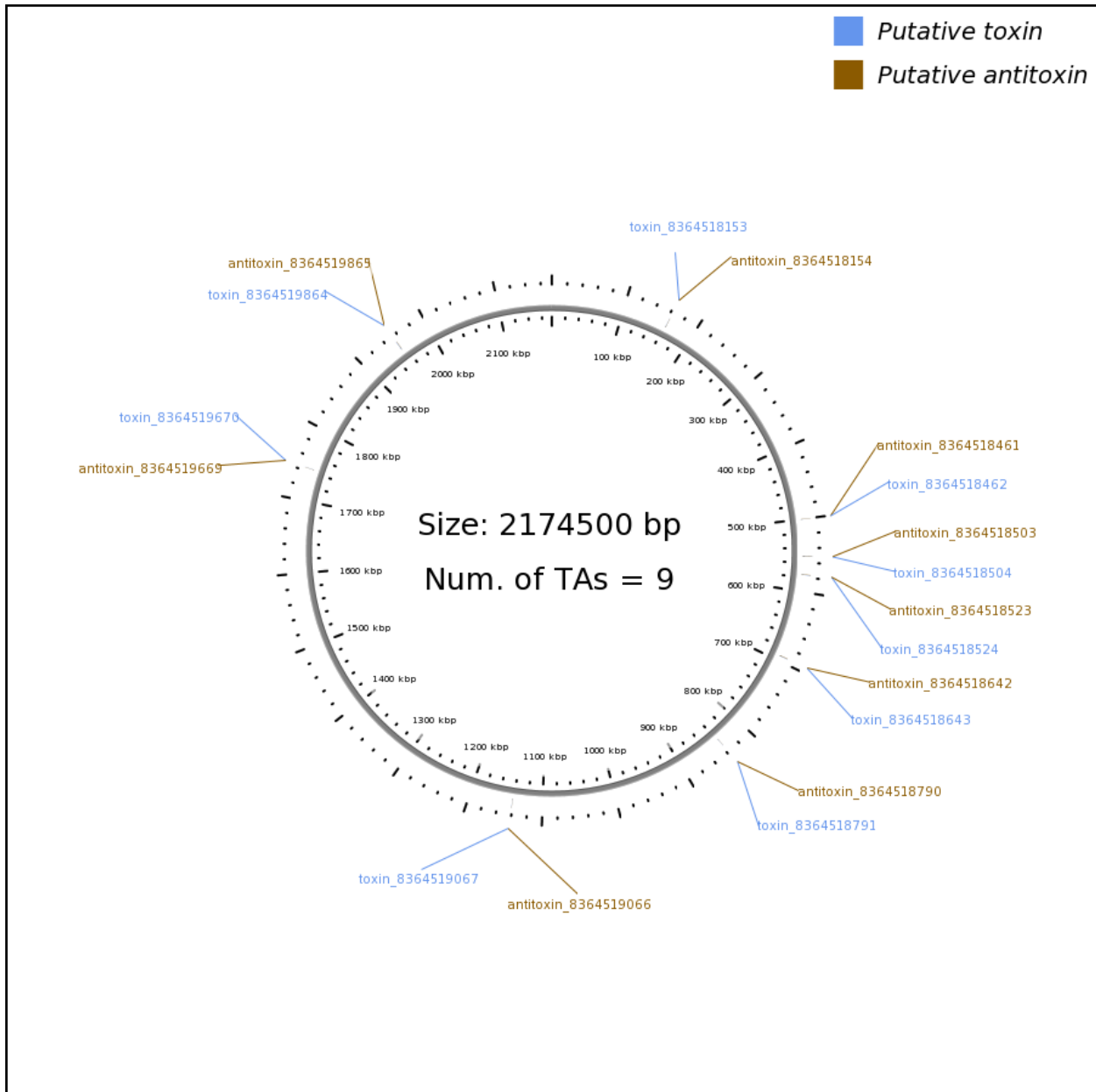


Figure 3-14: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Fusobacterium nucleatum* bacteria in the oral microbiome.

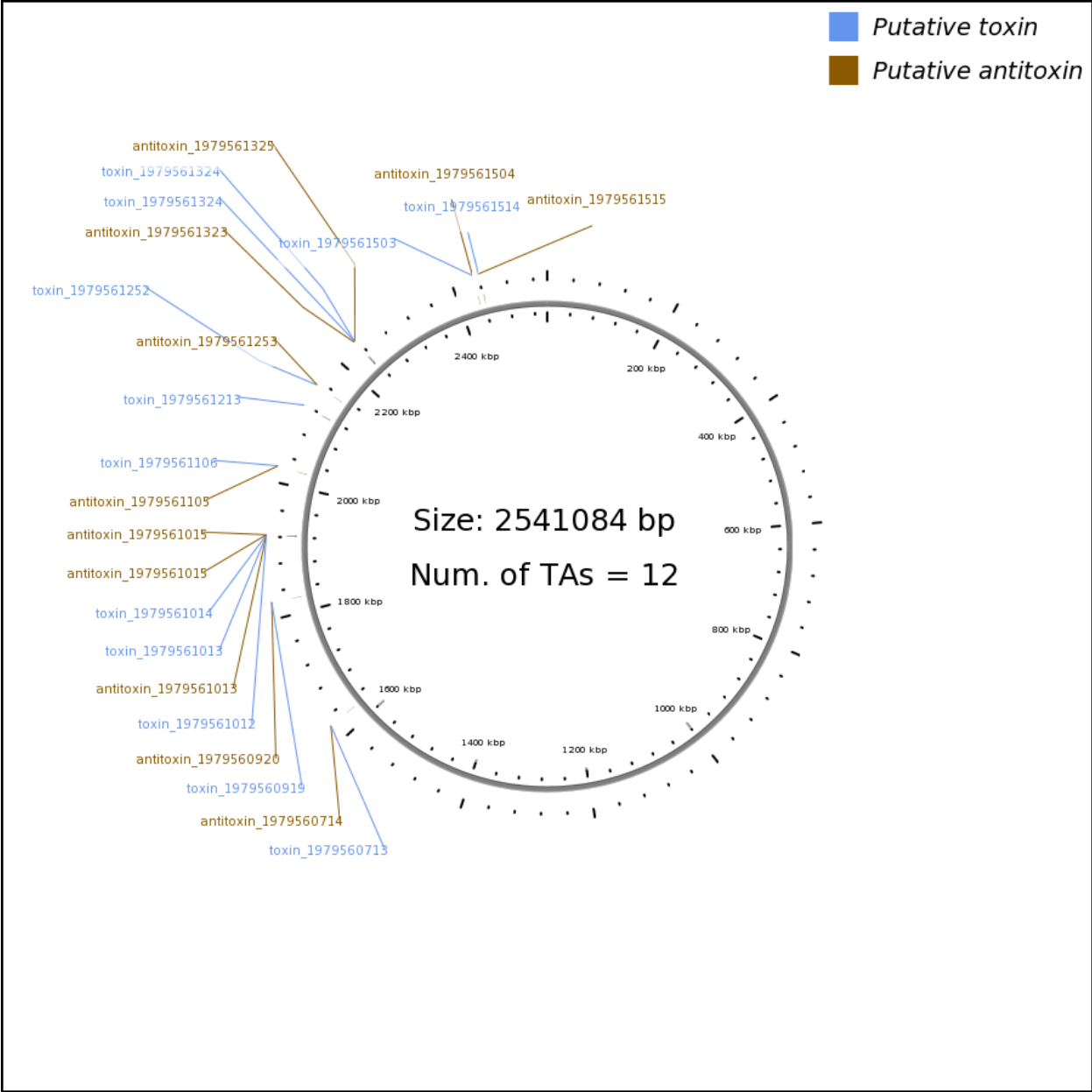


Figure 3-15: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Fusobacterium periodonticum* bacteria in the oral microbiome.

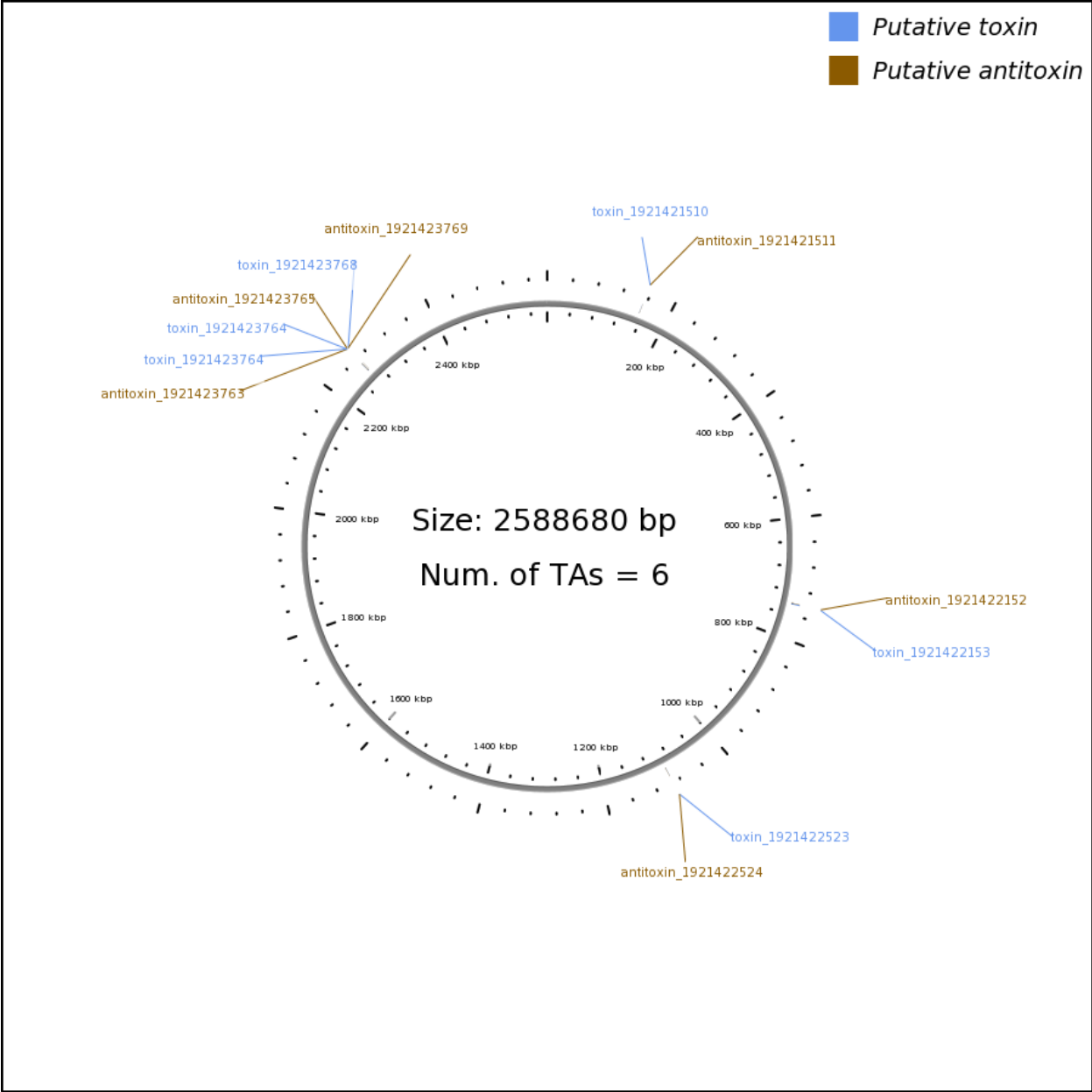


Figure 3-16: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Rothia aeria* bacteria in the oral microbiome.

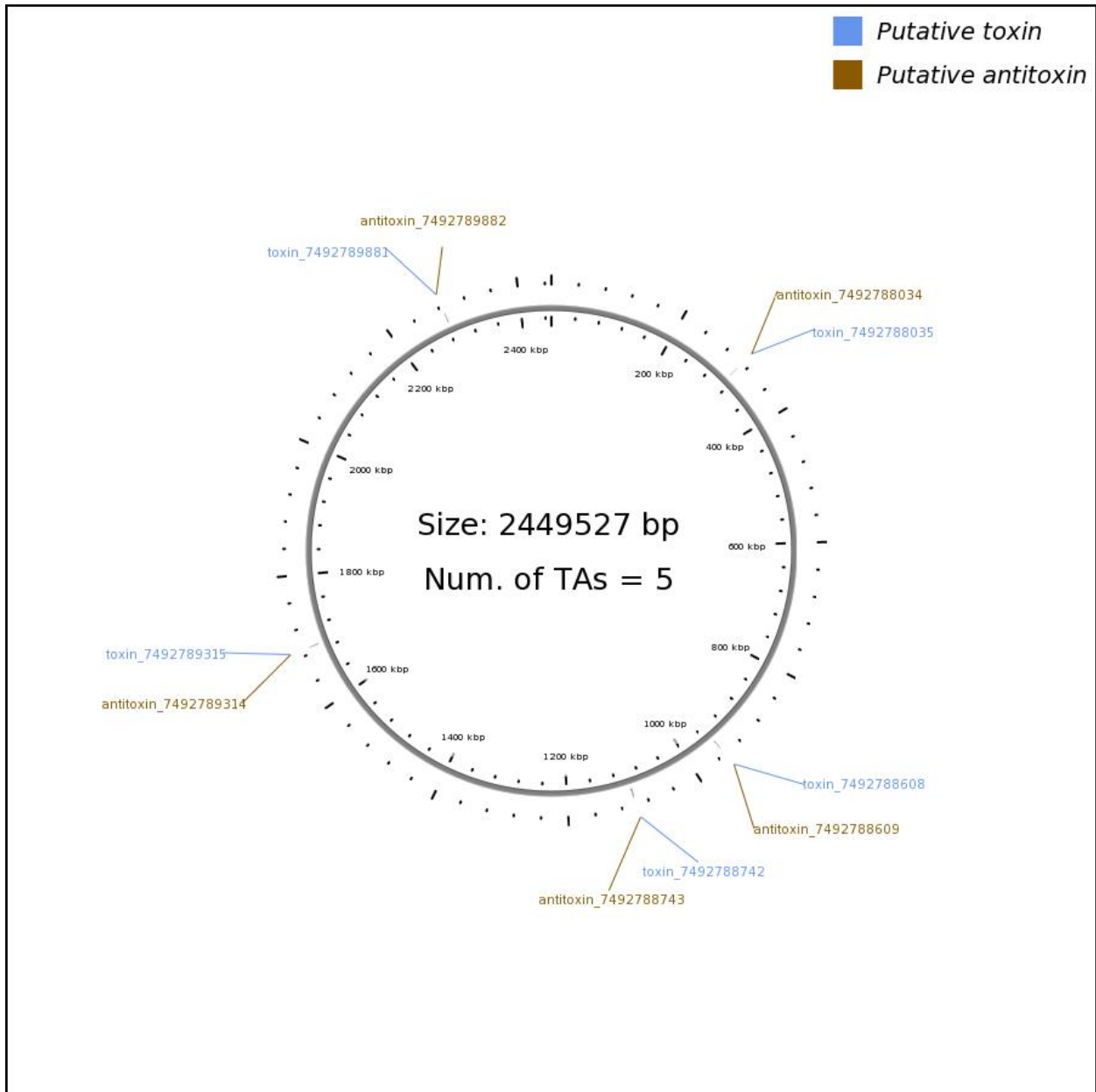


Figure 3-17: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus sanguinis* bacteria in the oral microbiome.



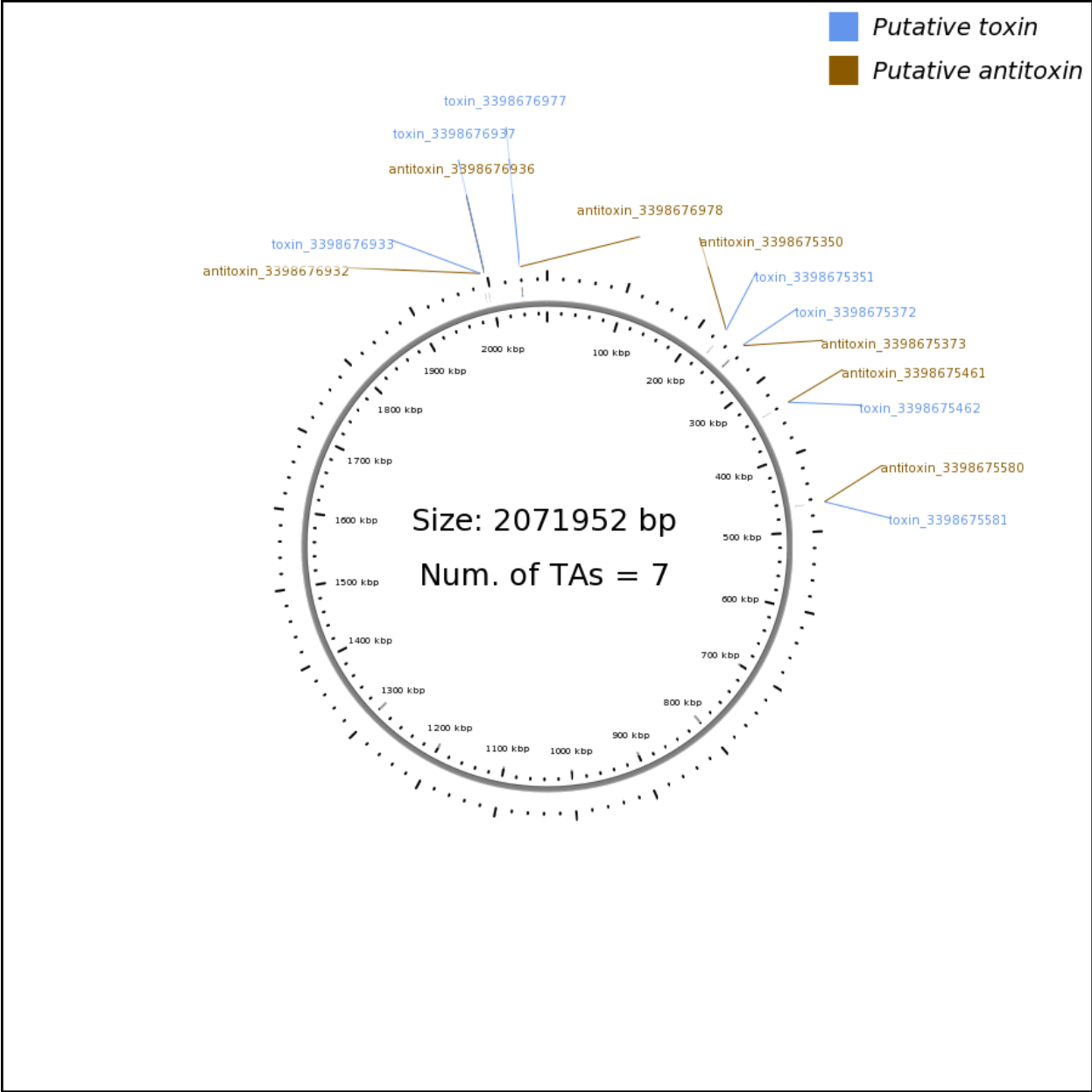


Figure 3-18: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Veillonella atypica* bacteria in the oral microbiome.

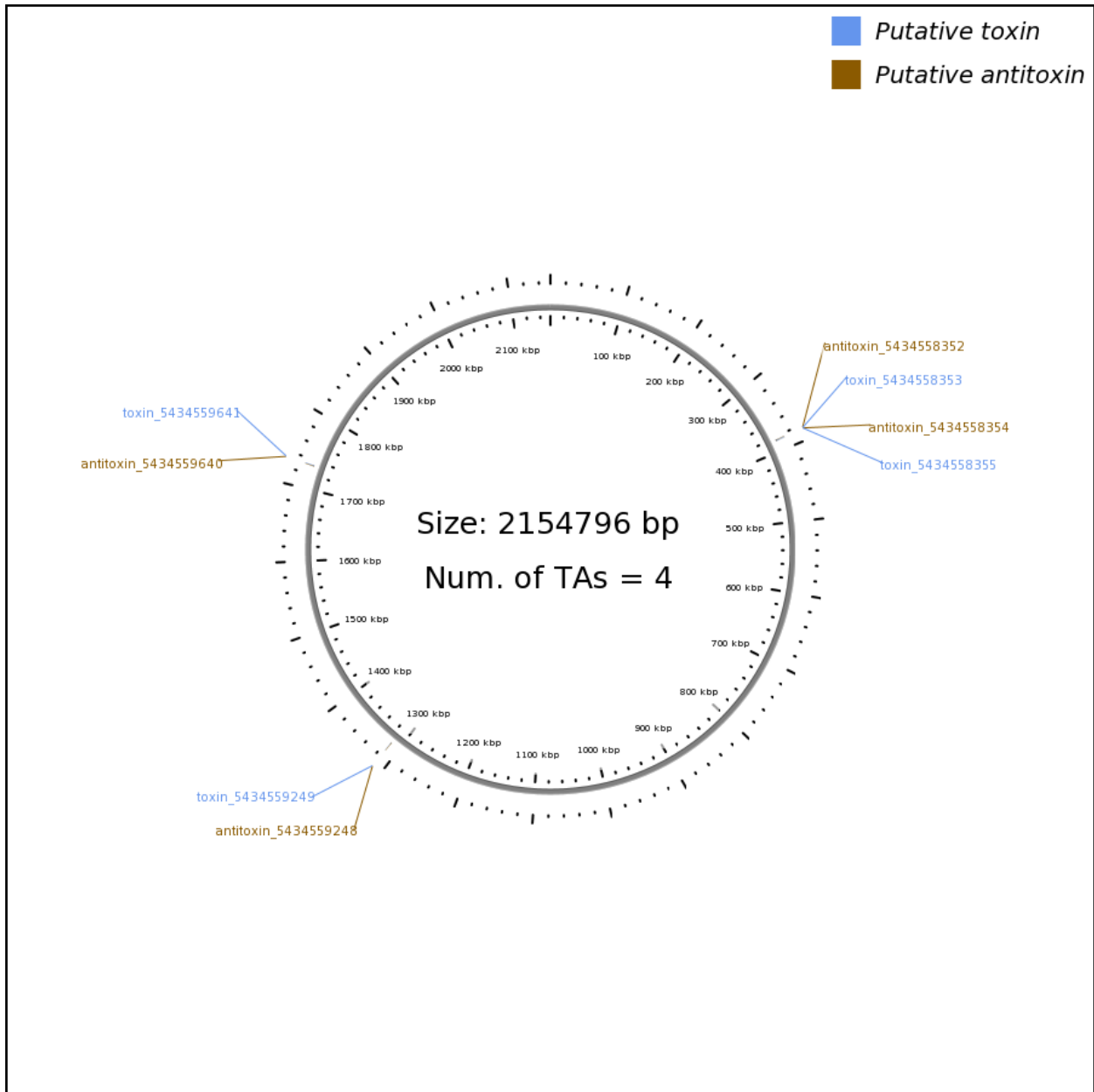


Figure 3-19: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus gordonii* bacteria in the oral microbiome.

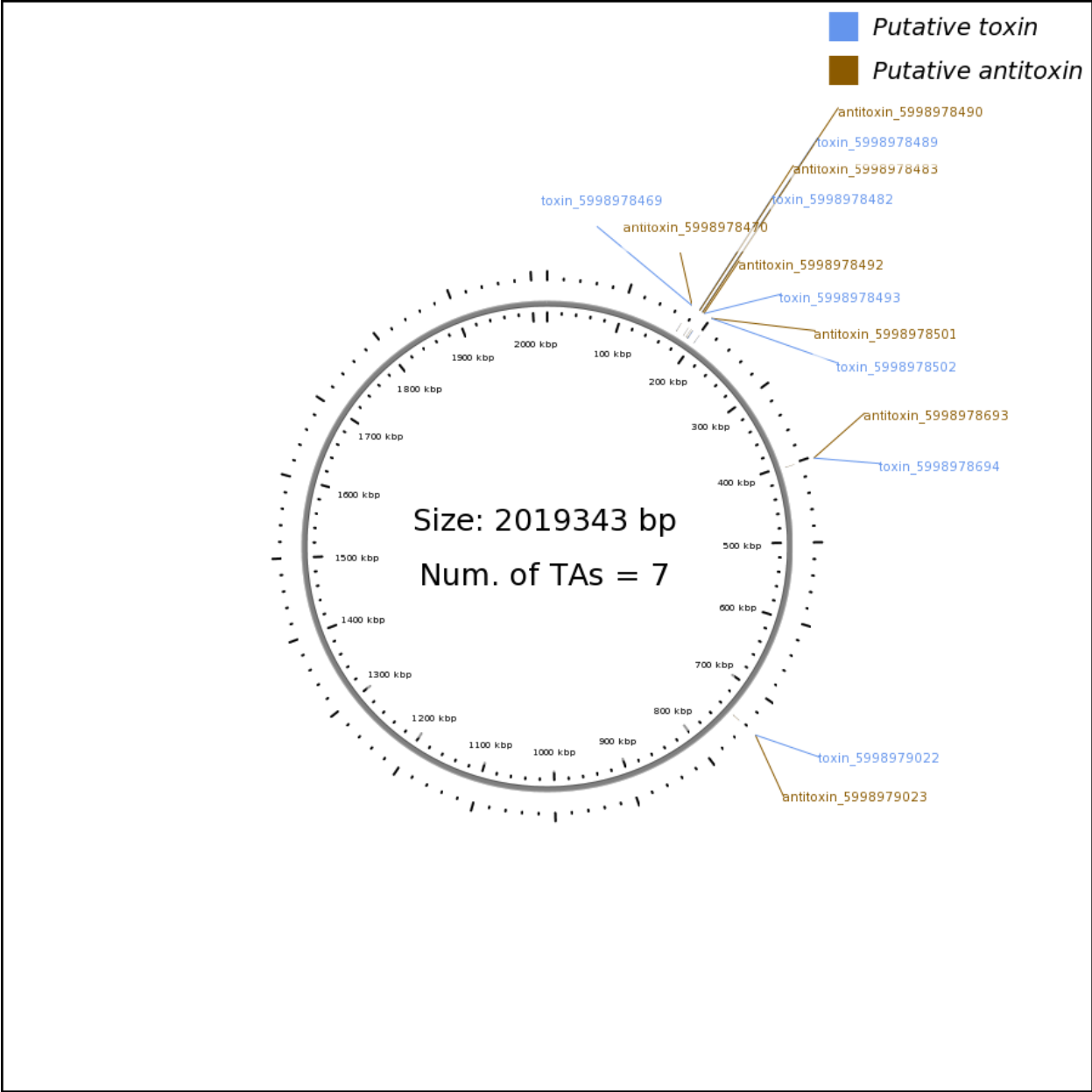


Figure 3-20: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus mutans* bacteria in the oral microbiome.

