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STUDYING THE GASTROINTESTINAL MICROBIOME OF A TRADITIONAL PERUVIAN COMMUNITY.

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NISHA B. PATEL
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STUDYING THE GASTROINTESTINAL MICROBIOME OF A TRADITIONAL PERUVIAN COMMUNITY.

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

_____
Dr. Paul A Lawson, Chair

_____
Dr. Krithivasan Sankaranarayanan, Co-Chair

_____
Dr. Ralph Tanner

_____
Dr. Cecil M. Lewis Jr.

_____
Dr. Christina Warinner

_____
Dr. Bradley Stevenson

I dedicate this dissertation to my parents, puppa & mota mommy, and my brother Akash without whose never-failing love, support and encouragement this work would not have been possible.

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Abstract

The human gastrointestinal (GI) tract is an ecosystem that is home to a plethora of bacteria, archaea, microeukaryotes, and viruses, collectively termed the "gut microbiota" or "microbiome" when referring to the genetic material of the microbiota. In order to better characterize global gut microbial diversity, non-industrialized population groups with distinctive lifestyles and diets should be included in gut microbiome studies. The microbial diversity of a traditional Peruvian community was explored using both culture dependent and independent approaches. First, an enrichment of a fecal sample from a community member was prepared using xylan as the primary substrate. Amongst the recovered isolates, two novel genera and one novel species belonging to the *Peptoniphilaceae* family, were identified and named as *Ezakiella peruensis*, *Citroniella saachavorans*, and *Peptoniphilus catoniae*. Second, the genomes of these novel bacteria were assembled and analyzed to examine the physiological potential of four phenotypic traits commonly used to discriminate these taxa from other members in the *Peptoniphilaceae* family. Finally, the use of Propidium Monoazide (PMA), was applied to human fecal samples for its ability to neutralize DNA from compromised bacterial cells, allowing for amplification of only DNA belonging to viable bacteria during downstream applications. While the proportion of live cells in each sample varied, PMA treatment did not significantly reduce microbial community richness and phylogenetic diversity. However, differences in the relative abundance of gut microbes, *Faecalibacterium*, *Holdemanella*, *Catenibacterium*, *Dorea*, and *Senegalemassilia* were observed.

Chapter 1: Introduction to the Human Gastrointestinal Tract

1.0 Diversity and Taxonomy

The human gastrointestinal (GI) tract is an ecosystem that is home to a plethora of bacteria, archaea, microeukaryotes, and viruses, collectively termed the "gut microbiota" (1). The number of microbes that inhabit the human gut has been estimated to exceed 100 trillion with a ratio between 1:1 and 1:20 bacterial to human cells (2). The GI tract is a complex ecosystem with cell densities ranging from $10^2 – 10^3$ cells/g in the proximal ileum and jejunum, to 10^{11} cells/g in the ascending colon (3). Microbial cell densities in the human gut are dependent upon chemical, nutritional, and immunological gradients along sections of the GI tract. For example, the small intestine is acidic with higher levels of oxygen than the large intestine, and contains antimicrobial peptides and bile acids, allowing for the relative abundance of facultative anaerobes such as members of the families *Lactobacillaceae* (*Firmicutes*) and *Enterobacteriaceae* (*Proteobacteria*) (4). In contrast, the microbial community in the large intestine is enriched in bacterial taxa belonging to *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae* (*Bacteroidetes*), *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* (*Firmicutes*), and *Verrucomicrobiaceae* (*Verrucomicrobia*) (4-6).

Multiple factors have been identified as impacting microbial community structure including diet (7-12), antibiotic use (13-15), different modes of birth (16, 17) and exposure to external microbial agents (18, 19). Despite these variations in community structure, *Bacteroides, Clostridium, Collinsella*, *Dorea, Eubacterium*, and *Roseburia*, are consistently observed in the human gut (20-23).

Gut microbiota have co-evolved with their human hosts to form complex mutualistic relationships (24). For example, degradation of dietary plant-derived polysaccharides (pectin, cellulose, hemicellulose and resistant starches) are driven by several 'keystone' species (those species upon which other species rely on for substrate degradation) to stabilize the gut ecosystem within the families *Bacteroidaceae, Lachnospiraceae*, and *Ruminococcaceae*. The byproducts resulting from these processes are readily utilized for energy by both microbial and human cells. Furthermore, microbial degradation of these substrates contribute to the production of short chain fatty acids (SCFAs) such as butyrate, acetate, succinate, propionate, and lactate (3), serving as an energy source for the host's colonic epithelial cells, playing a role in lipogenesis, and modulating the host immune system (3, 25). In addition to aiding in digestion, gut microbiota such as *Bifidobacterium* and *Lactobacilli* can contribute to vitamin synthesis (26). Only by studying the contributions and interactions of microbes with their human hosts can links be established between the microbiome and varying health and disease states, including immune/inflammatory responses.

1.1 Historical Perspective for Studying Gut Microbiota

Investigations of gut microbiota (collection of bacteria, archaea and eukarya) began in the late 1800s with the isolation of *Escherichia coli* from infant fecal samples (27, 28). Subsequent studies resulted in the recovery of key gastrointestinal bacterial groups including *Bacillus, Bacteroides*, and *Bifidobacterium* (29-31) with the latter two being reported as relatively more abundant taxa in the human gut (27). Methods for the isolation of anaerobic bacteria from mammalian sources were pioneered within ruminants and then applied to the isolation of individual microorganisms from the

human gut (32). This resulted in a landmark study by Holdeman & Moore in 1974, greatly expanded knowledge on dominant bacterial genera in the GI tract by describing previously unknown groups of *Bacteroides*, *Clostridium*, *Eubacterium*, *Veillonella*, *Ruminococcus*, *Bifidobacterium*, *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus*, and *Peptococcus* (33, 34). However, a key challenge to these early studies was the lack of high throughput approaches to properly characterize all recovered