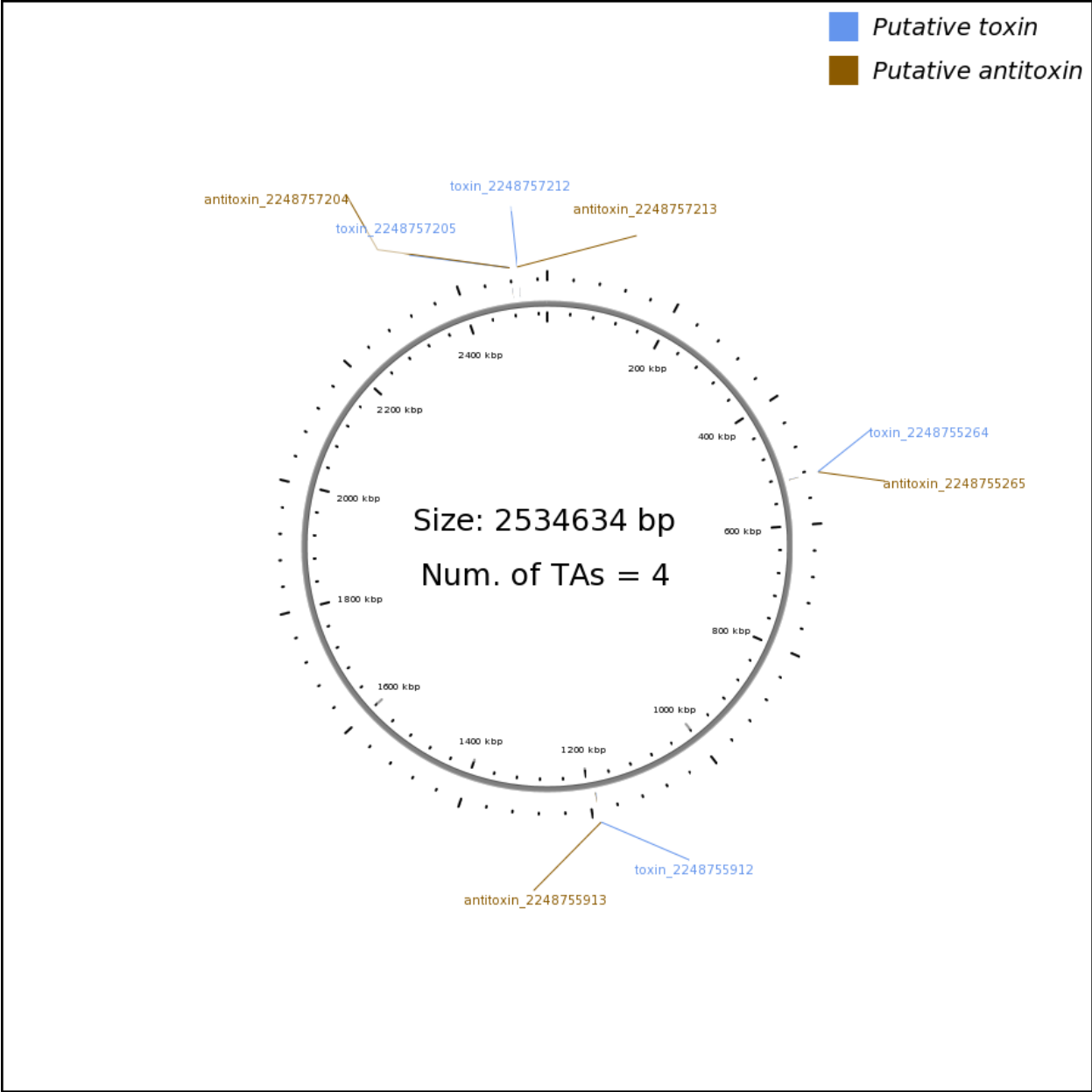


Figure 3-21: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Neisseria elongata* bacteria in the oral microbiome.

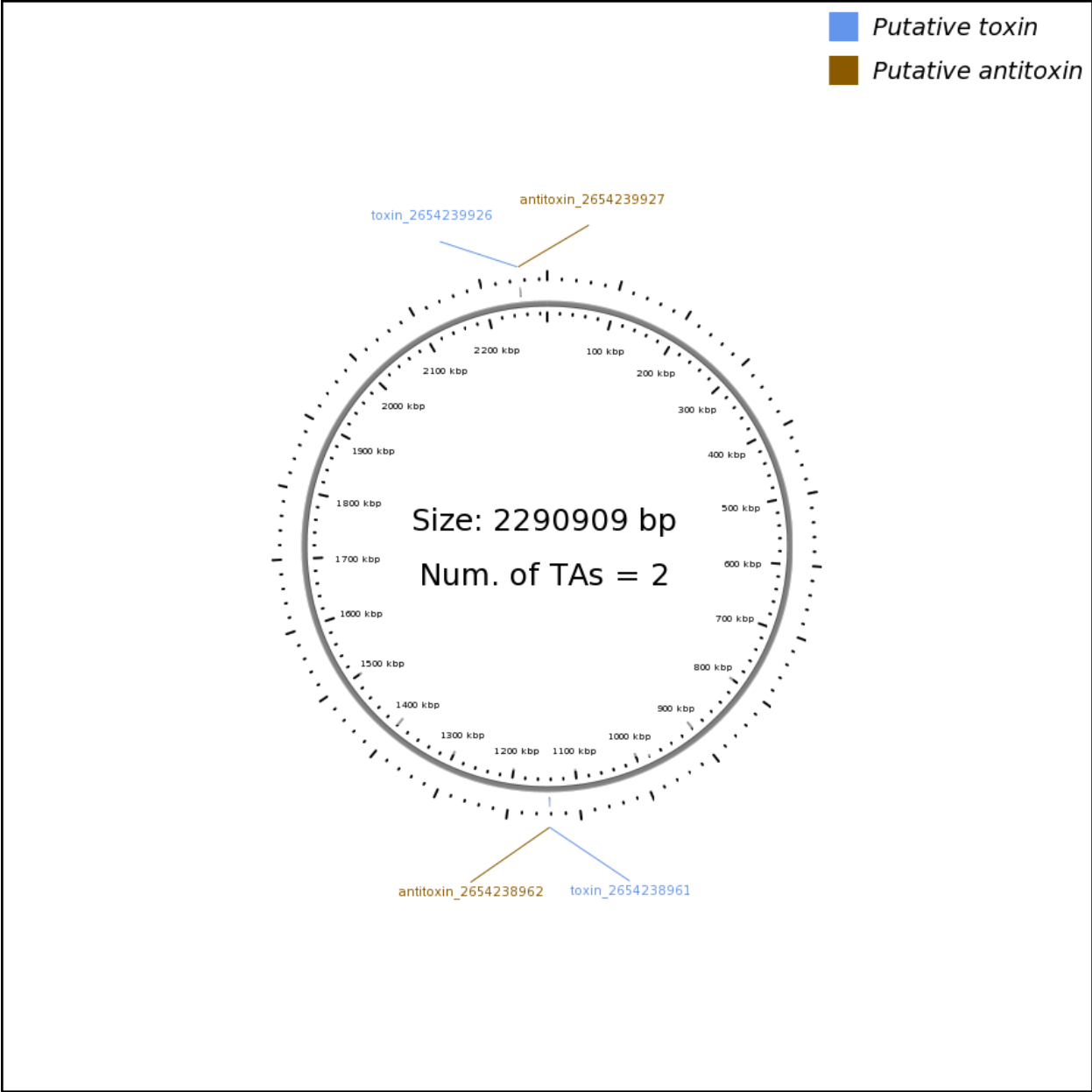


Figure 3-22: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Aggregatibacter aphrophilus* bacteria in the oral microbiome.

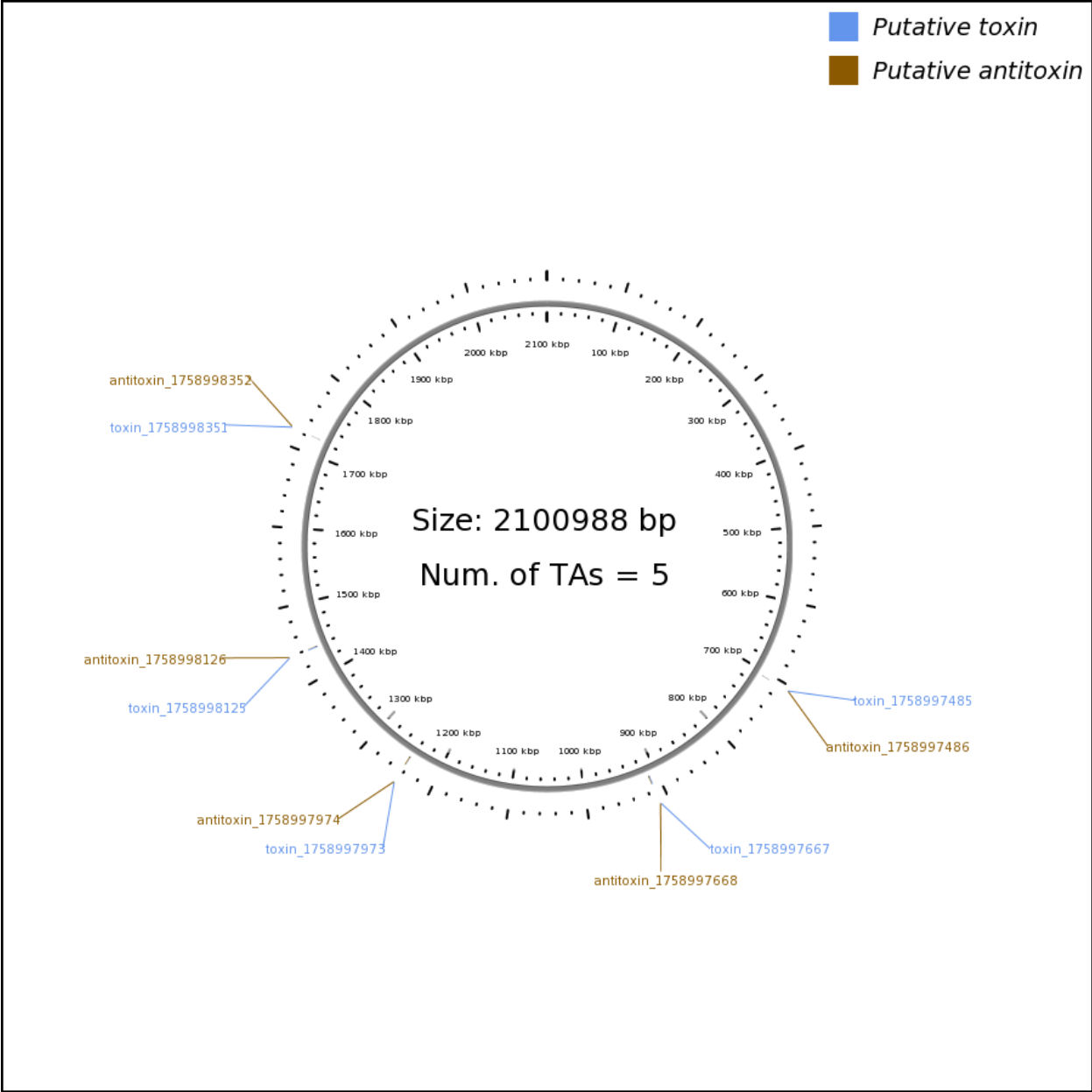


Figure 3-23: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus salivarius* bacteria in the oral microbiome.

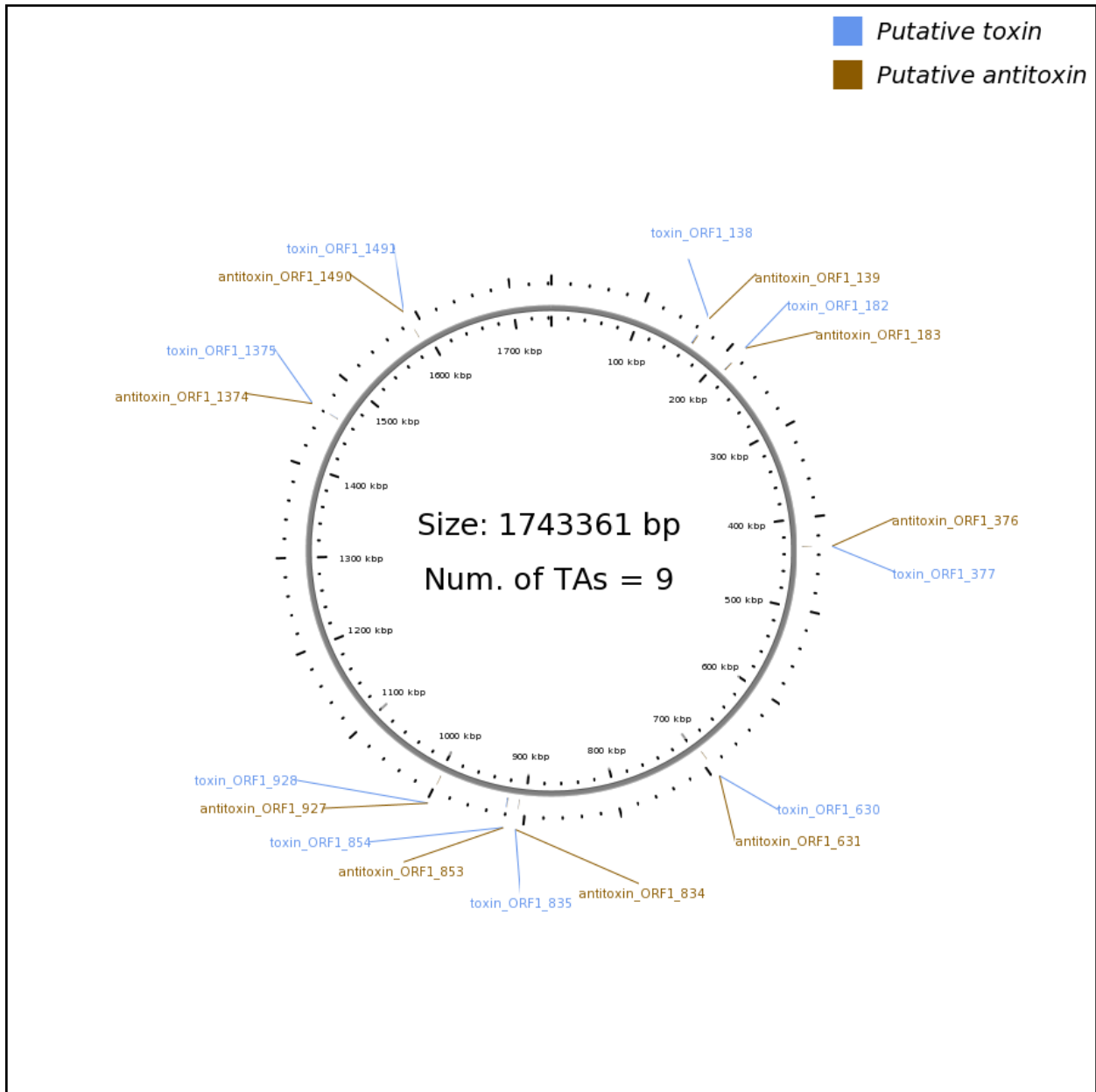


Figure 3-24: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Granulicatella elegans* bacteria in the oral microbiome.

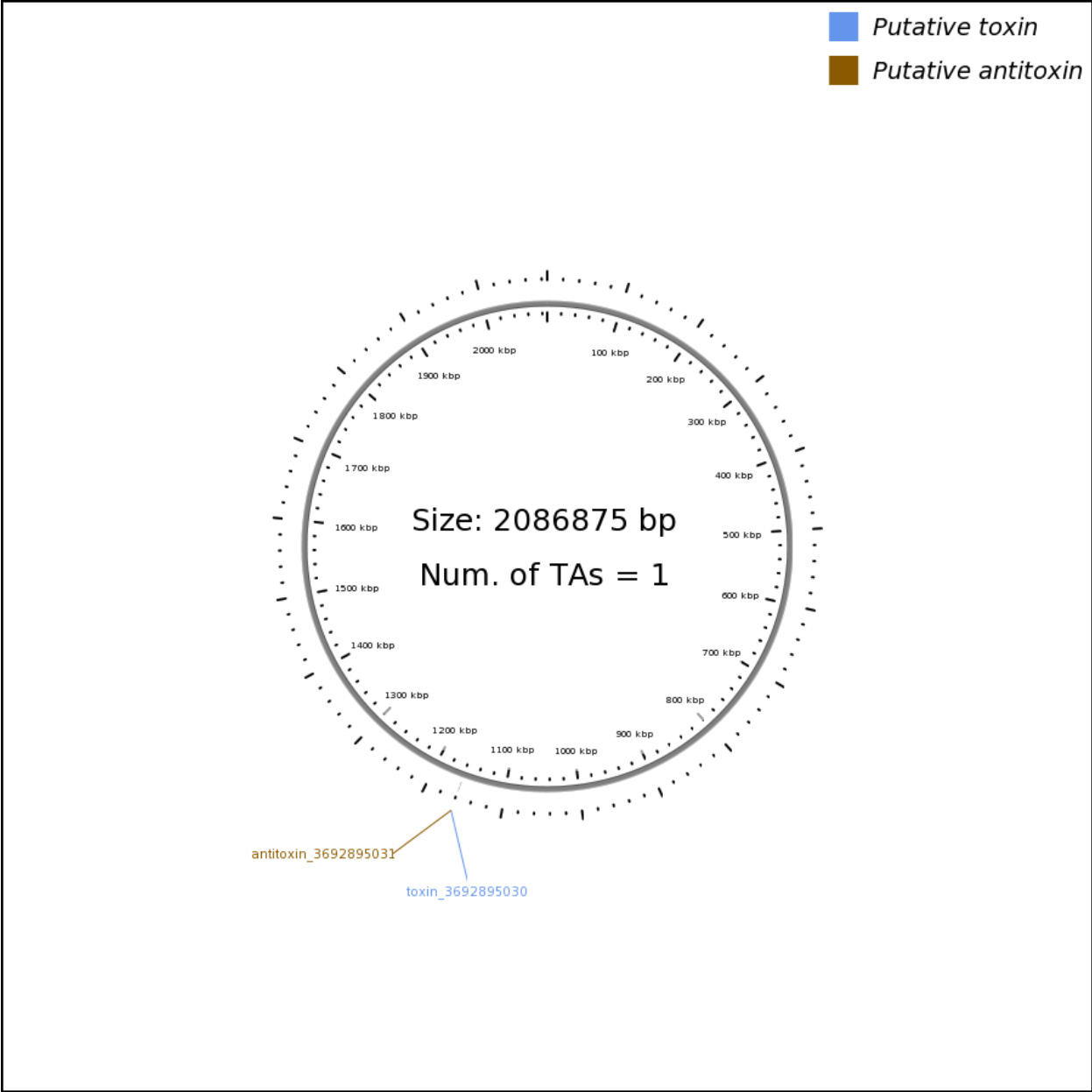


Figure 3-25: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus parainfluenzae* bacteria in the oral microbiome.



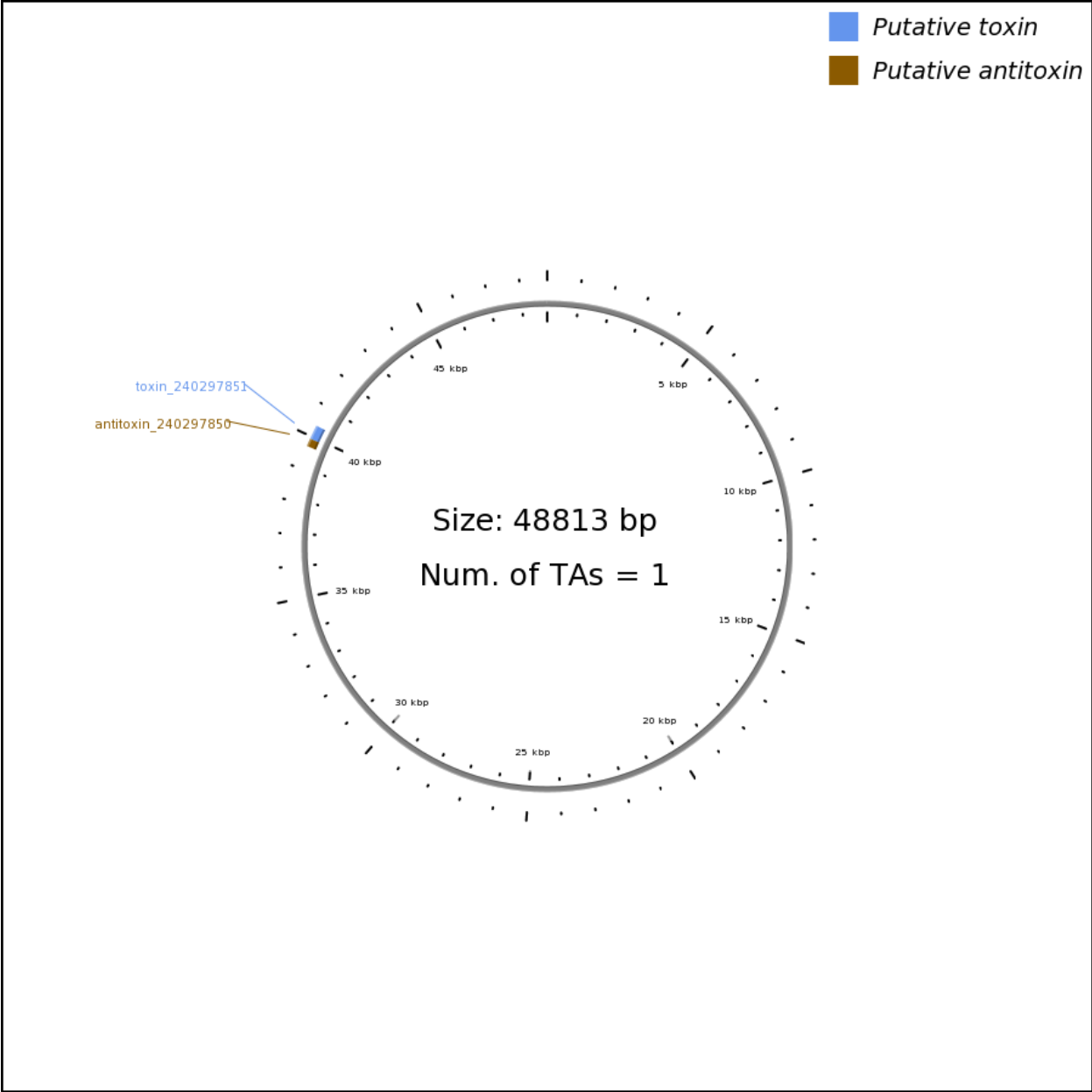


Figure 3-26 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Actinobacillus minor* bacteria in the oral microbiome.

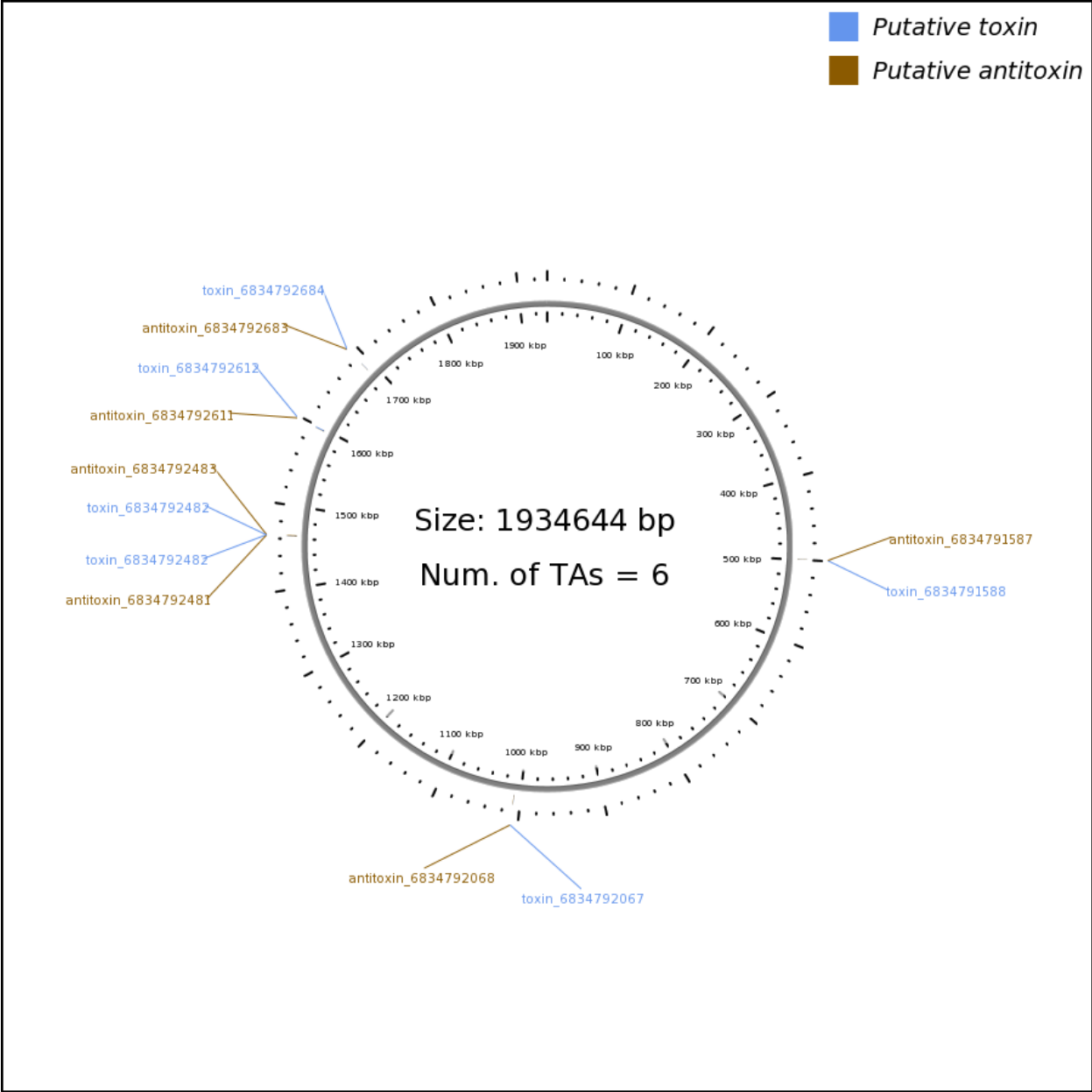


Figure 3-27: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus haemolyticus* bacteria in the oral microbiome.

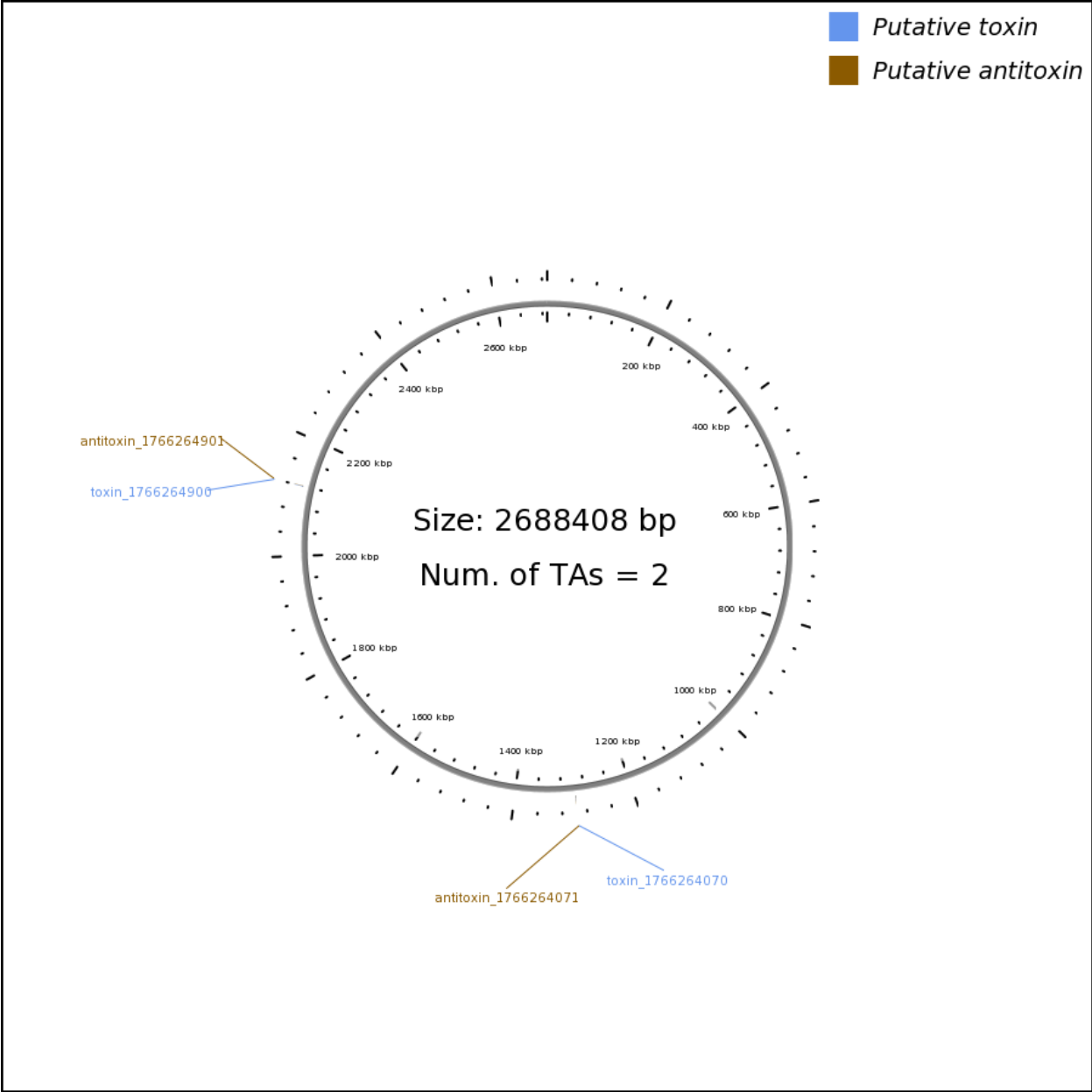


Figure 3-28: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Neisseria mucosa* bacteria in the oral microbiome.

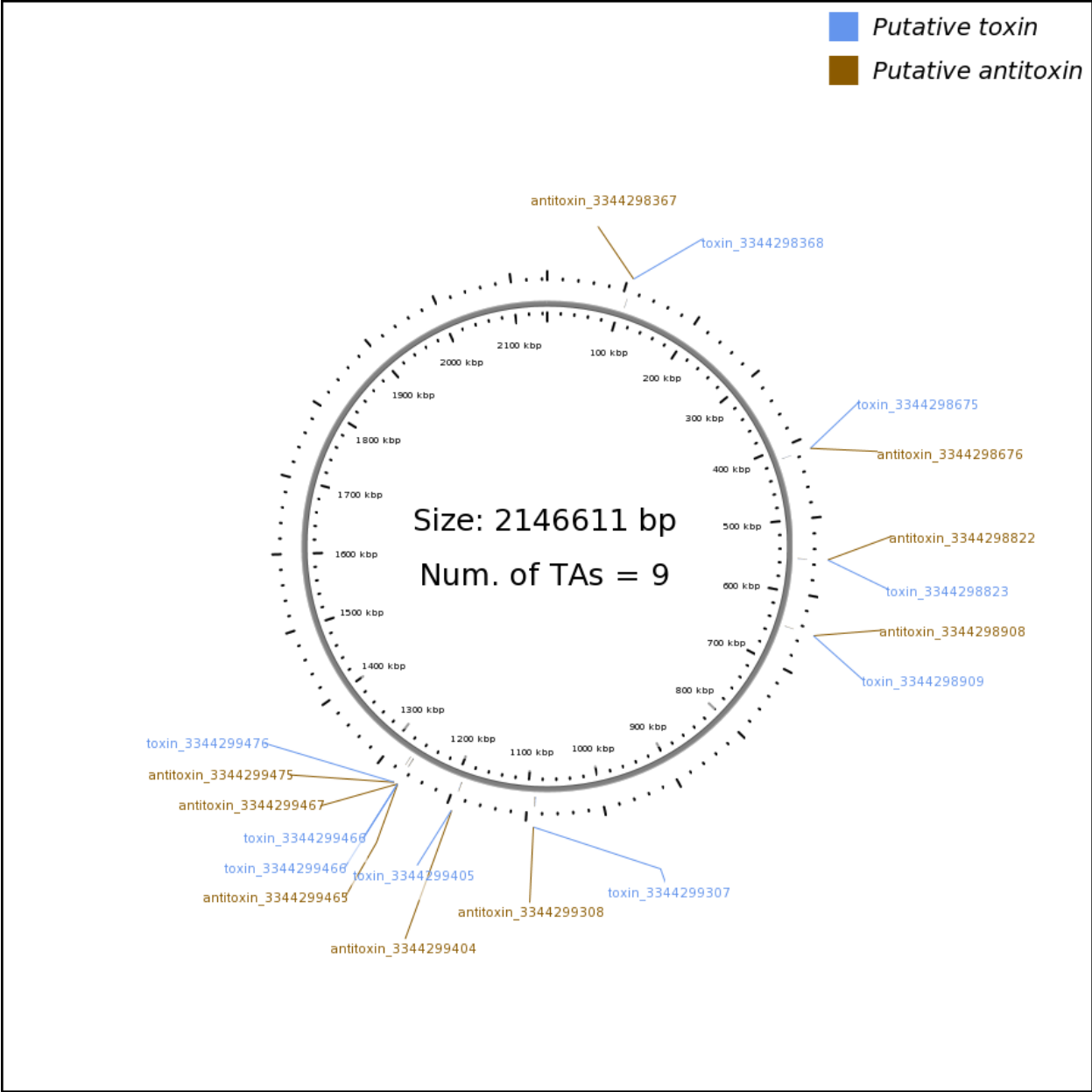


Figure 3-29: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus mitis* bacteria in the oral microbiome.

### 3.3. Toxin Antitoxin Systems Diversity, Abundance and Interaction Network

The abundance of the chromosomal type II toxin antitoxin systems is important to study. (Harms, Brodersen, Mitarai, & Gerdes, 2018c) Their unique character of altruistic selfishness has impact on the TAS distribution and abundance. (Melderer & Bast, 2009; Ramisetty & Santhosh, 2017) These become imperative as the TAS alters the plasticity of the genome and would affect it depending on the host environment and immune response. The abundance of the taxa used from the oral microbiome for the toxin antitoxin systems analysis is visualized from Figure 3-30 to Figure 3-33.

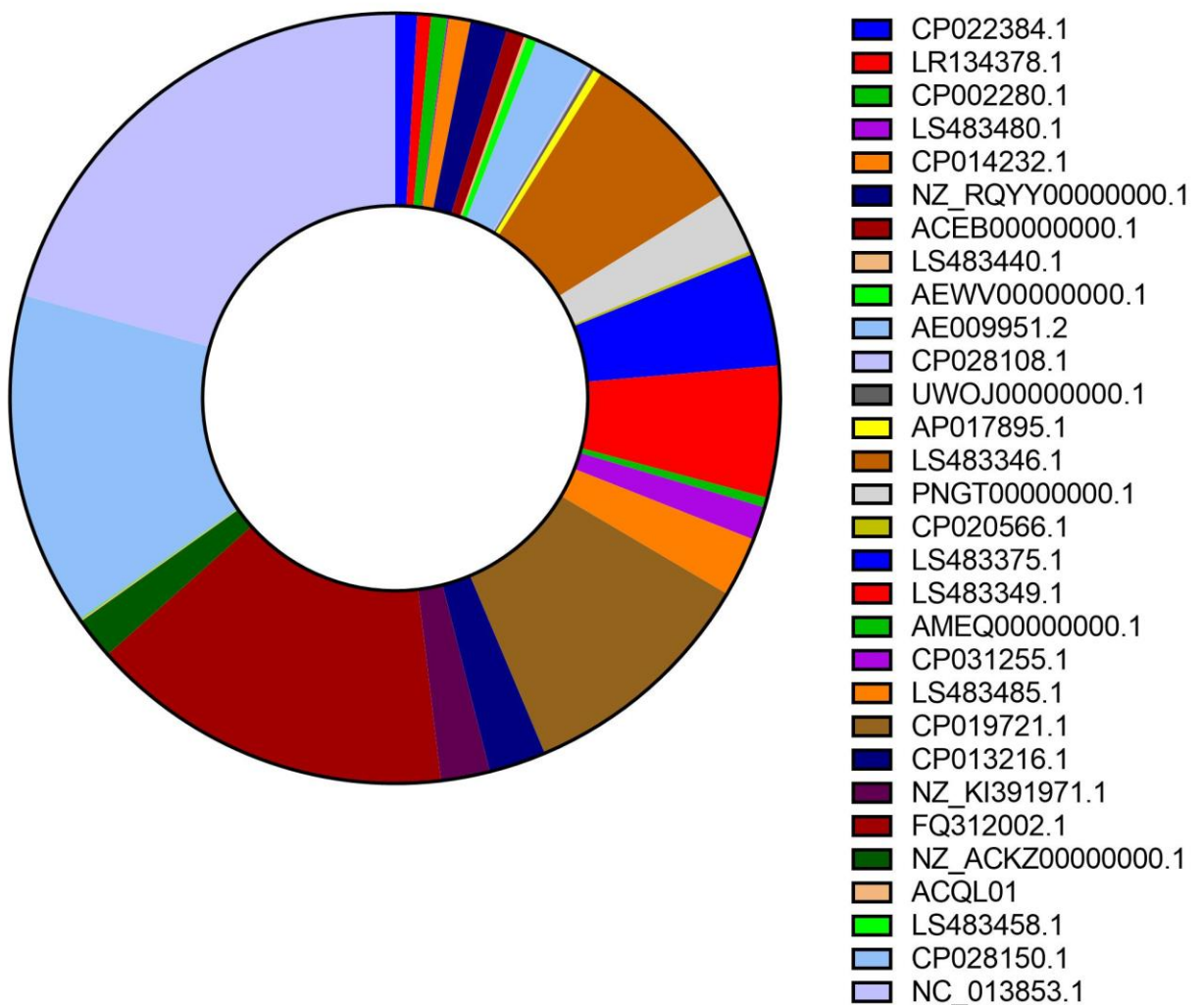


Figure 3-30: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 1. The total of the abundance of the taxa in this figure represents 81.854% of the total microbiome in sample 1.

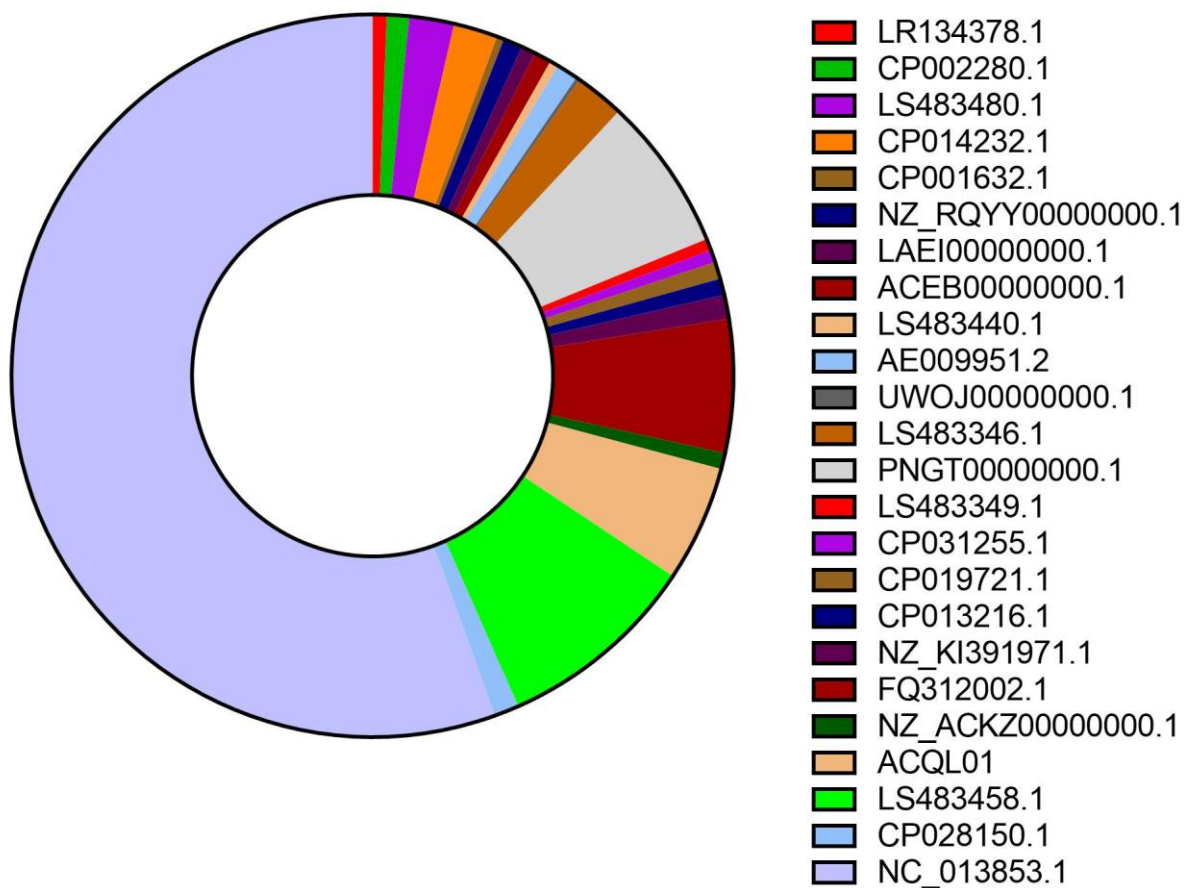


Figure 3-31: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 2. The total of the abundance of the taxa in this figure represents 89.7507% of the total microbiome in sample 2.

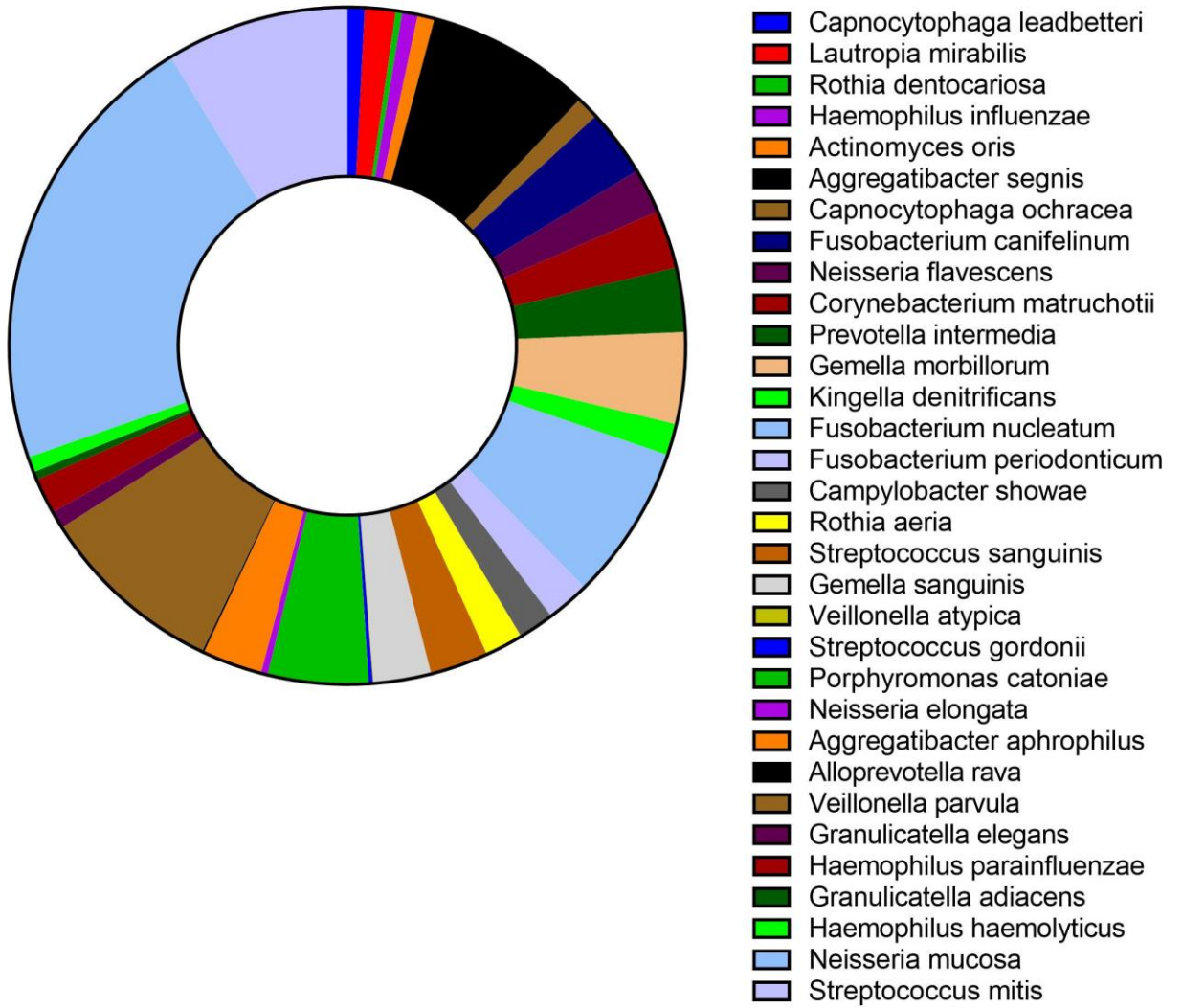


Figure 3-32: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 3. The total of the abundance of the taxa in this figure represents 61.1886% of the total microbiome in sample 3.

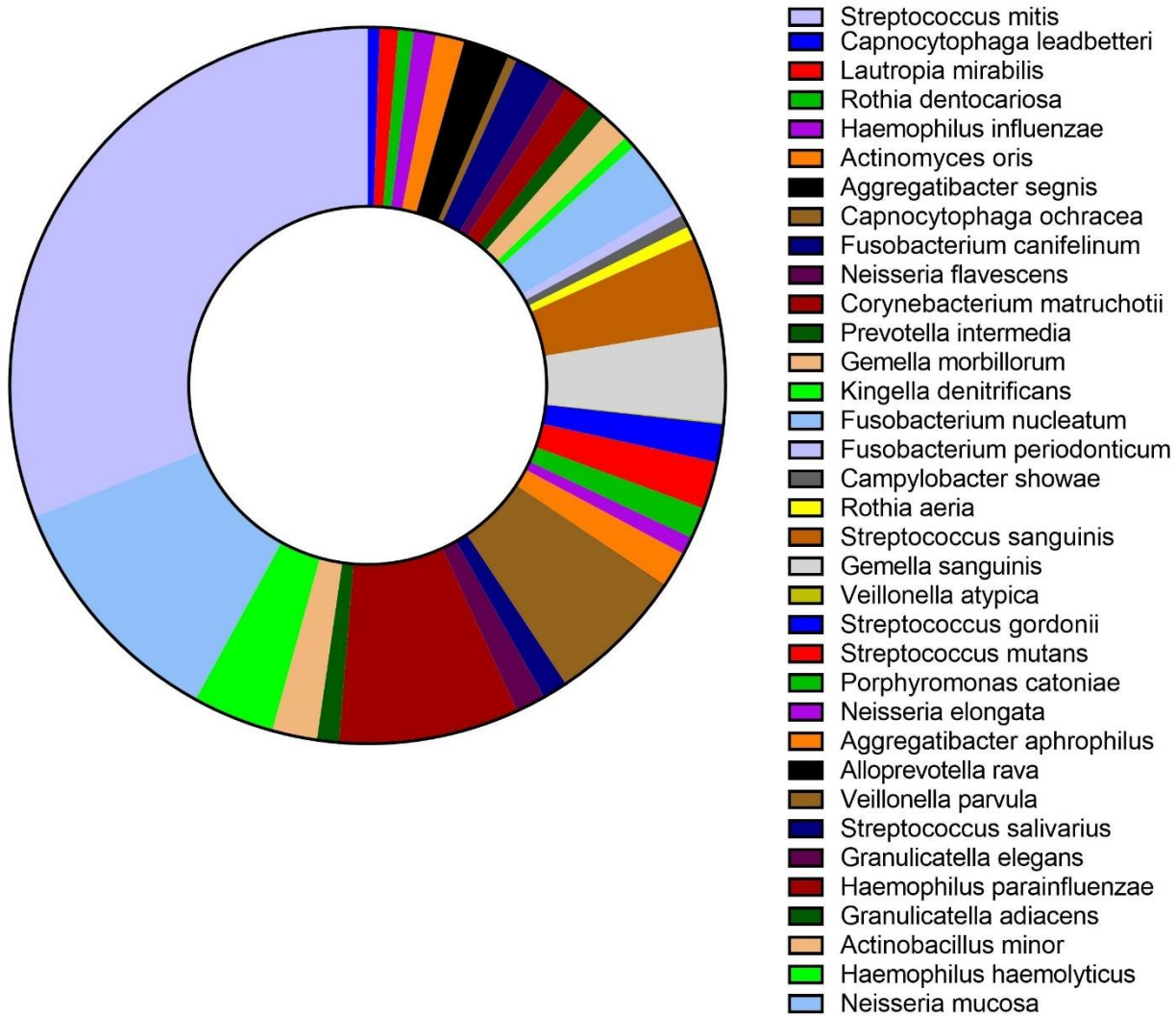


Figure 3-33: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis. The total of the abundance of the taxa in this figure represents 77.5978% of the total microbiome.



To calculate the abundance of the TAS in the oral microbiome, it has to be corrected for the abundance of the taxa in the microbiome. This is calculated by multiplying the TAS proportion by the OTUs abundance in the oral microbiome samples as shown in Table 3-3 and graphed in figures from Figure 3-37 to Figure 3-40. Another abundance study for the TAS genes is to account for the genome size and number of coding sequences (CDS) per taxa. This molecular abundance study treats the TAS as a molecular system as a unit of interest. This calculation has been applied, previously in many research studies, for prophages abundance calculations as molecular entity as compared to the context of the coding genes they are infecting (Nadeem & Wahl, 2017). These data are tabulated and visualized in Table 3-4 and from Figure 3-41 till Figure 3-44.

The frequency of the chromosomal type II Toxin Antitoxin Systems in the human oral microbiome is graphed from Figure 3-34 to Figure 3-36. It shows the highest frequency to the HTH-domain containing genes followed by RelE-like toxins. The RelE toxins are the most frequent type II TAS in the chromosomes in the database TADB (Xie et al., 2018a) However, the highly abundant HTH-domain containing proteins we observed in this project could have this higher frequency because they are a collection of different proteins that cannot be segmented through the domain and/or structural analysis applied.

A heatmap that is constructed based on the pairwise distance between frequencies of each Toxin Antitoxin family predicted in each Operational Taxonomic Unit of the taxa used in the analysis from the human oral microbiome (Figure 3-45). Another interesting tool in visualization of the toxin antitoxin systems frequencies in the oral microbiome is the Circos visualization tool (Figure 3-46). A directed Protein-Protein Interaction Network is constructed by CytoScape based on the degree centrality of the Toxin Antitoxin Systems interactions (Figure 3-47). The edges thickness corresponds to the frequencies of the interaction. The closeness and degree centralities for this network are charted in Table 3-5. The molecular diversity of the type II toxin antitoxin systems predicted from the oral microbiome is recorded in Table 3-6.

Table 3-3: denotes the count and corrected abundance of the Toxin Antitoxin Systems as described in the text.

<b>Operational Taxonomic Unit</b>	<b>Accession Assembly Number</b>	<b>or TAS Count</b>	<b>TAS %</b>	<b>Corrected Abundance of TAS in Oral 1</b>	<b>Corrected Abundance of TAS in Oral 2</b>	<b>Corrected Abundance of TAS in Oral 3</b>
<i>Capnocytophaga leadbetteri</i>	CP022384.1	4	2.877697842	0.000215926	0	0.000145064
<i>Lautropia mirabilis</i>	LR134378.1	0	0	0	0	0
<i>Rothia dentocariosa</i>	CP002280.1	8	5.755395683	0.000320858	0.000513028	0.000108798
<i>Haemophilus influenzae</i>	LS483480.1	11	7.913669065	3.8475E-05	0.001426798	0.000359967
<i>Actinomyces oris</i>	CP014232.1	16	11.51079137	0.000854378	0.002036623	0.000568921
<i>Aggregatibacter segnis</i>	LS483443.1	3	2.158273381	0	0	0.001041228
<i>Capnocytophaga ochracea</i>	CP001632.1	7	5.035971223	0	0.00014568	0.000337159
<i>Fusobacterium canifelinum</i>	NZ_RQYY00000000.1	0	0	0	0	0
<i>Neisseria flavescens</i>	LAEI00000000.1	0	0	0	0	0

<b>Operational Taxonomic Unit</b>	<b>Accession Assembly Number</b>	<b>or TAS Count</b>	<b>TAS %</b>	<b>Corrected Abundance of TAS in Oral 1</b>	<b>Corrected Abundance of TAS in Oral 2</b>	<b>Corrected Abundance of TAS in Oral 3</b>
<i>Corynebacterium matruchotii</i>	ACEB00000000.1	0	0	0	0	0
<i>Prevotella intermedia</i>	CP030094.1	0	0	0	0	0
<i>Gemella morbillorum</i>	LS483440.1	1	0.71942446	8.16137E-06	2.54094E-05	0.000192379
<i>Kingella denitrificans</i>	AEWV00000000.1	0	0	0	0	0
<i>Fusobacterium nucleatum</i>	AE009951.2	9	6.474820144	0.001277021	0.000559733	0.002936262
<i>Fusobacterium periodonticum</i>	CP028108.1	12	8.633093525	8.11473E-05	0	0.001093076
<i>Campylobacter showae</i>	UWOJ00000000.1	0	0	0	0	0
<i>Rothia aeria</i>	AP017895.1	6	4.316546763	0.000116824	0	0.00048024
<i>Streptococcus sanguinis</i>	LS483346.1	5	3.597122302	0.002098637	0.000715093	0.000611278

<b>Operational Taxonomic Unit</b>	<b>Accession Assembly Number</b>	<b>or TAS Count</b>	<b>TAS %</b>	<b>Corrected Abundance of TAS in Oral 1</b>	<b>Corrected Abundance of TAS in Oral 2</b>	<b>Corrected Abundance of TAS in Oral 3</b>
<i>Gemella sanguinis</i>	PNGT000000000.1	0	0	0	0	0
<i>Veillonella atypica</i>	CP020566.1	7	5.035971223	6.61071E-05	0	1.18997E-05
<i>Streptococcus gordonii</i>	LS483375.1	4	2.877697842	0.001116009	0	3.39993E-05
<i>Streptococcus mutans</i>	LS483349.1	7	5.035971223	0.002277021	0.00023546	0
<i>Porphyromonas catoniae</i>	AMEQ000000000.1	0	0	0	0	0
<i>Neisseria elongata</i>	CP031255.1	4	2.877697842	0.000331118	0.000144229	5.60988E-05
<i>Aggregatibacter aphrophilus</i>	LS483485.1	2	1.438848921	0.000294276	0	0.000251878
<i>Alloprevotella rava</i>	ACZK000000000.1	0	0	0	0	0
<i>Veillonella parvula</i>	CP019721.1	0	0	0	0	0

<b>Operational Taxonomic Unit</b>	<b>Accession Assembly Number</b>	<b>or TAS Count</b>	<b>TAS %</b>	<b>Corrected Abundance of TAS in Oral 1</b>	<b>Corrected Abundance of TAS in Oral 2</b>	<b>Corrected Abundance of TAS in Oral 3</b>
<i>Streptococcus salivarius</i>	CP013216.1	5	3.597122302	0.000704792	0.000237154	0
<i>Granulicatella elegans</i>	NZ_KI391971.1	9	6.474820144	0.001091291	0.000622893	0.000332768
<i>Haemophilus parainfluenzae</i>	FQ312002.1	1	0.71942446	0.000901481	0.000385255	7.47984E-05
<i>Granulicatella adiacens</i>	NZ_ACKZ000000000.1	0	0	0	0	0
<i>Actinobacillus minor</i>	ACQL01	1	0.71942446	4.54705E-06	0.000335888	0
<i>Haemophilus haemolyticus</i>	LS483458.1	6	4.316546763	1.18923E-05	0.00349488	0.000194646
<i>Neisseria mucosa</i>	CP028150.1	2	1.438848921	0.001651627	0.000143261	0.001901409
<i>Streptococcus mitis</i>	NC_013853.1	9	6.474820144	0.010968527	0.032255403	0.00346665
<b>Total</b>	Total	139	100	0.024430118	0.043276785	0.014198518

Table 3-4: denotes the molecular abundance of the Toxin Antitoxin Systems in relation to the gene counts.

<b>Operational Taxonomic Units</b>	<b>Accession Assembly Number</b>	<b>or Genome Size</b>	<b>Gene Count</b>	<b>Molecular Abundance of TAS in Oral 1</b>	<b>Molecular Abundance of TAS in Oral 2</b>	<b>Molecular Abundance of TAS in Oral 3</b>	<b>Molecular Abundance of TAS in the Oral Microbiome</b>
<i>Capnocytophaga leadbetteri</i>	CP022384.1	2,504,023	2,253	1.33217E-05	0	8.94977E-06	0.001775411
<i>Lautropia mirabilis</i>	LR134378.1	3,172,010	2,595	0	0	0	0
<i>Rothia dentocariosa</i>	CP002280.1	2,492,820	2,217	2.0117E-05	3.21655E-05	6.82132E-06	0.00360848
<i>Haemophilus influenzae</i>	LS483480.1	1,846,600	1,863	2.87065E-06	0.000106455	2.68575E-05	0.005904455
<i>Actinomyces oris</i>	CP014232.1	3,104,690	2,459	4.82955E-05	0.000115124	3.21594E-05	0.00650671
<i>Aggregatibacter segnis</i>	LS483443.1	1,965,660	1,896	0	0	7.63347E-05	0.001582278
<i>Capnocytophaga ochracea</i>	CP001632.1	2,658,650	2,171	0	9.32731E-06	2.15869E-05	0.003224321
<i>Fusobacterium canifelinum</i>	NZ_RQYY000000000.1	2,224,460	2,204	0	0	0	0

<b>Operational Taxonomic Units</b>	<b>Accession Assembly Number</b>	<b>or Genome Size</b>	<b>Gene Count</b>	<b>Molecular Abundance of TAS in Oral 1</b>	<b>Molecular Abundance of TAS in Oral 2</b>	<b>Molecular Abundance of TAS in Oral 3</b>	<b>Molecular Abundance of TAS in the Oral Microbiome</b>
<i>Neisseria flavescens</i>	LAEI000000000.1	2,291,790	1,994	0	0	0	0
<i>Corynebacterium matruchotii</i>	ACEB000000000.1	2,867,410	3,144	0	0	0	0
<i>Prevotella intermedia</i>	CP030094.1	2,777,800	1,762	0	0	0	0
<i>Gemella morbillorum</i>	LS483440.1	1,756,930	1,656	6.85042E-07	2.13279E-06	1.61478E-05	0.000603865
<i>Kingella denitrificans</i>	AEWV000000000.1	2,220,450	2,522	0	0	0	0
<i>Fusobacterium nucleatum</i>	AE009951.2	2,408,530	2,067	8.58761E-05	3.76405E-05	0.000197455	0.004354136
<i>Fusobacterium periodonticum</i>	CP028108.1	2,477,380	2,540	4.44074E-06	0	5.9818E-05	0.004724409
<i>Campylobacter showae</i>	UWOJ000000000.1	2,213,800	2,392	0	0	0	0
<i>Rothia aeria</i>	AP017895.1	2,603,190	2,799	5.80156E-06	0	2.3849E-05	0.002143623

<b>Operational Taxonomic Units</b>	<b>Accession Assembly Number</b>	<b>or Genome Size</b>	<b>Gene Count</b>	<b>Molecular Abundance of TAS in Oral 1</b>	<b>Molecular Abundance of TAS in Oral 2</b>	<b>Molecular Abundance of TAS in Oral 3</b>	<b>Molecular Abundance of TAS in the Oral Microbiome</b>
<i>Streptococcus sanguinis</i>	LS483346.1	2,361,220	2,314	0.000126063	4.2955E-05	3.6719E-05	0.002160761
<i>Gemella sanguinis</i>	PNGT00000000.1	1,836,490	1,893	0	0	0	0
<i>Veillonella atypica</i>	CP020566.1	2,071,950	1,887	4.86957E-06	0	8.76558E-07	0.003709592
<i>Streptococcus gordonii</i>	LS483375.1	2,190,540	2,051	7.56339E-05	0	2.30419E-06	0.001950268
<i>Streptococcus mutans</i>	LS483349.1	1,962,000	1,885	0.000167908	1.73629E-05	0	0.003713528
<i>Porphyromonas catoniae</i>	AMEQ00000000.1	2,070,830	1,853	0	0	0	0
<i>Neisseria elongata</i>	CP031255.1	2,397,850	2,574	1.78809E-05	7.78856E-06	3.02942E-06	0.001554002
<i>Aggregatibacter aphrophilus</i>	LS483485.1	2,332,870	2,097	1.95061E-05	0	1.66958E-05	0.000953743



<b>Operational Taxonomic Units</b>	<b>Accession Assembly Number</b>	<b>or Genome Size</b>	<b>Gene Count</b>	<b>Molecular Abundance of TAS in Oral 1</b>	<b>Molecular Abundance of TAS in Oral 2</b>	<b>Molecular Abundance of TAS in Oral 3</b>	<b>Molecular Abundance of TAS in the Oral Microbiome</b>
<i>Alloprevotella rava</i>	ACZK00000000.1	2,593,870	2,177	0	0	0	0
<i>Veillonella parvula</i>	CP019721.1	2,142,200	2,036	0	0	0	0
<i>Streptococcus salivarius</i>	CP013216.1	2,236,020	1,903	5.14798E-05	1.73224E-05	0	0.00262743
<i>Granulicatella elegans</i>	NZ_KI391971.1	1,743,360	1,629	9.31182E-05	5.31505E-05	2.83946E-05	0.005524862
<i>Haemophilus parainfluenzae</i>	FQ312002.1	2,074,140	1,993	6.2873E-05	2.68692E-05	5.21675E-06	0.000501756
<i>Granulicatella adiacens</i>	NZ_ACKZ00000000.1	1,947,170	1,889	0	0	0	0
<i>Actinobacillus minor</i>	ACQL01	2,276,210	2,411	2.62148E-07	1.93648E-05	0	0.000414766
<i>Haemophilus haemolyticus</i>	LS483458.1	1,914,630	1,774	9.31807E-07	0.000273838	1.52513E-05	0.003382187

<b>Operational Taxonomic Units</b>	<b>Accession Assembly Number</b>	<b>or Genome Size</b>	<b>Gene Count</b>	<b>Molecular Abundance of TAS in Oral 1</b>	<b>Molecular Abundance of TAS in Oral 2</b>	<b>Molecular Abundance of TAS in Oral 3</b>	<b>Molecular Abundance of TAS in the Oral Microbiome</b>
<i>Neisseria mucosa</i>	CP028150.1	2,492,650	2,734	8.39708E-05	7.28355E-06	9.667E-05	0.000731529
<i>Streptococcus mitis</i>	NC_013853.1	1,989,190	2,002	0.000761551	0.002239511	0.000240691	0.004495504
<b>Total</b>	Accession or Assembly Number	80,219,383	75,636	0.001647457	0.00300829	0.000915829	0.066147616

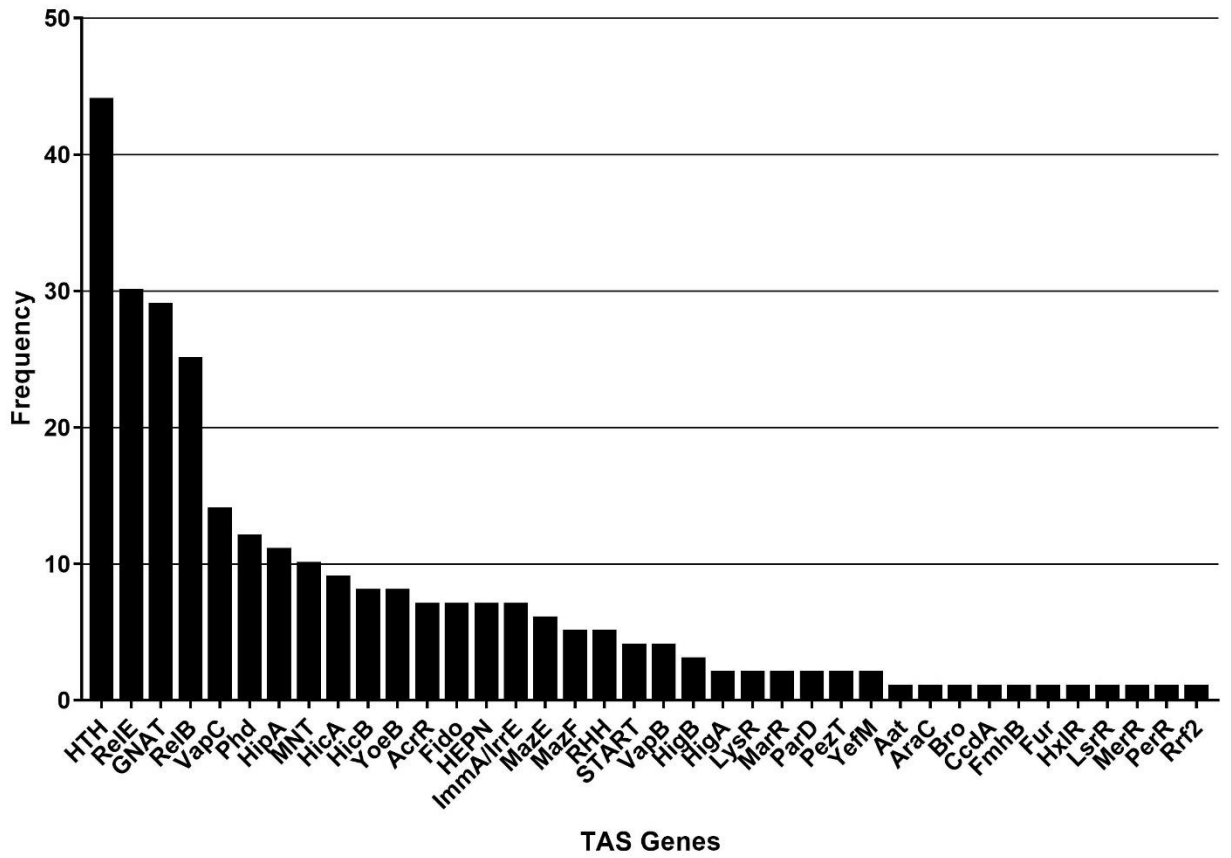


Figure 3-34: represents a histogram to visualize the frequencies of the chromosomal type II toxin antitoxin systems predicted in the human oral microbiome.

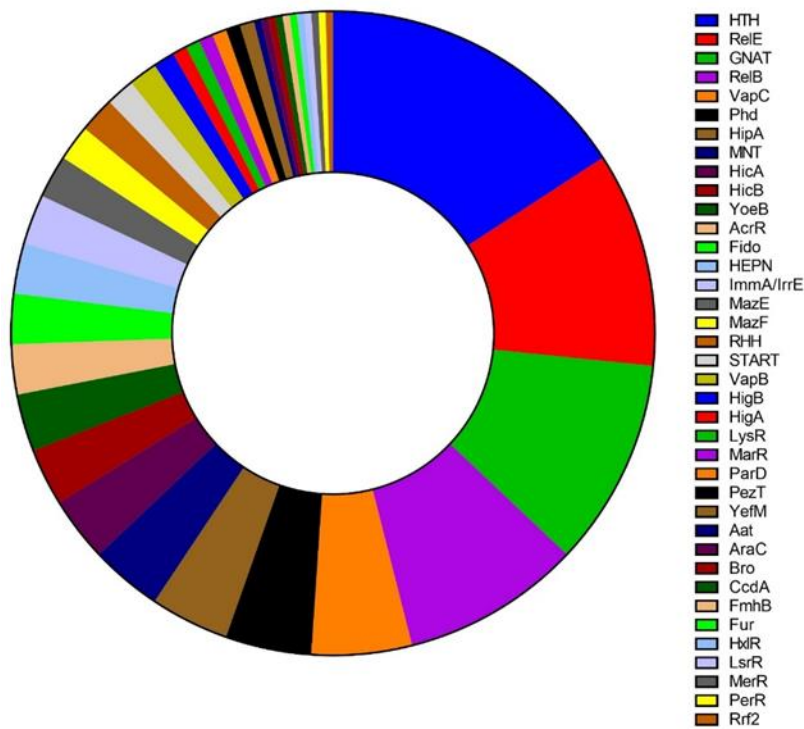


Figure 3-35: A pie chart that visualizes the frequencies of the Toxin Antitoxin Systems families predicted in the human oral microbiome.

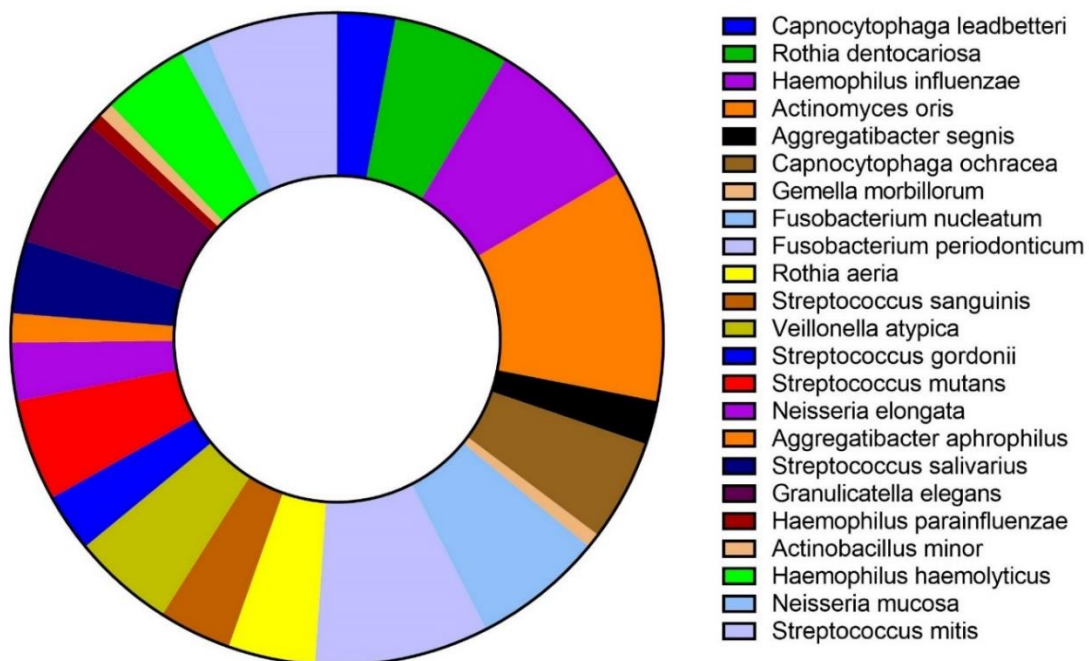


Figure 3-36: A pie chart shows the percentages of each family of the type II Toxin Antitoxin Systems in each of the Operational Taxonomic Units used in this study from the human oral microbiome.

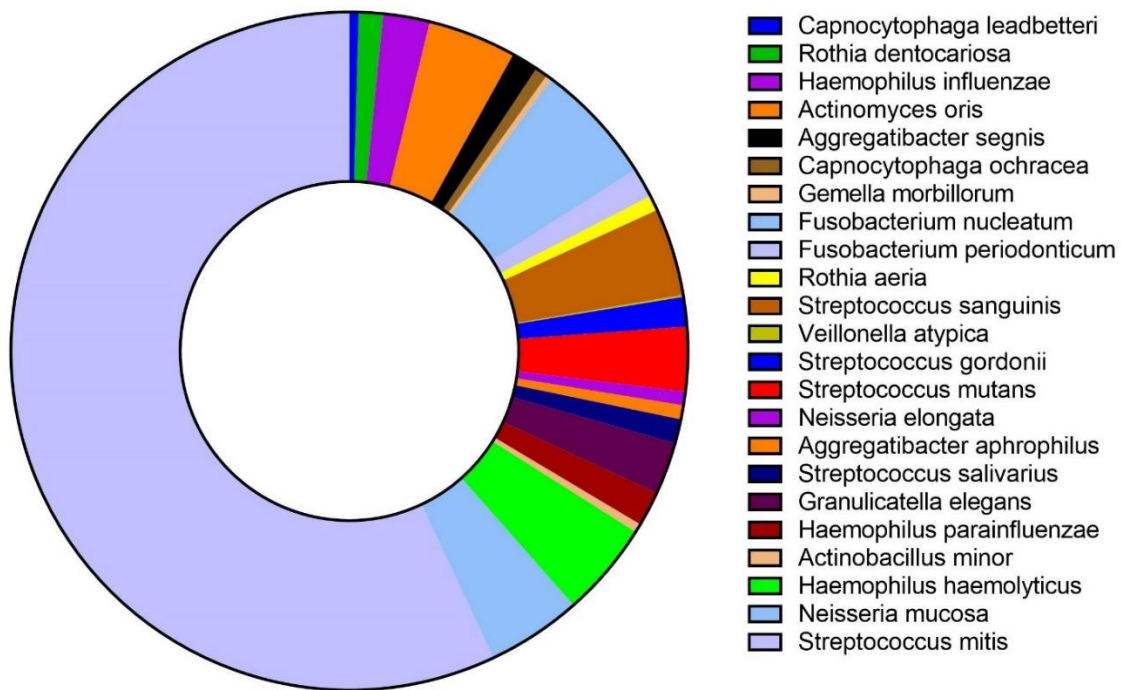


Figure 3-37: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon that are used for the analysis from the human oral microbiome.

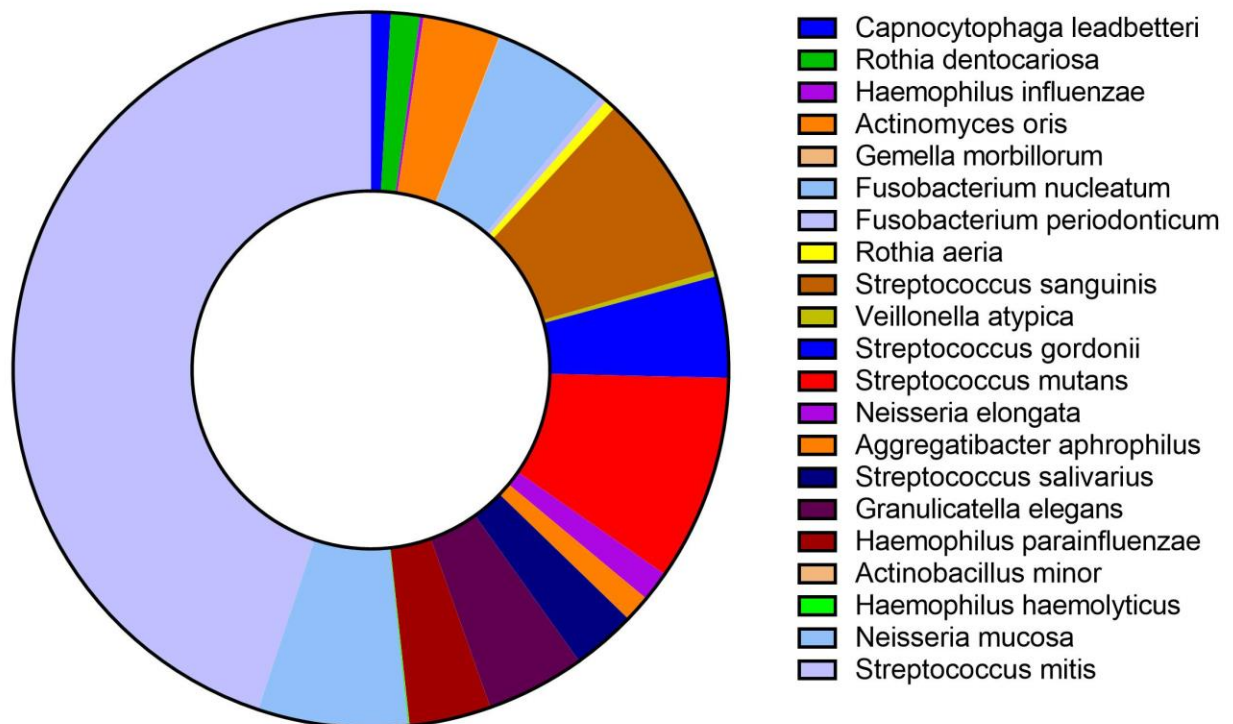


Figure 3-38: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 1 sample that are used for the analysis from the human oral microbiome.

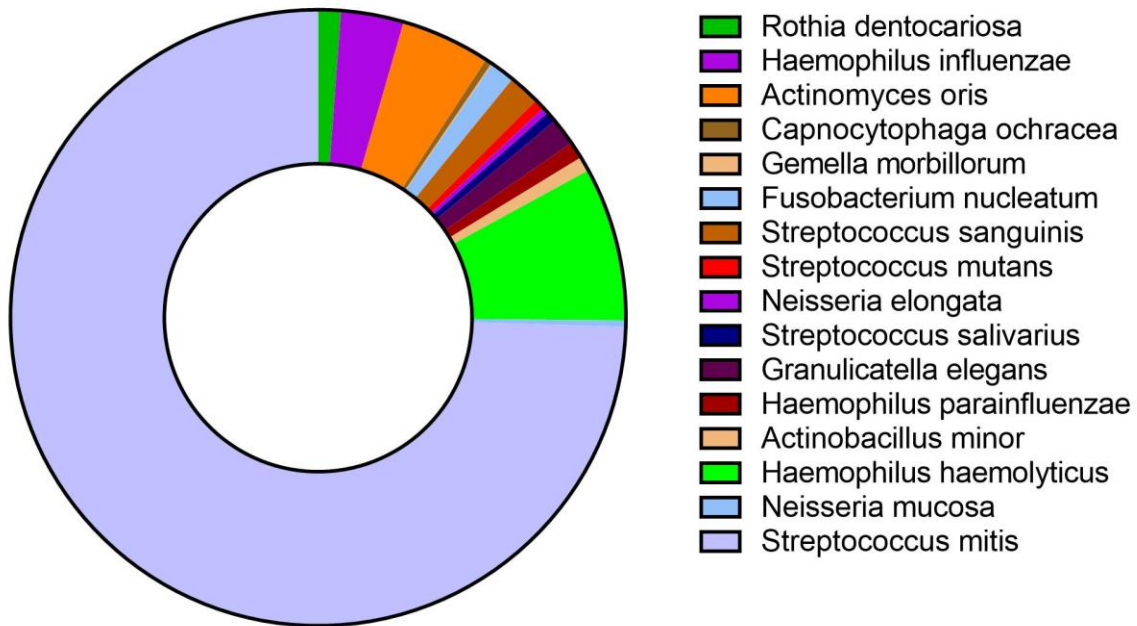


Figure 3-39: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 2 sample that are used for the analysis from the human oral microbiome.

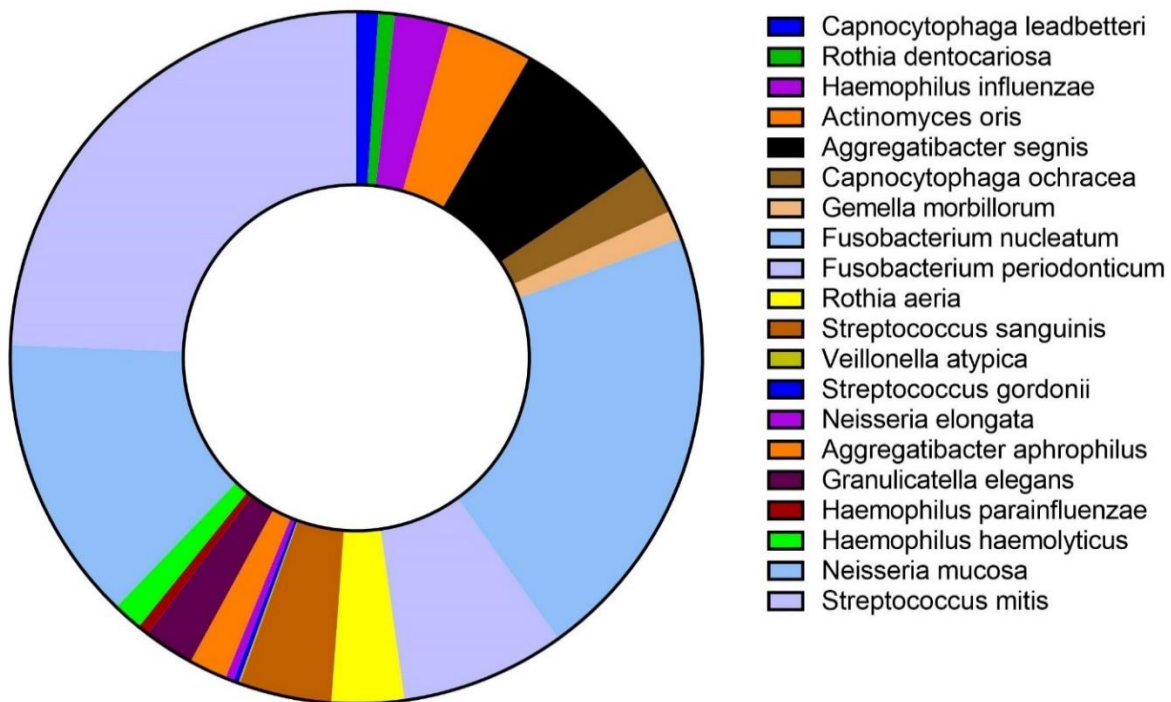


Figure 3-40: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 3 sample that are used for the analysis from the oral microbiome.

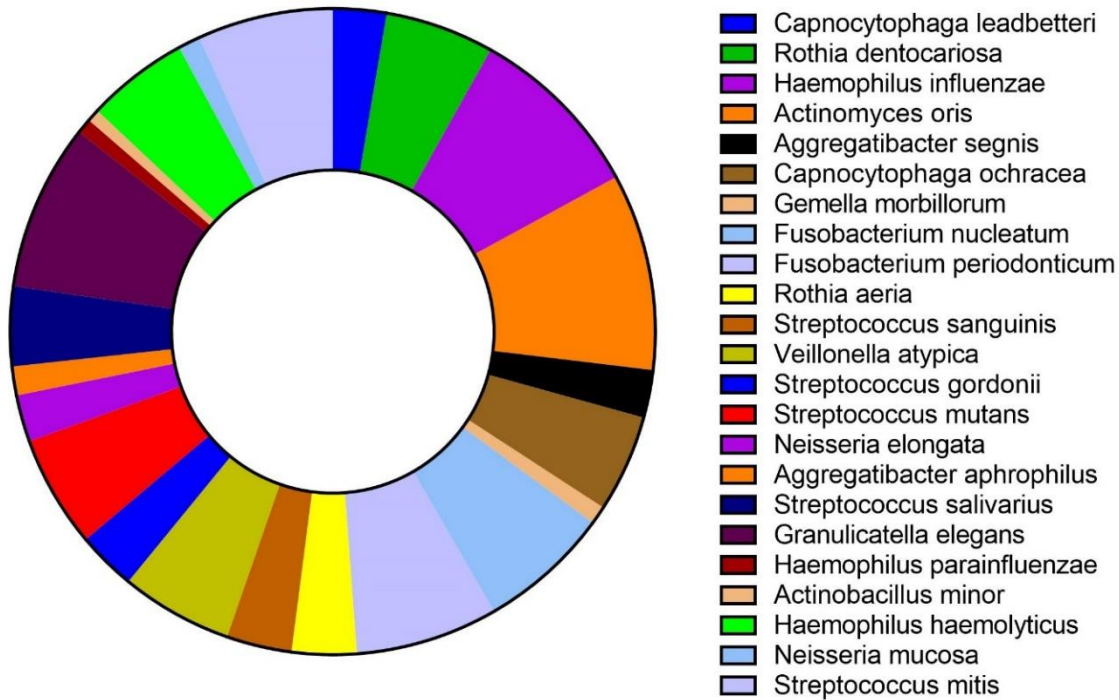


Figure 3-41: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon that are used for the analysis from the human oral microbiome.

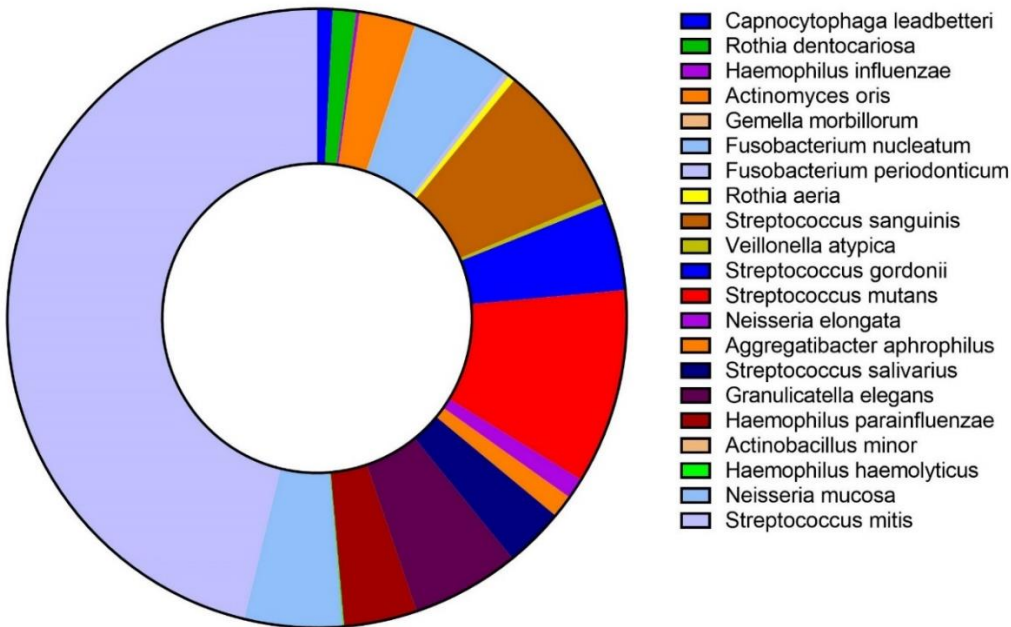


Figure 3-42: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 1 sample that are used for the analysis from the human oral microbiome.

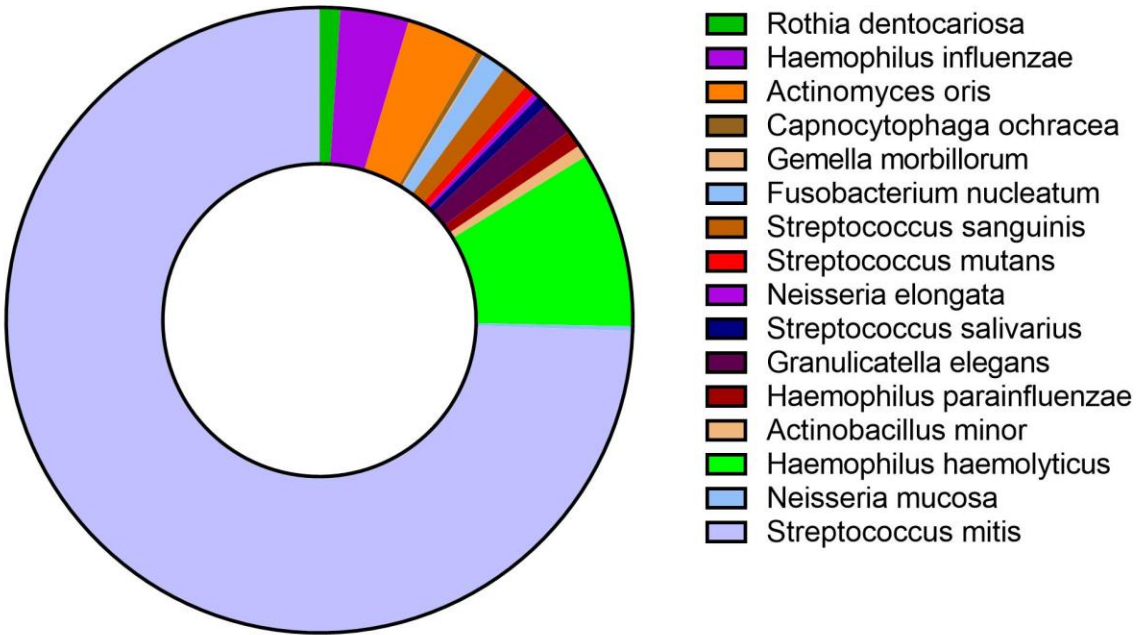


Figure 3-43 A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 2 sample that are used for the analysis from the human oral microbiome.

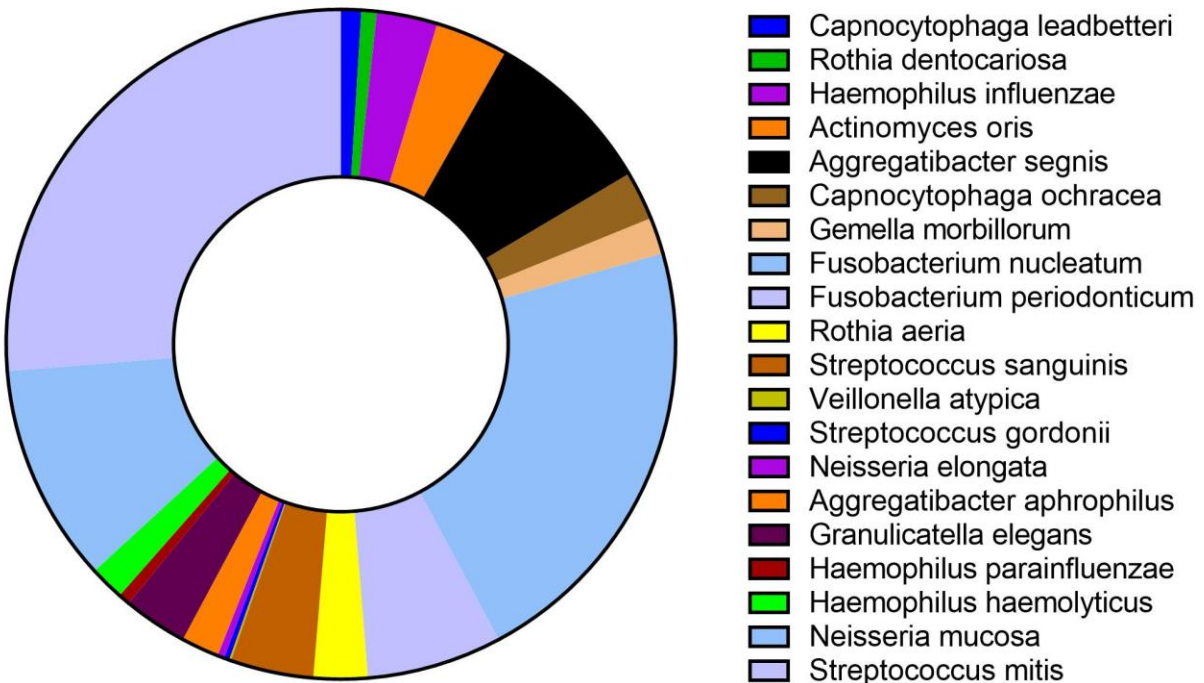


Figure 3-44: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 3 sample that are used for the analysis from the human oral microbiome.



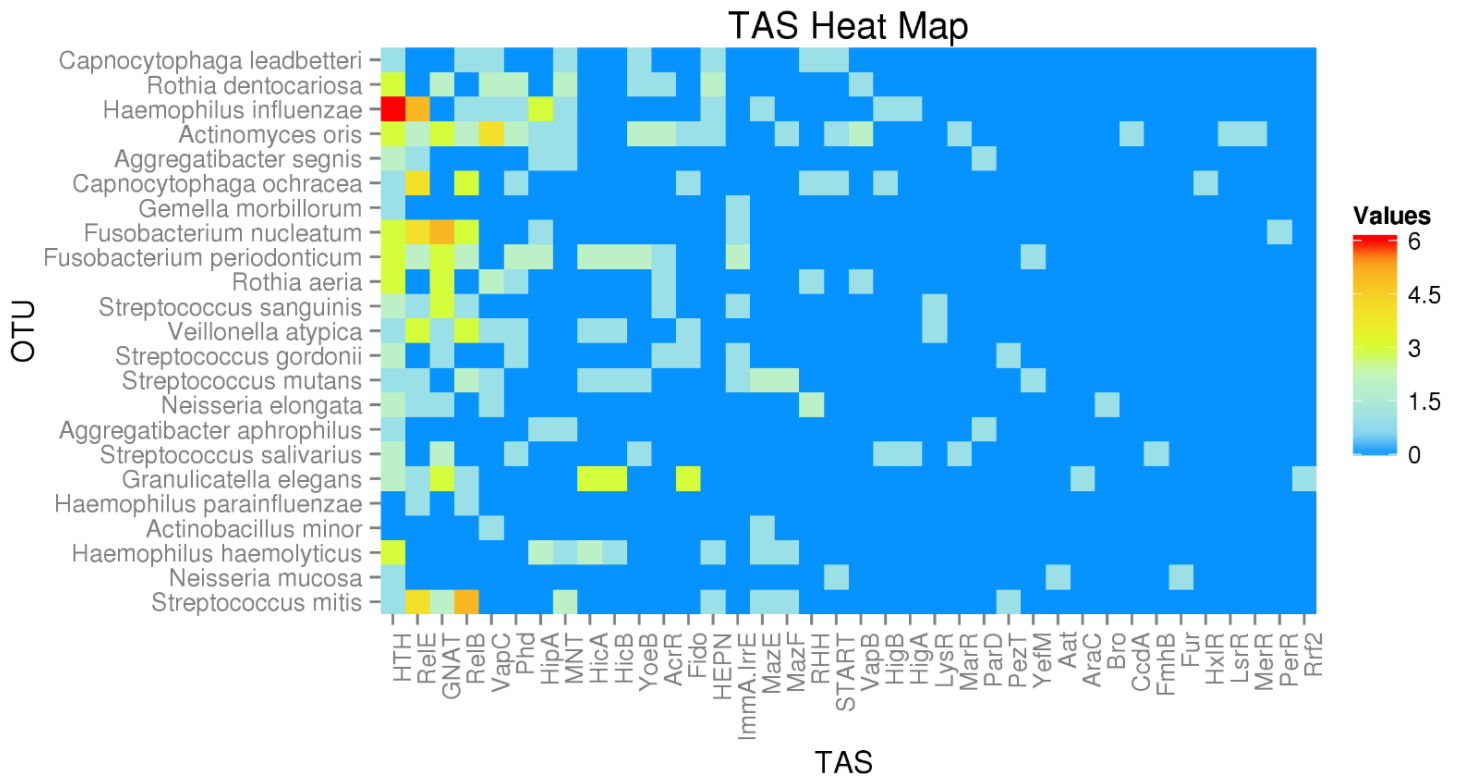


Figure 3-45: A heatmap visualizes the frequencies of the Type II Toxin Antitoxin Systems predicted in each Operational Taxonomic Unit for the taxa used in the analysis for the human oral microbiome.

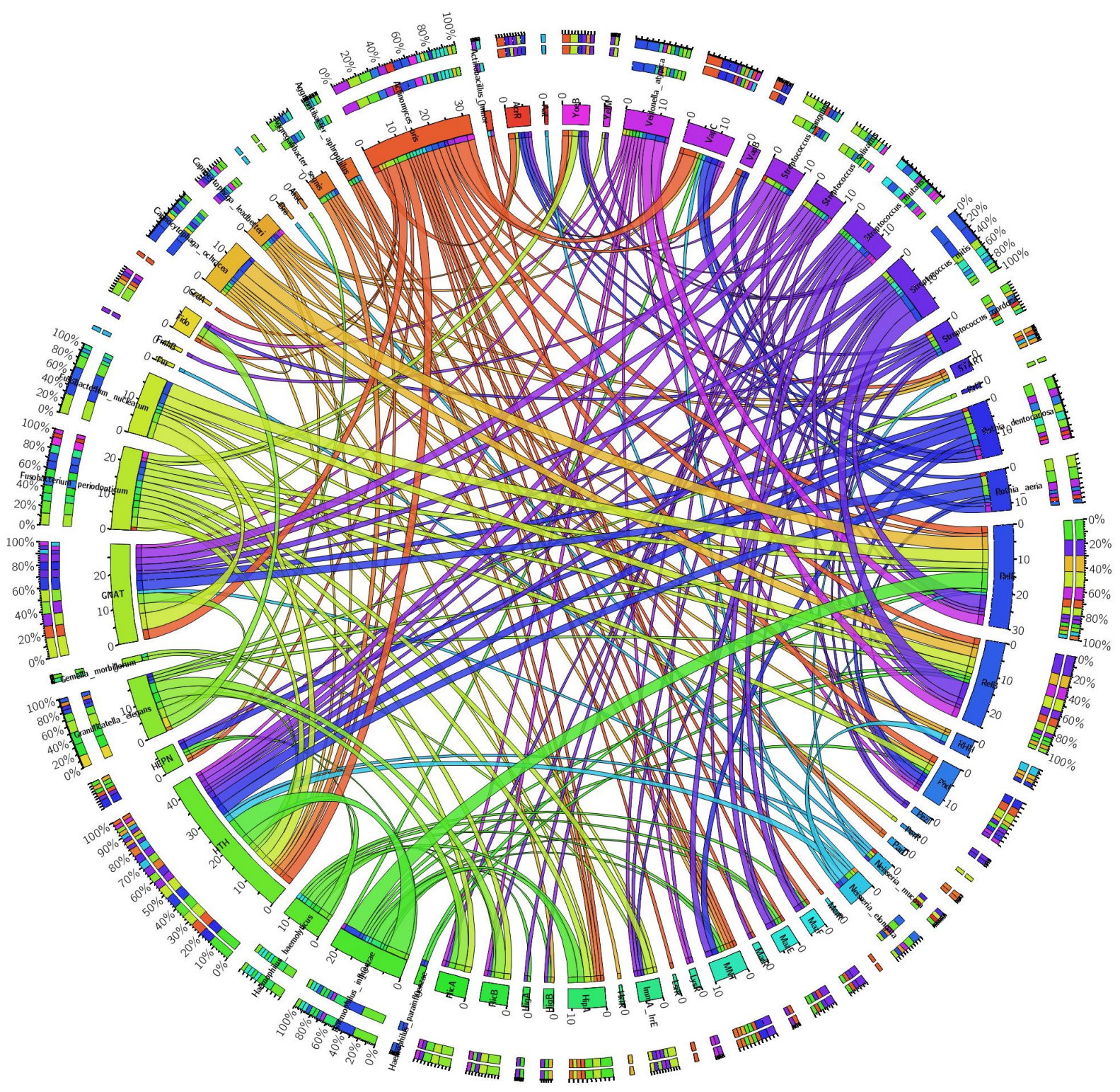


Figure 3-46: A Circos map that visualizes the frequencies of the Type II Toxin Antitoxin Systems predicted in each Operational Taxonomic Unit for the taxa used in the analysis for the human oral microbiome. The bands connect the toxin-antitoxin genes with their respective species. Thickness of the bands represents the frequency.

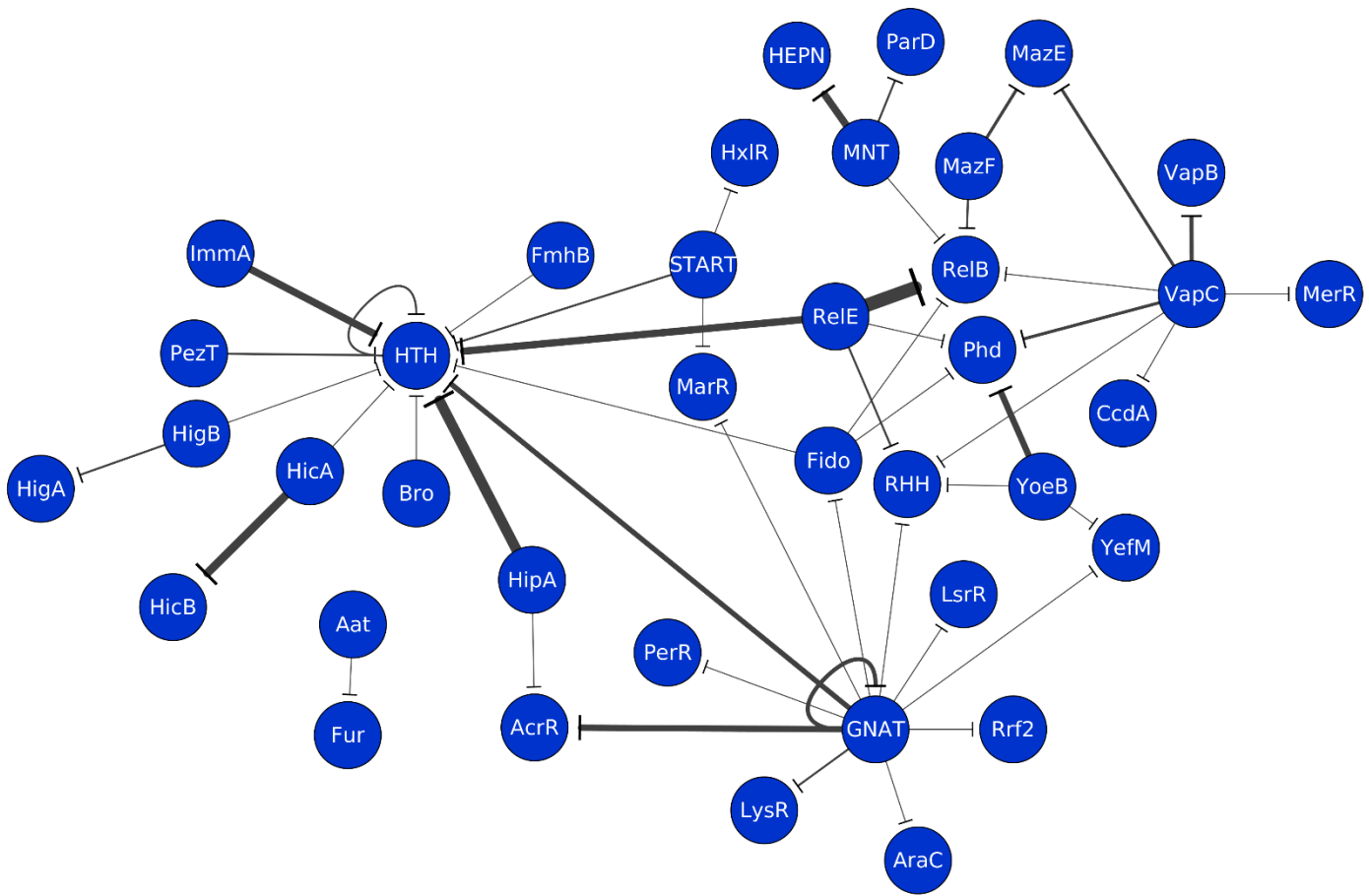


Figure 3-47: A directed protein-protein interaction network visualizes cross talk of the chromosomal Type II Toxin Antitoxin Systems that are predicted in the human oral microbiome. The edges thickness corresponds to the frequencies of the toxin-antitoxin interactions.

Table 3-5: The closeness and degree centralities of the protein-protein interaction network depicted in Figure 3-47.

<b>Toxin Antitoxin System</b>	<b>Degree</b>	<b>Closeness</b>
<b>HTH</b>	44	0.000794
<b>RelE</b>	30	0.000893
<b>GNAT</b>	29	0.001239
<b>RelB</b>	25	0.000794
<b>VapC</b>	14	0.000985
<b>Phd</b>	12	0.000794
<b>HipA</b>	11	0.00084
<b>MNT</b>	10	0.000866
<b>HicA</b>	9	0.00084
<b>HicB</b>	8	0.000794
<b>YoeB</b>	8	0.000866
<b>AcrR</b>	7	0.000794
<b>Fido</b>	7	0.000866
<b>HEPN</b>	7	0.000794
<b>ImmA/IrrE</b>	7	0.000816
<b>MazE</b>	6	0.000794
<b>MazF</b>	5	0.00084
<b>RHH</b>	5	0.000794
<b>START</b>	4	0.000866
<b>VapB</b>	4	0.000794
<b>HigB</b>	3	0.00084
<b>HigA</b>	2	0.000794
<b>LysR</b>	2	0.000794
<b>MarR</b>	2	0.000794
<b>ParD</b>	2	0.000794
<b>PezT</b>	2	0.000816
<b>YefM</b>	2	0.000794
<b>AraC</b>	1	0.000794

<b>Toxin Antitoxin System</b>	<b>Degree</b>	<b>Closeness</b>
<b>Bro</b>	1	0.000816
<b>CcdA</b>	1	0.000794
<b>FmhB</b>	1	0.000816
<b>HxlR</b>	1	0.000794
<b>LsrR</b>	1	0.000794
<b>MerR</b>	1	0.000794
<b>PerR</b>	1	0.000794
<b>Rrf2</b>	1	0.000794

Table 3-6: The molecular diversity of the type II toxin antitoxin systems predicted in the oral microbiome. TAS stands for Toxin Antitoxin Systems. OM stands for Oral Microbiome.

<b>Diversity Index</b>	<b>Berger-Parker index</b>	<b>Margalef index</b>	<b>Simpson index <math>\lambda</math></b>	<b>Simpson index D</b>	<b>Simpson index Dr</b>	<b>Shannon index</b>	<b>Pielou index</b>
	d	dMa	$\lambda$	D	Dr	He	J Je
<b>TAS in</b>	6.318181	6.574714	0.066903	0.933096	14.94681	3.038007	0.835171
<b>OM</b>	818	12	878	122	677	498	282

### 3.4. Evolutionary and Phylogenetic Analysis

During population bottlenecks in dynamic environments, like the oral microbiome, the stress response and Horizontal Gene Transfer play critical roles (Koonin, 2016; L. Liu et al., 2012; Pinilla-Redondo et al., 2018) The fact that one genetic system has both capabilities for stress response and modulating horizontal transfer is quite unique from the evolutionary point of view. To expand, the protein-protein interactions is believed to be evolved from promiscuous intermediates that enables it for the “mixing and matching” phenomenon among different families and super-families of the toxins and the antitoxins (Aakre et al., 2015; Gillis & Pavlidis, 2012; Goeders & Van Melderen, 2014) This is visualized by making a phylogenetic analysis of all the toxin and antitoxin families (see appendix). The results show a mosaic nature where nearly different families of different species are not clustered in a clade. By evaluating the evolutionary grades, it emphasizes the para-speciation style of the oral microbiome (Flintoft, 2013) This means that the genetic drift is prominent where genetic variations are extensive which drives the exaptation and provide background for adaptation (Harms et al., 2017) Under extreme stress, natural selection is the driving force (Martinez Jose L., 2009) However, in benevolent quasi stressful nature, the genetic drift mediated by the altruistic selfish systems is probably the main drive force for evolution. For example, neither the HTH-containing genes and GNAT containing genes are not clustered together nor the species are clustered together. Moreover, the divergence timing of the phylogenetic analysis was close, yet showed different evolutionary rates as measured by the molecular clock test. The tests of molecular clocks using the Maximum Likelihood method showed lnL Parameters With Clock equals to -104601.156 297 43.851 and Without Clock equals to -102026.798 573 16.3. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ( $P = 0.000E+000$ ). This demote the notion of lateral gene transfer of the chromosomal type II toxin antitoxin systems as agreed in the literature (Harms et al., 2018c)

Molecular allometry analysis is an analysis to compare the genome size, coding genes and the Toxin Antitoxin Systems (Figure 3-48 and Figure 3-49). The results demonstrate the persistence and maintenance of the TAS throughout all genome sizes despite the natural decline in the number of coding genes as the genome size decreases. The impact of the chromosomal type II toxin antitoxin systems in the evolution of the oral microbiome is one of the questions that need further investigations to make us appreciate the vast functionality of the TAS.

### Genome Size correlation with CDS

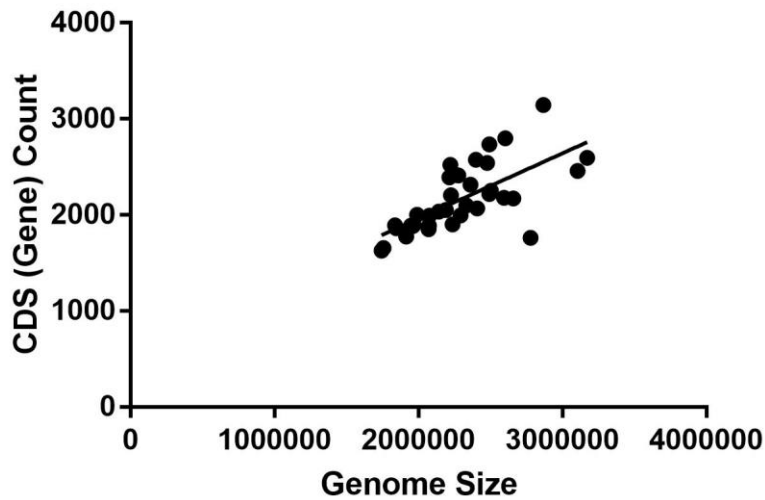


Figure 3-48: represents Pearson's correlation with regression analysis for the genome size for all genomes used in this study against the number of gene coding sequences for each respective genome. The analysis has R square value of 0.4729. The line equation is  $Y = 0.0006787 * X + 605.4$ . The deviation from zero is significant with P value  $< 0.0001$ . CDS stands for CoDing Sequences.

### Genome Size Correlations with TAS

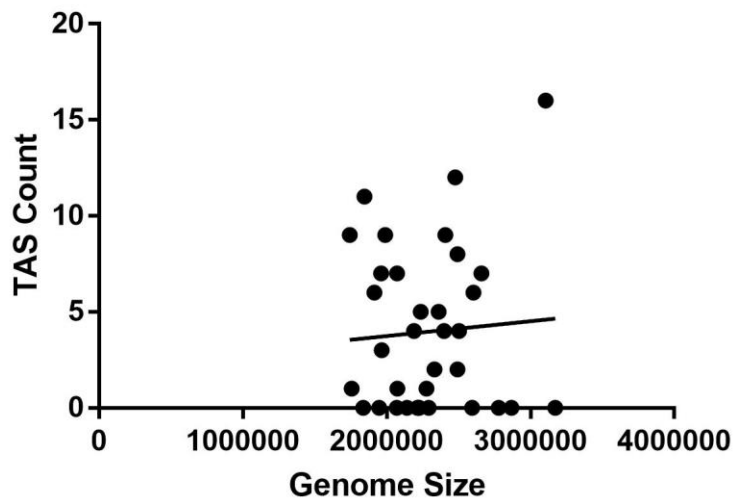


Figure 3-49: represents Pearson's correlation with regression analysis for the genome size for all genomes used in this study against the number of predicted chromosomal type II toxin antitoxin systems for each respective genome. The analysis has R square value of 0.00418. The line equation is  $Y = 7.743e-007 * X + 2.197$ . The deviation from zero is insignificant with p value equals to 0.7121 implying a slope of near zero value of  $7.743e-007 \pm 2.08e-006$ . TAS stands for Toxin Antitoxin Systems.

#### 4. CONCLUSION

The predictions of potentially novel chromosomal type II toxin antitoxin systems in the oral microbiome had opened questions more than just resolved ones. There have been 278 sequences that are revealed that are putatively functional due to the similarity in the domain architecture. This highlights the need in better functional prediction tools that can sense small protein coding genes. The distribution of the toxin antitoxin systems throughout the species in the oral microbiome is magnitude. Uneven intra-genomic and inter-genomic distribution as well as abundance are evident all over the microbial communities in the buccal cavity. The cross-talk network of the toxin antitoxin systems is, probably, shaping the co-evolutions that affects the bacterial adaptations.

Experimental validations for the predicted novel toxin antitoxin systems is a logical step. Yet, the functional analysis assays are difficult. The ectopic expression of proteins affects the fitness of the host bacterium. Thus, it is always debatable to assess the cytotoxicity of the protein that is attenuated by another co-expressed protein. The research group that first experimentally tested the toxin antitoxin systems published recent paper that antagonizes their methodology expressing this challenge in the functional assay (Song & Wood, 2018b)

On the other hand, this strikingly valuable system is a core for designing genetic networks of synthetic organisms. This could be applied in the post-antibiotic era where clinician could use organisms of beneficial characters addicted as a tool for competing against pathogens instead of the chemotherapy (Rugbjerg, Sarup-Lytzen, Nagy, & Sommer, 2018) The overall value for such endeavor is the ice-berg of understanding how the nature hold the answer when it comes to surviving communicable, as well as non-communicable, diseases.



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APPENDIX

S1 table: This table contains the abundance data for all unique Operational Taxonomic Units assigned in the oral microbiome analysis

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>Other</b>	Other	0.00035653512 6813	0.0	0.00295368620 038
<b>g__Actinomyces</b>	s__cardiffensis	0.0	0.0	0.00029536862 0038
<b>g__Actinomyces</b>	s__dentalis- orihominis	0.0	0.0	0.00277646502 836
<b>g__Actinomyces</b>	s__georgiae	0.0	0.0	0.00498188405 797
<b>g__Actinomyces</b>	s__gerencseria e	0.00084271939 0649	0.0	0.0
<b>g__Actinomyces</b>	s__israelii	0.0	0.0	0.00167375551 355
<b>g__Actinomyces</b>	s__massiliensi s	0.00106960538 044	0.0	0.00098456206 6793
<b>g__Actinomyces</b>	s__meyeri- odontolyticus	0.00149096507 576	0.0	0.0
<b>g__Actinomyces</b>	s__naeslundii	0.00764929908 435	0.0	0.01313405797 1
<b>g__Actinomyces</b>	s__naeslundii- oris	0.00312778543 068	0.0	0.00354442344 045
<b>g__Actinomyces</b>	s__odontolytic us	0.0	0.0	0.00604521109 011
<b>g__Actinomyces</b>	s__oris	0.00429462766 388	0.0	0.00112240075 614
<b>g__Actinomyces</b>	s__oris- viscosus	0.0	0.01769316155 94	0.0

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Actinomyces</b>	s__sp4769	0.00405153553 197	0.0	0.0
<b>g__Actinomyces</b>	s__viscosus	0.00118304837 533	0.0	0.0
<b>g__NA</b>	s__sp4816	0.00330605299 408	0.0	0.0
<b>g__Bifidobacterium</b>	s__adolescenti s	0.0	0.0	0.00031505986 1374
<b>g__Bifidobacterium</b>	s__angulatum	0.0	0.0	0.00027567737 8702
<b>g__Bifidobacterium</b>	s__ruminantiu m	0.0	0.00037000908 2041	0.00027567737 8702
<b>g__Corynebacteriu m</b>	s__durum	0.00056721497 4475	0.00148003632 816	0.00500157529 931
<b>g__Corynebacteriu m</b>	s__matruchotii	0.00619074629 284	0.00689562380 168	0.01697385003 15
<b>g__Rothia</b>	s__aeria	0.00270642573 535	0.0	0.01112555135 48
<b>g__Rothia</b>	s__dentocarios a	0.00557491289 199	0.00891385515 826	0.00189035916 824
<b>g__Rothia</b>	s__mucilagino sa	0.00093995624 3416	0.0	0.00112240075 614
<b>g__Propionibacteriu m</b>	s__propionicu m	0.00097236852 7672	0.00151367351 744	0.00057104599 874
<b>g__Propionibacteriu m</b>	s__sp7795	0.0	0.0	0.00064981096 4083
<b>g__Atopobium</b>	s__parvulum	0.0	0.0	0.00151622558 286
<b>g__Olsenella</b>	s__uli	0.0	0.0	0.00049228103 3396

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Bacteroides</b>	s__fragilis	0.0	0.00104275286 757	0.00086641461 8778
<b>g__Porphyromonas</b>	s__catoniae	0.00337087756 26	0.0	0.02939902331 44
<b>g__Porphyromonas</b>	s__endodontal is	0.0	0.0	0.00679347826 087
<b>g__Porphyromonas</b>	s__sp13364- sp13375	0.01003160197 71	0.0	0.0
<b>g__Porphyromonas</b>	s__sp13375	0.01531480431 08	0.00450738336 305	0.0
<b>g__Porphyromonas</b>	s__sp13380	0.0	0.00322917017 054	0.00275677378 702
<b>g__Tannerella</b>	s__forsythia	0.0	0.00080729254 2635	0.00515910522 999
<b>g__Alloprevotella</b>	s__rava	0.0	0.0	0.00055135475 7404
<b>g__Alloprevotella</b>	s__sp13491	0.04164978526 86	0.02135961519 06	0.03711798991 81
<b>g__Alloprevotella</b>	s__sp13512- sp13517	0.00191232477 109	0.0	0.00250078764 965
<b>g__Alloprevotella</b>	s__sp13514	0.0	0.0	0.00864445494 644
<b>g__Alloprevotella</b>	s__tannerae	0.0	0.0	0.00385948330 183
<b>g__NA</b>	s__sp13865	0.0	0.0	0.01451244486 45
<b>g__NA</b>	s__sp13879	0.0	0.0	0.00127993068 683
<b>g__NA</b>	s__sp13918	0.0	0.0	0.00096487082 5457

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__NA</b>	s__sp13920	0.0	0.0	0.00047258979 206
<b>g__NA</b>	s__sp13921	0.0	0.0	0.00133900441 084
<b>g__Paraprevotella</b>	s__clara	0.0	0.0	0.00108301827 347
<b>g__Prevotella</b>	s__baroniae	0.0	0.0	0.00055135475 7404
<b>g__Prevotella</b>	s__copri	0.0	0.0	0.00070888468 8091
<b>g__Prevotella</b>	s__histicola- jejuni	0.0	0.0	0.00452898550 725
<b>g__Prevotella</b>	s__intermedia	0.0	0.0	0.01874606175 17
<b>g__Prevotella</b>	s__loescheii	0.00051859654 8092	0.0	0.00578922495 274
<b>g__Prevotella</b>	s__marshii	0.0	0.0	0.00049228103 3396
<b>g__Prevotella</b>	s__melaninoge nica	0.00042135969 5325	0.0	0.00222511027 095
<b>g__Prevotella</b>	s__micans	0.0	0.0	0.00122085696 282
<b>g__Prevotella</b>	s__nanceiensis	0.00341949598 898	0.00074001816 4082	0.00039382482 6717
<b>g__Prevotella</b>	s__nigrescens	0.00022688598 979	0.0	0.00224480151 229
<b>g__Prevotella</b>	s__oris	0.0	0.0	0.00027567737 8702
<b>g__Prevotella</b>	s__pallens	0.00090754395 9161	0.0	0.0

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Prevotella</b>	s__pleuritidis	0.0	0.0	0.00053166351 6068
<b>g__Prevotella</b>	s__saccharolyt ica	0.0	0.0	0.00232356647 763
<b>g__Prevotella</b>	s__shahii	0.0	0.0	0.00066950220 5419
<b>g__Prevotella</b>	s__veroralis	0.00050239040 5964	0.0	0.0
<b>g__NA</b>	s__sp14445	0.0	0.0	0.00033475110 271
<b>g__Bergeyella</b>	s__cardium	0.00042135969 5325	0.0	0.00063011972 2747
<b>g__Bergeyella</b>	s__sp16466	0.0	0.00067274378 5529	0.0
<b>g__Bergeyella</b>	s__sp16471	0.00468357507 495	0.00087456692 1188	0.00220541902 962
<b>g__Capnocytophaga</b>	s__gingivalis	0.00243092131 918	0.0	0.00543478260 87
<b>g__Capnocytophaga</b>	s__granulosa	0.00032412284 2557	0.00380100238 824	0.00878229363 579
<b>g__Capnocytophaga</b>	s__haemolytic a	0.00050239040 5964	0.0	0.0
<b>g__Capnocytophaga</b>	s__leadbetteri	0.00750344380 52	0.0	0.00504095778 198
<b>g__Capnocytophaga</b>	s__ochracea	0.0	0.00289279827 778	0.00669502205 419
<b>g__Capnocytophaga</b>	s__sp16491	0.00046997812 1708	0.0	0.00120116572 149
<b>g__Capnocytophaga</b>	s__sp16511	0.00333846527 834	0.00043728346 0594	0.00527725267 801



<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Capnocytophaga</b>	s__sp16514	0.00948059314 48	0.0	0.01476843100 19
<b>g__Capnocytophaga</b>	s__sp16515	0.00037274126 8941	0.0	0.00456836798 992
<b>g__NA</b>	s__sp19416	0.0	0.0	0.00049228103 3396
<b>g__NA</b>	s__sp19423	0.0	0.0	0.00120116572 149
<b>g__Olivibacter</b>	s__jilunii	0.0	0.00053819502 8423	0.00027567737 8702
<b>g__Gemella</b>	s__haemolysa ns-sanguinis- taiwanensis	0.02140831375 09	0.06182515389 01	0.01681632010 08
<b>g__Gemella</b>	s__morbilloru m	0.00113442994 895	0.00353190487 403	0.02674070573 41
<b>g__Abiotrophia</b>	s__sp28088	0.0	0.0	0.00504095778 198
<b>g__Granulicatella</b>	s__adiacens	0.01437484806 74	0.00642470315 18	0.00238264020 164
<b>g__Granulicatella</b>	s__elegans	0.01685438781 3	0.00962023613 307	0.00513941398 866
<b>g__Lactobacillus</b>	s__fermentum	0.0	0.00121093881 395	0.00049228103 3396
<b>g__Streptococcus</b>	s__NA	0.16940280366 3	0.49816677318 4	0.05354048519 22
<b>g__Streptococcus</b>	s__anginosus	0.0	0.0	0.00076795841 2098
<b>g__Streptococcus</b>	s__anginosus- constellatus- intermedius	0.00050239040 5964	0.0	0.00088610586 0113

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Streptococcus</b>	s__gordonii	0.03878129811 2	0.0	0.00118147448 015
<b>g__Streptococcus</b>	s__intermediu s	0.00032412284 2557	0.0	0.0
<b>g__Streptococcus</b>	s__mutans	0.04521513653 67	0.00467556930 943	0.0
<b>g__Streptococcus</b>	s__salivarius- vestibularis	0.01959322583 26	0.00659288909 819	0.0
<b>g__Streptococcus</b>	s__sanguinis	0.05834211166 03	0.01987957886 24	0.01699354127 28
<b>g__Streptococcus</b>	s__sinensis	0.0	0.0	0.00474558916 194
<b>g__Parvimonas</b>	s__micra	0.0	0.0	0.00208727158 16
<b>g__Mogibacterium</b>	s__neglectum	0.0	0.0	0.00131931316 95
<b>g__NA</b>	s__sp31630	0.0	0.0	0.00084672337 7442
<b>g__NA</b>	s__sp31635	0.0	0.0	0.00023629489 603
<b>g__NA</b>	s__sp31682	0.0	0.0	0.00037413358 5381
<b>g__Butyrivibrio</b>	s__sp32116	0.0	0.0	0.00078764965 3434
<b>g__Catonella</b>	s__morbi	0.00030791670 0429	0.0	0.00342627599 244
<b>g__Johnsonella</b>	s__ignava	0.0	0.00087456692 1188	0.0
<b>g__Johnsonella</b>	s__sp32278	0.0	0.00104275286 757	0.00511972274 732

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Johnsonella</b>	s__sp32283	0.0	0.0	0.00049228103 3396
<b>g__Johnsonella</b>	s__sp32285	0.0	0.00070638097 4806	0.0
<b>g__Lachnoanaeroba culum</b>	s__saburreum	0.0	0.00074001816 4082	0.00277646502 836
<b>g__Lachnoanaeroba culum</b>	s__sp32292	0.0	0.0	0.00161468178 954
<b>g__Lachnoanaeroba culum</b>	s__sp32293	0.0	0.0	0.00704946439 824
<b>g__Lachnoanaeroba culum</b>	s__umeaense	0.00059962725 8731	0.0	0.00324905482 042
<b>g__NA</b>	s__rectale	0.0	0.00131185038 178	0.00070888468 8091
<b>g__NA</b>	s__sp33423	0.0	0.0	0.00029536862 0038
<b>g__Oribacterium</b>	s__sp33063	0.0	0.00057183221 77	0.0
<b>g__NA</b>	s__sp30555	0.00025929827 4046	0.0	0.0
<b>g__Peptococcus</b>	s__sp34118	0.0	0.0	0.00448960302 457
<b>g__Filifactor</b>	s__alocis	0.0	0.0	0.00057104599 874
<b>g__Peptoclostridium</b>	s__sp34347	0.0	0.0	0.00212665406 427
<b>g__Peptoclostridium</b>	s__sp34351	0.0	0.0	0.00206758034 026
<b>g__Peptostreptococ cus</b>	s__anaerobius- stomatis	0.0	0.0	0.00490311909 263

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__NA</b>	s__sp35348	0.0	0.0	0.00181159420 29
<b>g__NA</b>	s__sp35352	0.0	0.0	0.00112240075 614
<b>g__Solobacterium</b>	s__moorei	0.0	0.0	0.00108301827 347
<b>g__Dialister</b>	s__invisus	0.0	0.00107639005 685	0.00224480151 229
<b>g__Selenomonas</b>	s__artemidis	0.00027550441 6174	0.0	0.00232356647 763
<b>g__Selenomonas</b>	s__infelix	0.00055100883 2347	0.0	0.00567107750 473
<b>g__Selenomonas</b>	s__noxia	0.0	0.0	0.00218572778 828
<b>g__Selenomonas</b>	s__sp37070	0.0	0.0	0.00116178323 882
<b>g__Selenomonas</b>	s__sp37070- sp37072	0.0	0.0	0.00175252047 889
<b>g__Selenomonas</b>	s__sp37072	0.0	0.0	0.00043320730 9389
<b>g__Veillonella</b>	s__atypica	0.00131269751 236	0.0	0.0
<b>g__Veillonella</b>	s__atypica- parvula	0.0	0.0	0.00023629489 603
<b>g__Veillonella</b>	s__denticariosi	0.0	0.00581923374 483	0.0
<b>g__Veillonella</b>	s__denticariosi -parvula- tobetsuensis	0.00341949598 898	0.0	0.0

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Veillonella</b>	s__parvula	0.07984766226 4	0.00686198661 24	0.05434782608 7
<b>g__Veillonella</b>	s__sp37198	0.03374118791 02	0.02226781930 1	0.00082703213 6106
<b>g__Veillonella</b>	s__tobetsuensi s	0.0	0.00581923374 483	0.00248109640 832
<b>g__Fusobacterium</b>	s__canifelinu m-nucleatum	0.01262458471 76	0.00773655353 359	0.01975031505 99
<b>g__Fusobacterium</b>	s__nucleatum	0.00709829025 201	0.00090820411 0465	0.02392485822 31
<b>g__Fusobacterium</b>	s__nucleatum- periodonticum	0.0	0.0	0.00167375551 355
<b>g__Fusobacterium</b>	s__periodontic um	0.00093995624 3416	0.0	0.01098771266 54
<b>g__Fusobacterium</b>	s__sp37444	0.0	0.00228732887 08	0.0
<b>g__Leptotrichia</b>	s__buccalis	0.00218782918 726	0.0	0.00527725267 801
<b>g__Leptotrichia</b>	s__buccalis- hofstadii	0.0	0.0	0.00322936357 908
<b>g__Leptotrichia</b>	s__hofstadii	0.0	0.0	0.00157529930 687
<b>g__Leptotrichia</b>	s__shahii	0.00372741268 941	0.00225369168 152	0.00185097668 557
<b>g__Leptotrichia</b>	s__sp37510	0.00019447370 5534	0.0	0.0
<b>g__Leptotrichia</b>	s__sp37518	0.00831375091 16	0.00174913384 238	0.01795841209 83
<b>g__Leptotrichia</b>	s__trevisanii	0.00087513167 4905	0.0	0.0

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g_Leptotrichia</b>	s_wadei	0.00095616238 5544	0.0	0.0
<b>g_Streptobacillus</b>	s_hongkongensis	0.0	0.00090820411 0465	0.0
<b>g_NA</b>	s_sp38230	0.0	0.0	0.00555293005 671
<b>g_NA</b>	s_sp19816	0.00051859654 8092	0.00050455783 9147	0.01466997479 52
<b>g_Lautropia</b>	s_mirabilis	0.00478081192 772	0.00568468498 772	0.00897920604 915
<b>g_Comamonas</b>	s_sp48939	0.00058342111 6603	0.0	0.00124054820 416
<b>g_Eikenella</b>	s_corrodens	0.00056721497 4475	0.0	0.00580891619 408
<b>g_Kingella</b>	s_denitrificans	0.00338708370 472	0.0	0.00921550094 518
<b>g_Kingella</b>	s_oralis	0.00262539502 471	0.00077365535 3359	0.00098456206 6793
<b>g_Neisseria</b>	s_bacilliformis	0.00097236852 7672	0.0	0.0
<b>g_Neisseria</b>	s_cinerea	0.00847581233 287	0.00319553298 126	0.00061042848 1411
<b>g_Neisseria</b>	s_elongata	0.01150636091 08	0.00501194120 219	0.00194943289 225
<b>g_Neisseria</b>	s_flavescens	0.0	0.00575195936 628	0.01317344045 37
<b>g_Neisseria</b>	s_macacae-mucosa-sicca	0.11431812657	0.00370009082 041	0.13214792060 5

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Neisseria</b>	s__mucosa- perflava- subflava	0.00046997812 1708	0.00625651720 542	0.0
<b>g__Neisseria</b>	s__oralis	0.00048618426 3836	0.00669380066 602	0.00129962192 817
<b>g__Neisseria</b>	s__shayeganii	0.0	0.0	0.00364287964 713
<b>g__Neisseria</b>	s__sp49957	0.00064824568 5115	0.0	0.0
<b>g__Simonsiella</b>	s__muelleri	0.0	0.0	0.00025598613 7366
<b>g__Bilophila</b>	s__wadsworthi a	0.0	0.0	0.00041351606 8053
<b>g__Campylobacter</b>	s__concisus	0.00354914512 6	0.0	0.00419423440 454
<b>g__Campylobacter</b>	s__curvus	0.0	0.0	0.00031505986 1374
<b>g__Campylobacter</b>	s__gracilis	0.00108581152 257	0.0	0.00076795841 2098
<b>g__Campylobacter</b>	s__rectus- showae	0.00118304837 533	0.00151367351 744	0.01047574039 07
<b>g__Cardiobacterium</b>	s__hominis	0.00066445182 7243	0.0	0.00163437303 088
<b>g__Cardiobacterium</b>	s__valvarum	0.00034032898 4685	0.0	0.00023629489 603
<b>g__Escherichia- Shigella</b>	s__coli	0.0	0.00090820411 0465	0.00063011972 2747
<b>g__Actinobacillus</b>	s__sp62066	0.00063203954 2987	0.04668841871 57	0.0

<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__Aggregatibacter</b>	s__aphrophilu s	0.02045215136 54	0.0	0.01750551354 76
<b>g__Aggregatibacter</b>	s__segnis	0.0	0.0	0.04824354127 28
<b>g__Aggregatibacter</b>	s__sp62087	0.00034032898 4685	0.00578559655 555	0.01205103969 75
<b>g__Haemophilus</b>	s__haemolytic us	0.00027550441 6174	0.08096471458 84	0.00450929426 591
<b>g__Haemophilus</b>	s__haemolytic us-influenzae	0.00048618426 3836	0.01802953345 22	0.00454867674 858
<b>g__Haemophilus</b>	s__haemolytic us-quentini	0.00037274126 8941	0.0	0.0
<b>g__Haemophilus</b>	s__influenzae	0.0	0.0	0.00137838689 351
<b>g__Haemophilus</b>	s__parainfluen zae	0.12530589093 3	0.05355040532 81	0.01039697542 53
<b>g__Haemophilus</b>	s__pittmaniae	0.0	0.00050455783 9147	0.00206758034 026
<b>g__*Saccharimonas</b>	s__sp65946	0.0	0.0	0.00066950220 5419
<b>g__*Saccharimonas</b>	s__sp65955	0.0	0.0	0.00409577819 786
<b>g__*Saccharimonas</b>	s__sp65958	0.0	0.0	0.00096487082 5457
<b>g__*Saccharimonas</b>	s__sp65962	0.0	0.0	0.00823093887 839
<b>g__NA</b>	s__sp65941	0.00046997812 1708	0.0	0.00427299936 988
<b>g__NA</b>	s__sp66013	0.0	0.0	0.00240233144 297

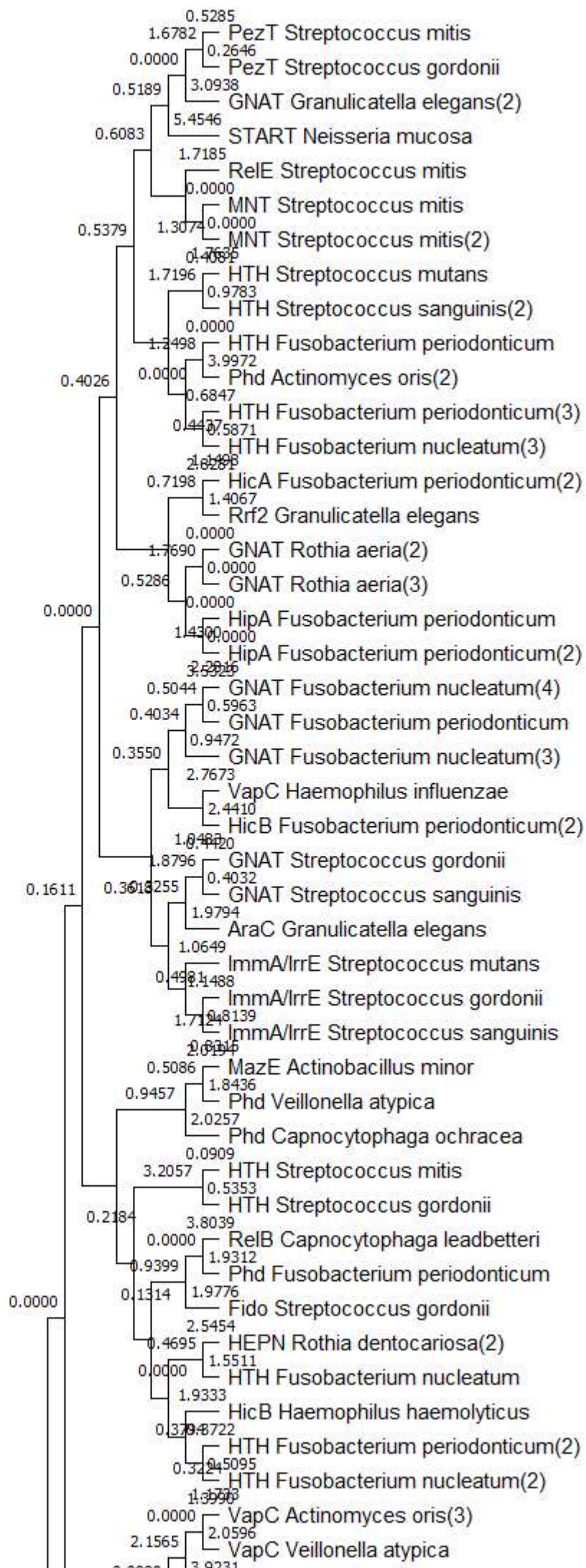


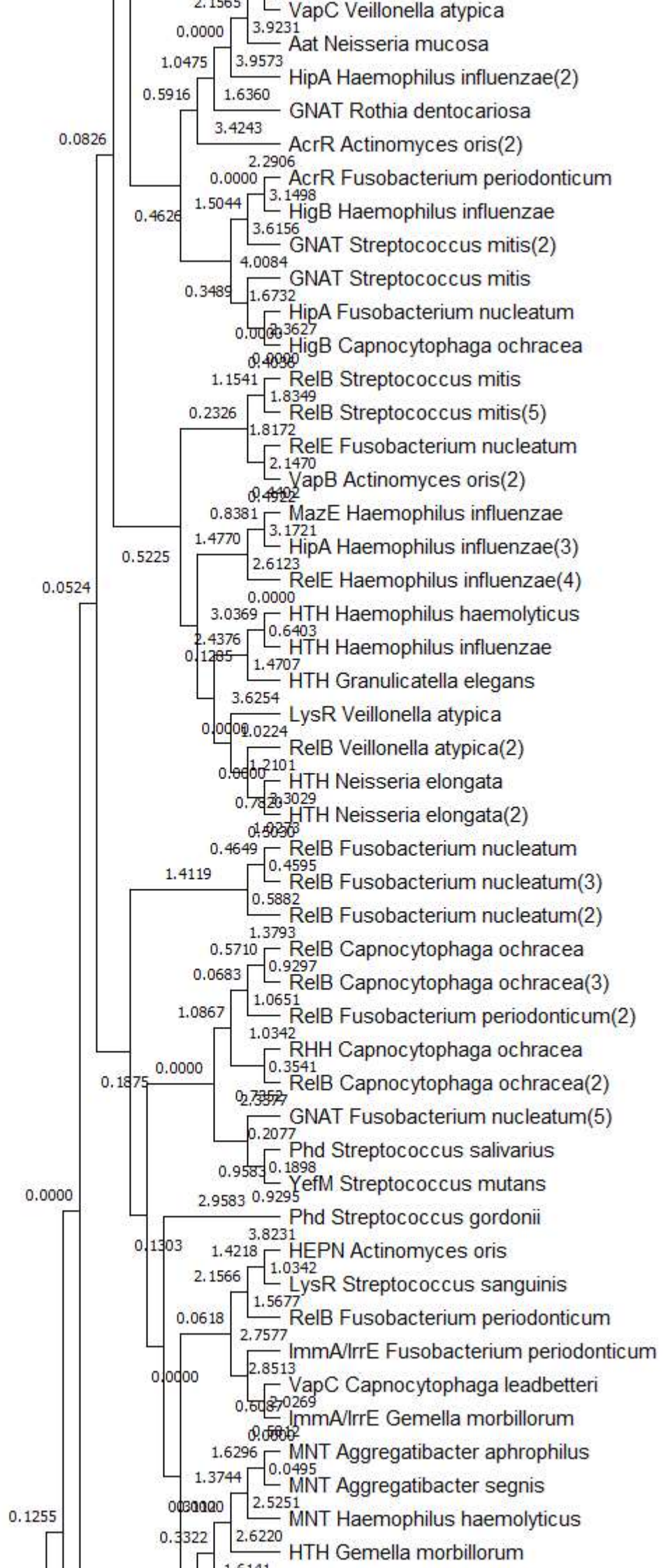
<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__NA</b>	s__sp66034	0.00021067984 7662	0.0	0.00133900441 084
<b>g__NA</b>	s__sp66108	0.0	0.00117730162 468	0.0
<b>g__NA</b>	s__sp66127	0.0	0.0	0.01435491493 38
<b>g__NA</b>	s__sp66606	0.0	0.0	0.00163437303 088
<b>g__NA</b>	s__sp66623	0.0	0.00067274378 5529	0.0
<b>g__NA</b>	s__sp66675	0.0	0.0	0.00102394454 946
<b>g__NA</b>	s__sp66707	0.0	0.0	0.00031505986 1374
<b>g__Treponema</b>	s__denticola	0.0	0.0	0.00057104599 874
<b>g__Treponema</b>	s__maltophilu m	0.0	0.0	0.00037413358 5381
<b>g__Treponema</b>	s__medium	0.0	0.0	0.00057104599 874
<b>g__Treponema</b>	s__medium- vincentii	0.0	0.0	0.00055135475 7404
<b>g__Treponema</b>	s__socranskii	0.0	0.0	0.00151622558 286
<b>g__Treponema</b>	s__sp66800	0.0	0.0	0.00029536862 0038
<b>g__Treponema</b>	s__vincentii	0.0	0.0	0.00135869565 217
<b>g__Fretibacterium</b>	s__sp67092	0.0	0.0	0.00035444234 4045

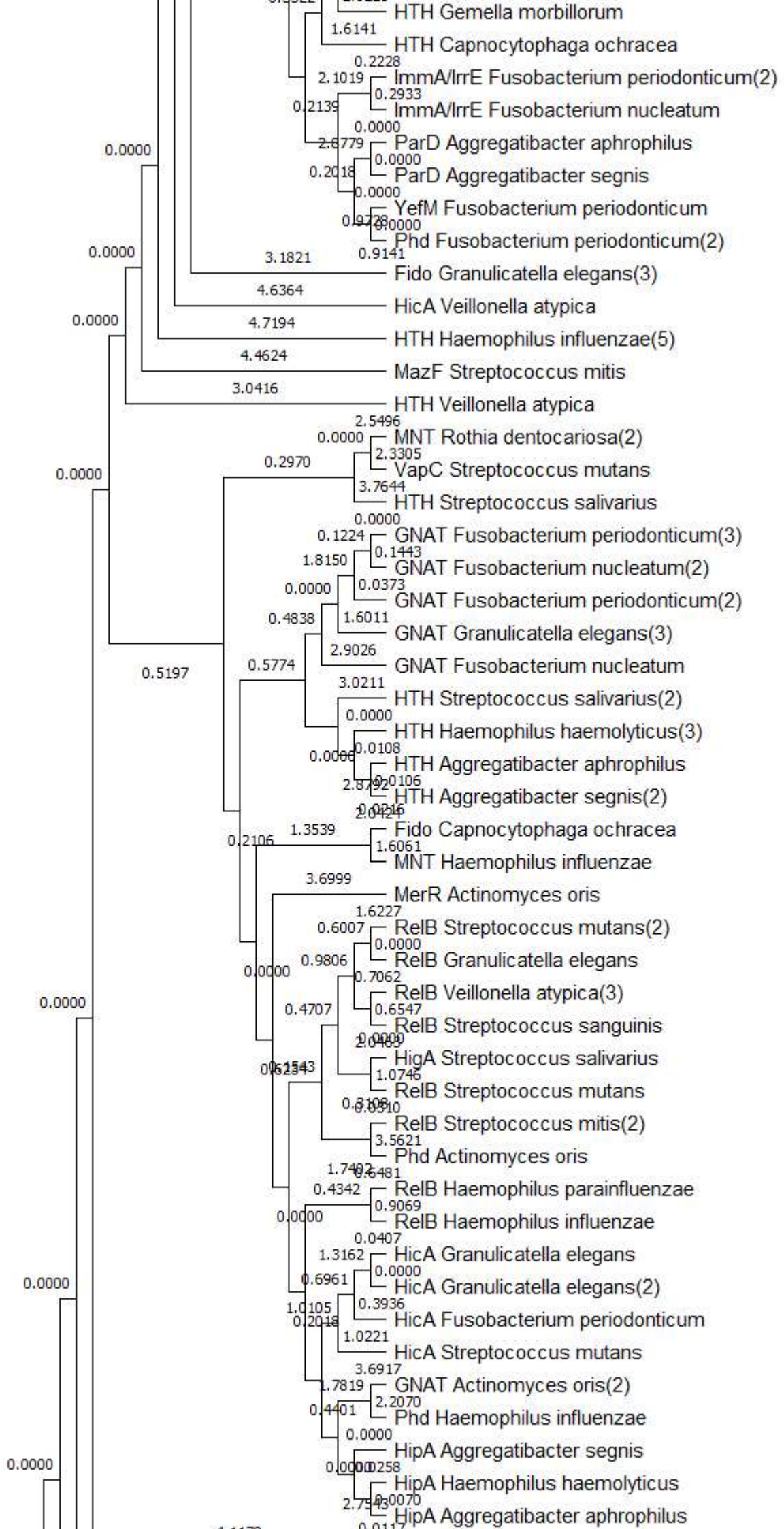
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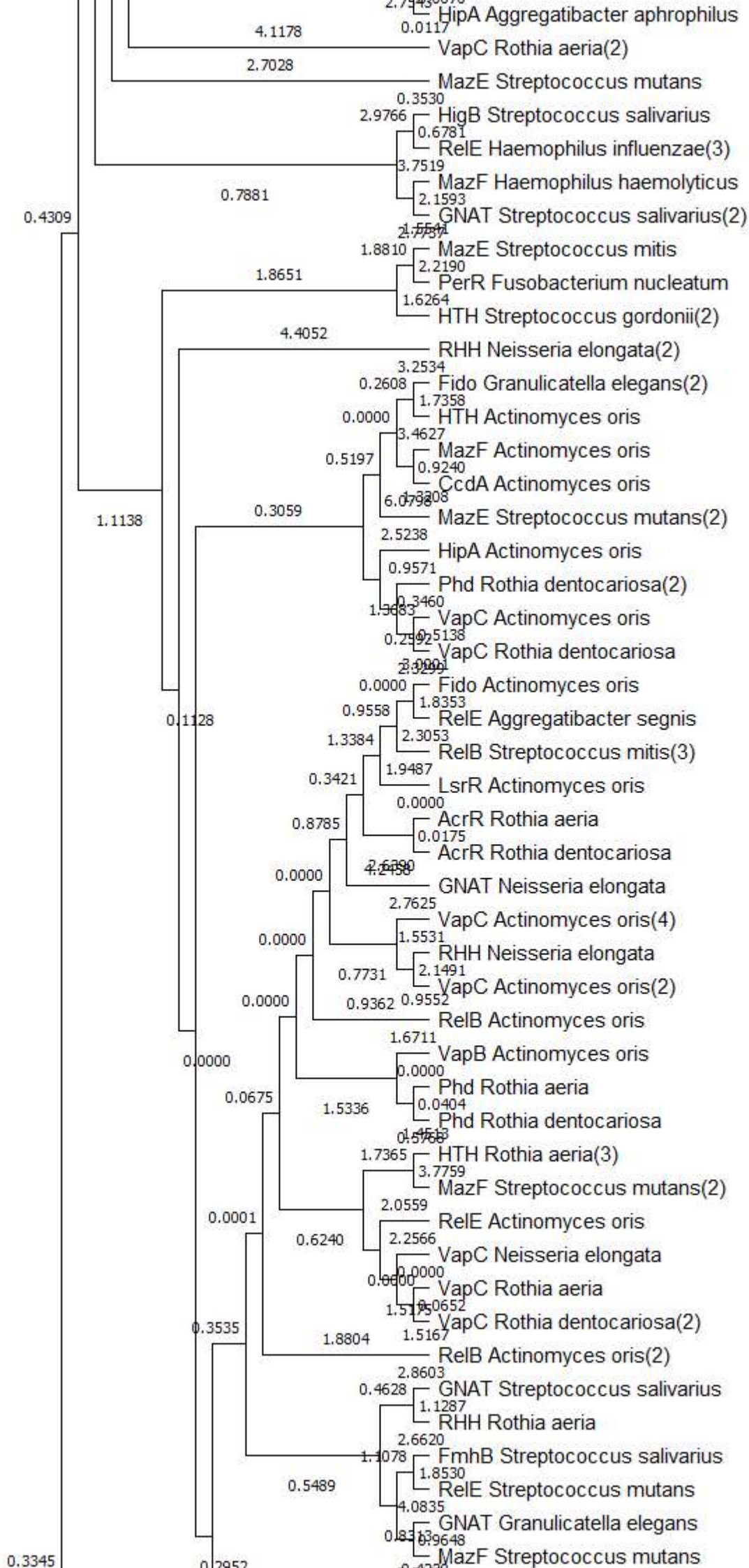
<b>Genus</b>	<b>Species</b>	<b>Oral 1</b>	<b>Oral 2</b>	<b>Oral 3</b>
<b>g__NA</b>	s__sp67855	0.0	0.0	0.00039382482 6717

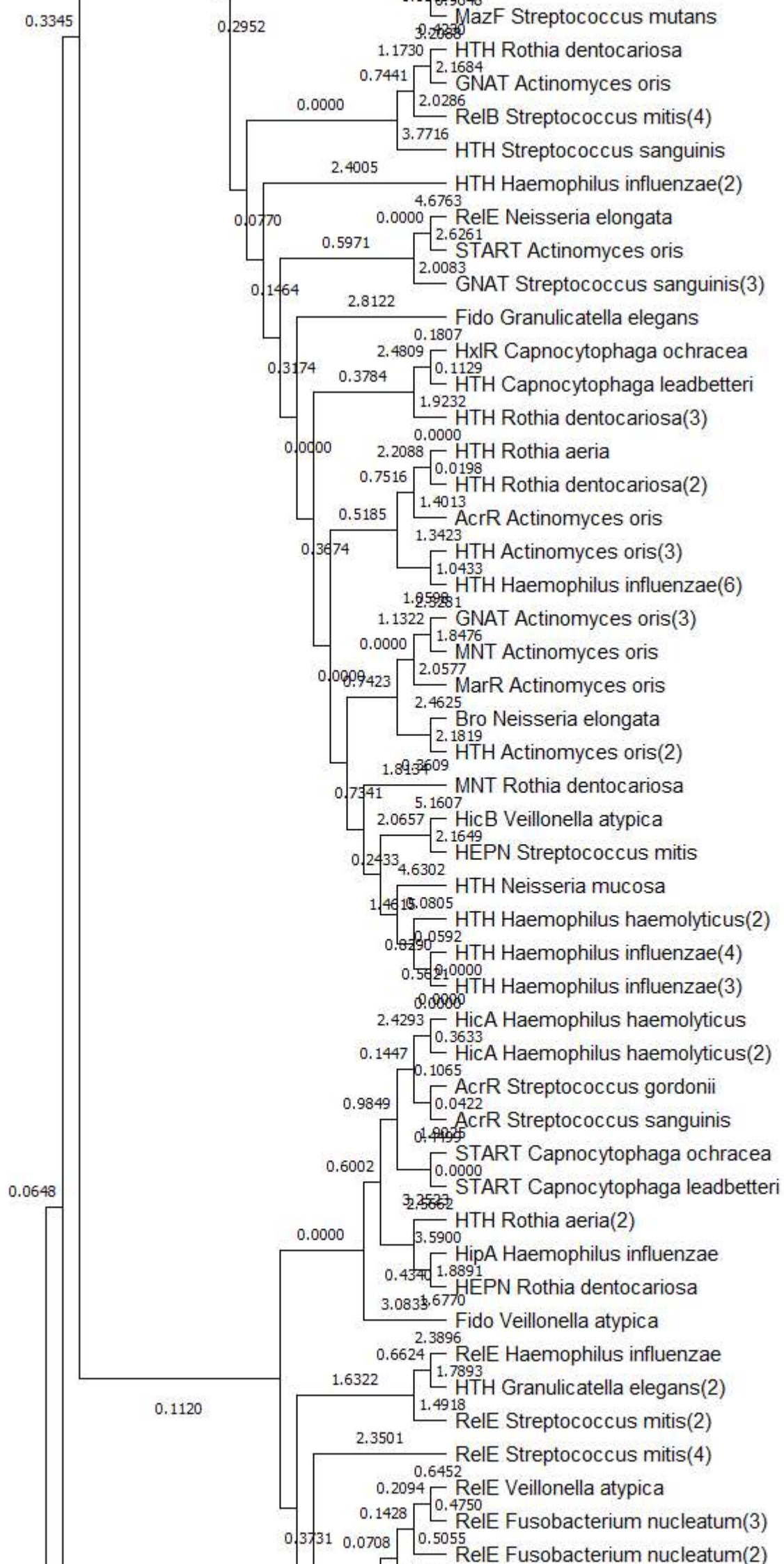
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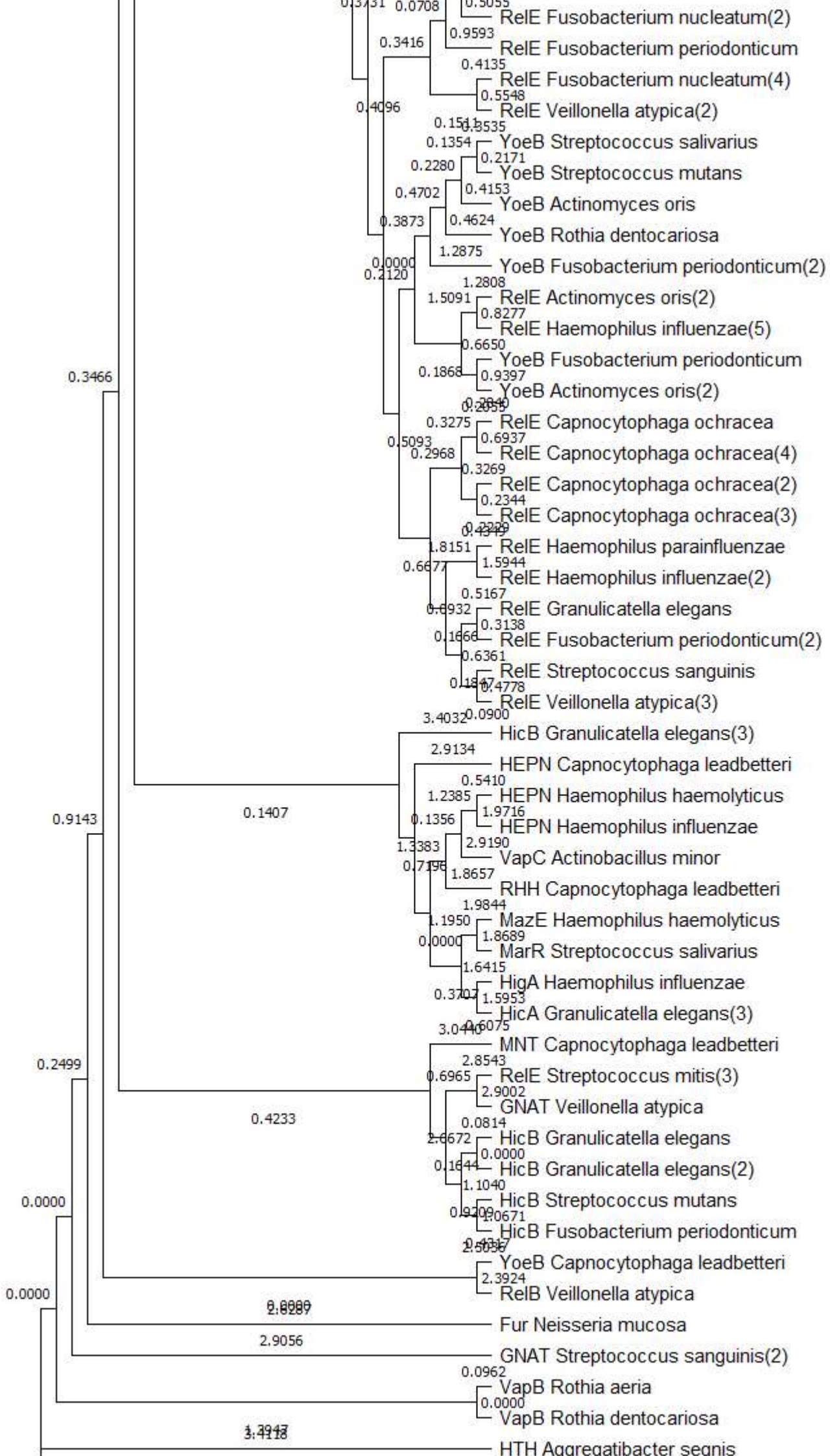














3.4118

HTH *Aggregatibacter segnis*

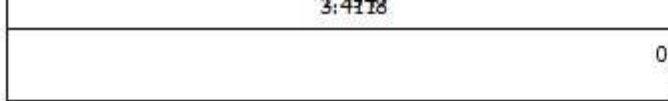
0.0327

GNAT *Rothia aerea*

0.0118

GNAT *Rothia dentocariosa*(2)

20.6767

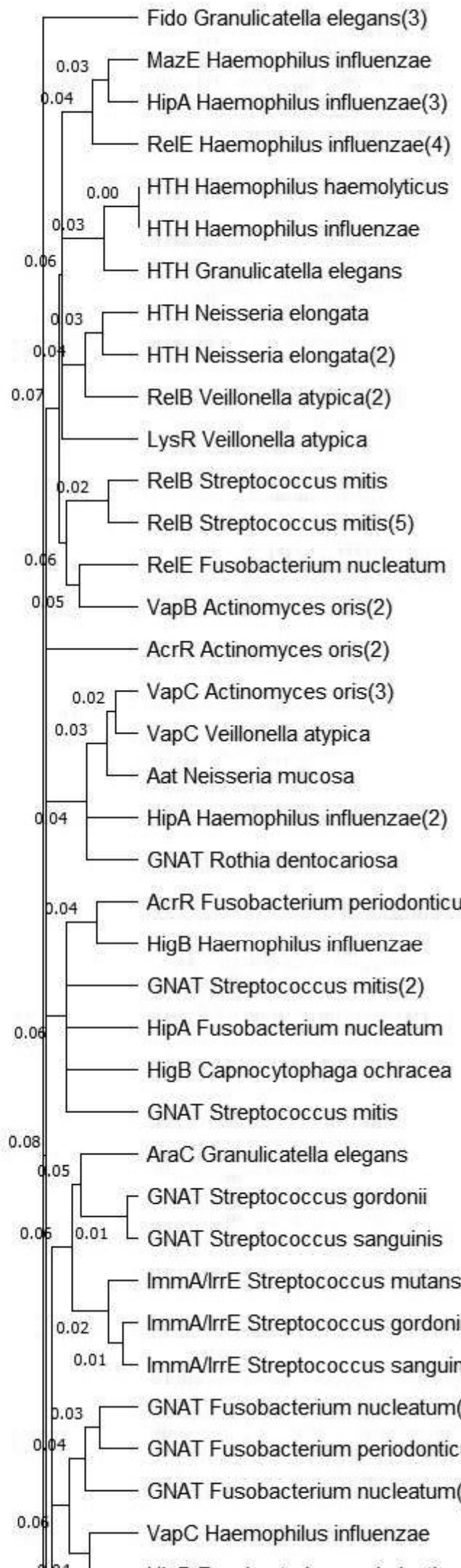


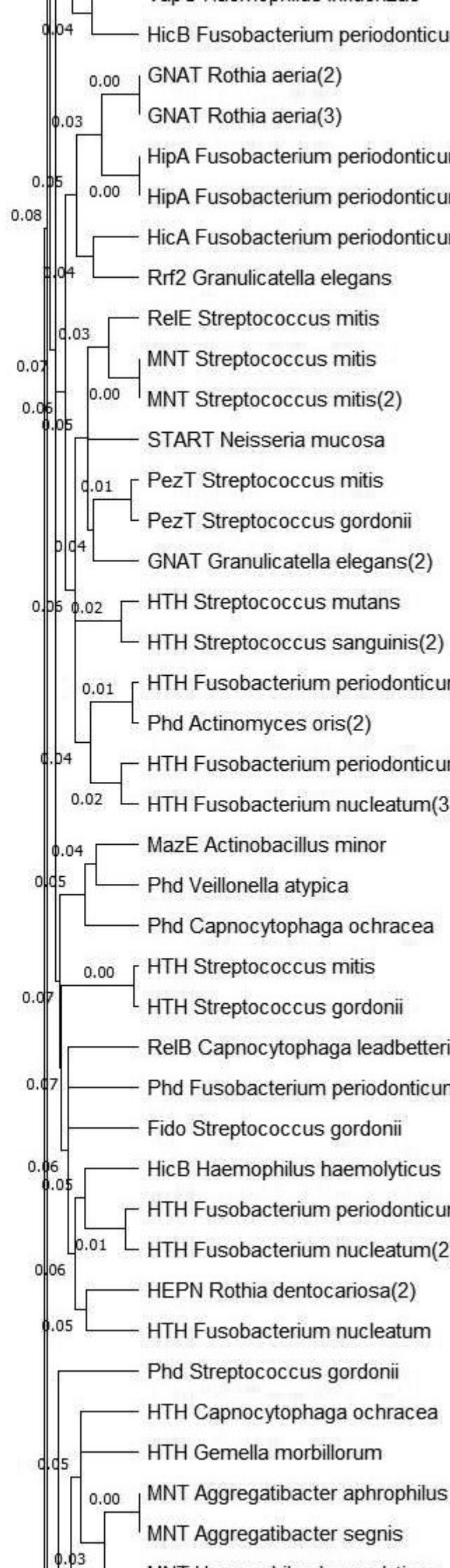
## Figure. Molecular Phylogenetic analysis by Maximum Likelihood method

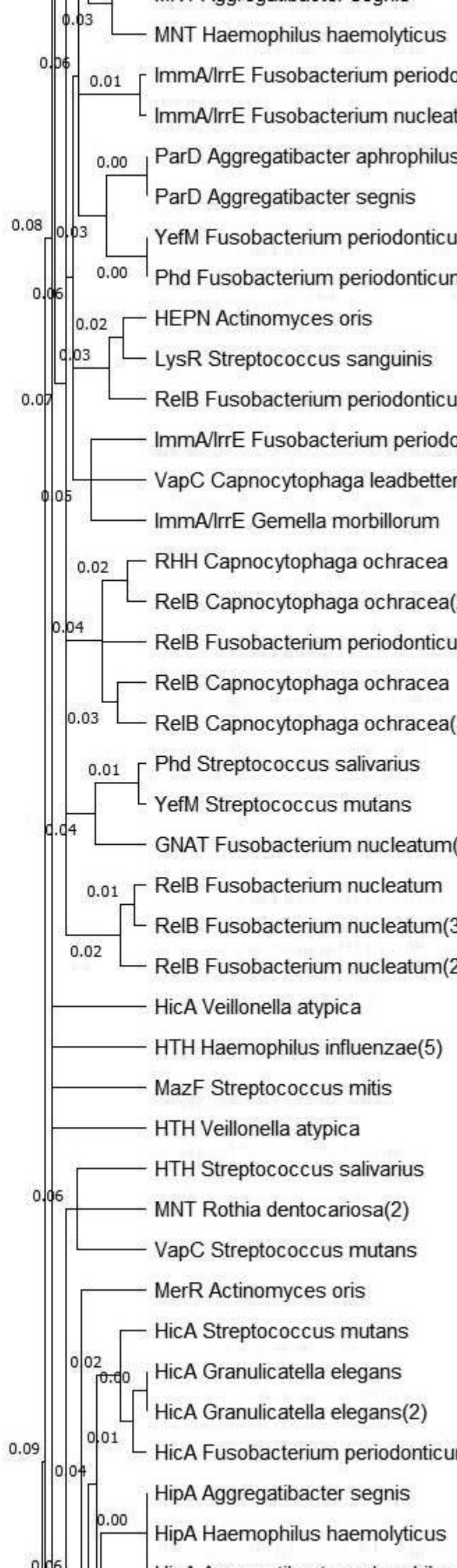
The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model [1]. The tree with the highest log likelihood (-103010.66) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 13.2539)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 277 amino acid sequences. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

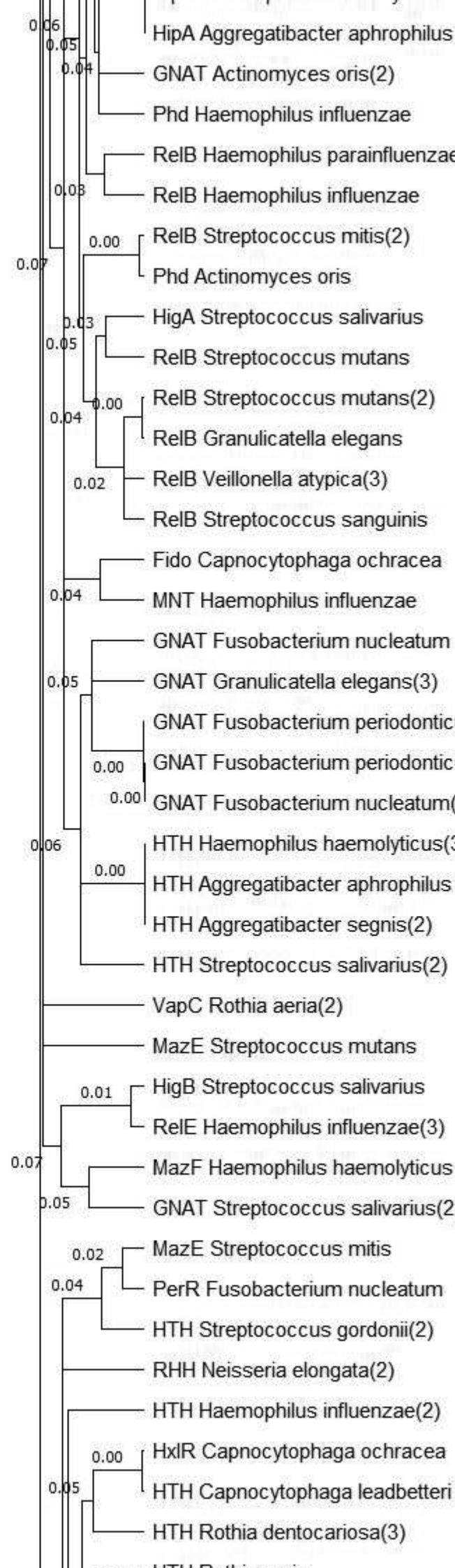
1. Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Molecular Biology and Evolution* **18**:691-699.
2. Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* **35**:1547-1549.

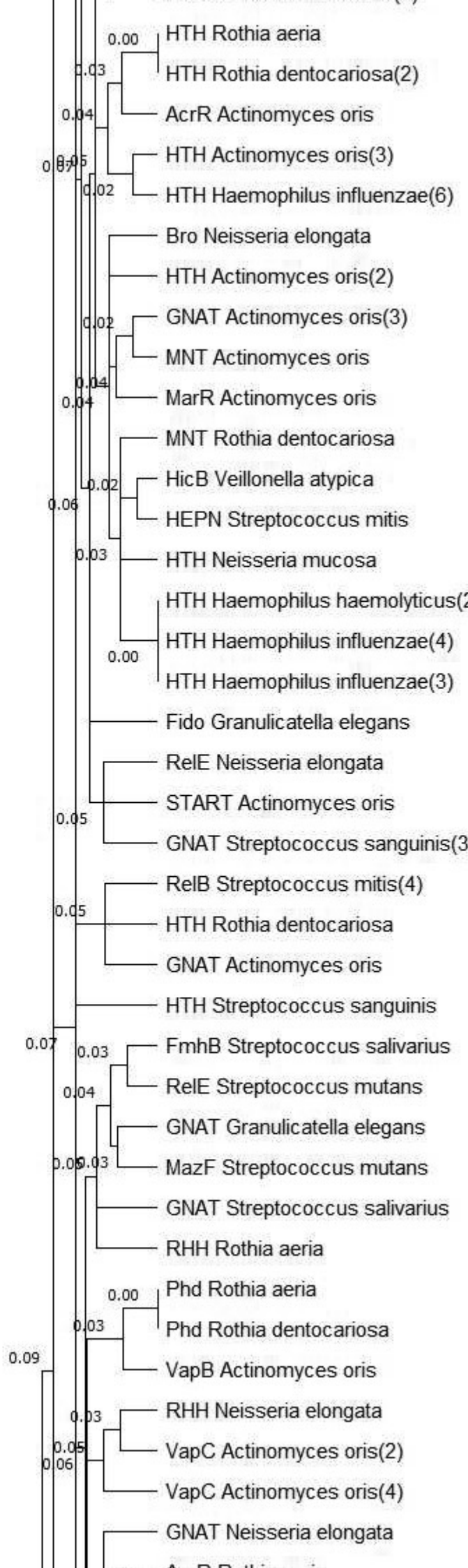
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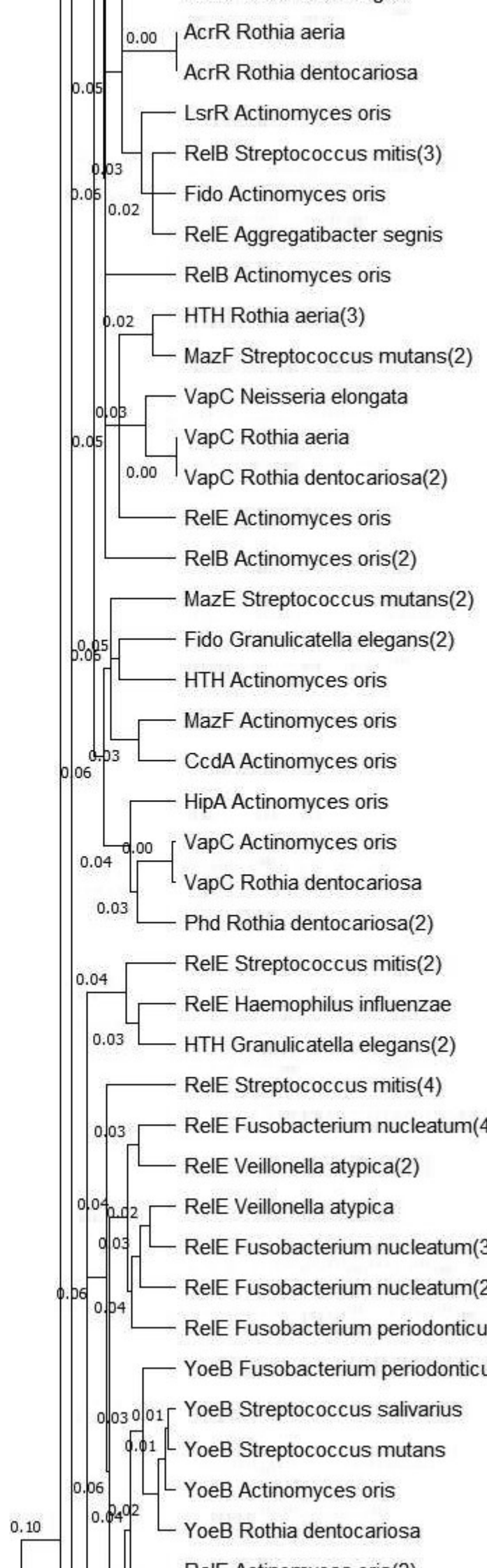




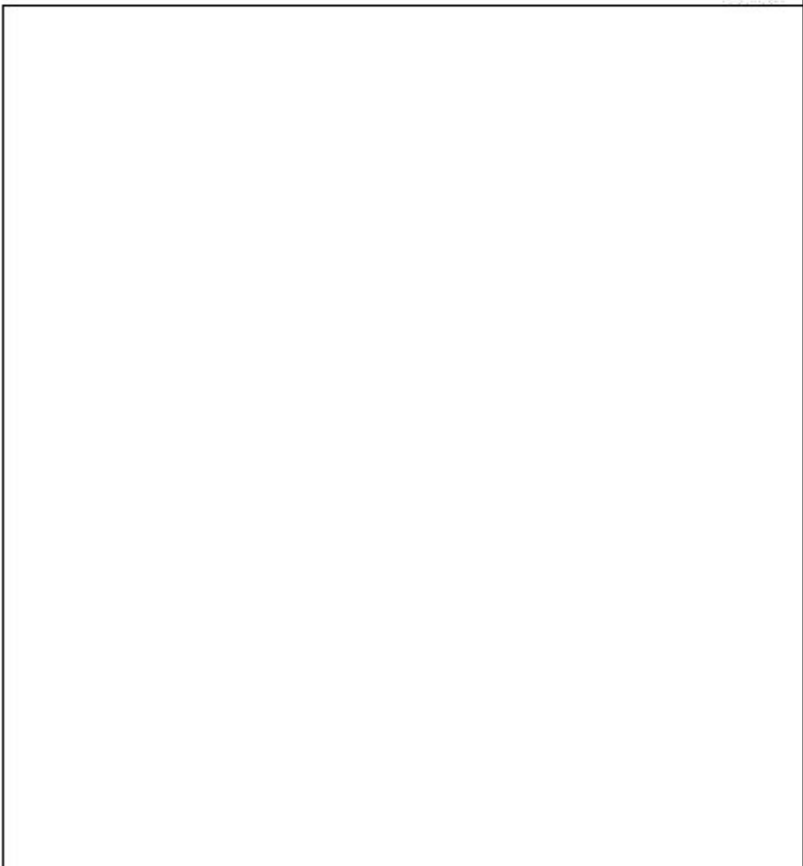
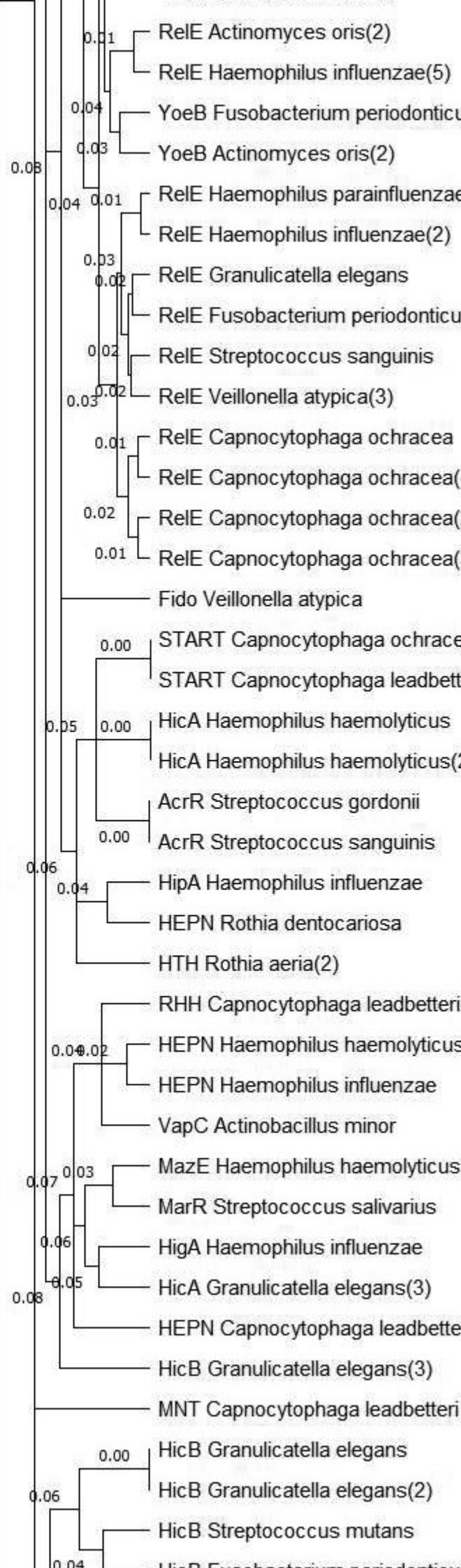




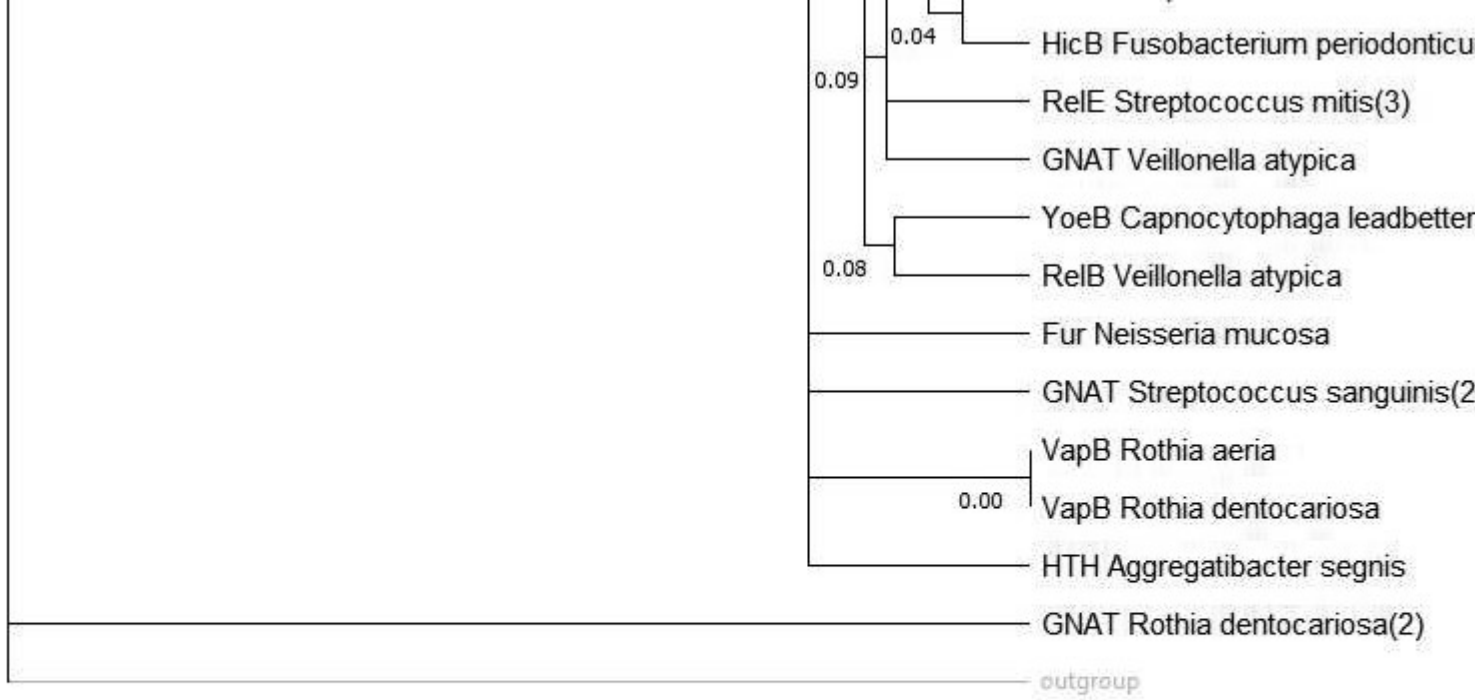








0.13



## Figure. Timetree analysis using the RelTime method

A timetree inferred using the Reltime method [1][2] and the Whelan And Goldman + Freq. model [3]. The estimated log likelihood value is -103039.01. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 24.5210)). The analysis involved 277 amino acid sequences. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

1. Tamura K., Battistuzzi FU, Billing-Ross P, Murillo O, Filipowski A, and Kumar S. (2012). Estimating Divergence Times in Large Molecular Phylogenies. *Proceedings of the National Academy of Sciences* 109:19333-19338.
2. Tamura K., Qiqing T., and Kumar S. (2018). Theoretical Foundation of the RelTime Method for Estimating Divergence Times from Variable Evolutionary Rates. *Molecular Biology and Evolution* 35: 1770-1782.
3. Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Molecular Biology and Evolution* 18:691-699.
4. Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

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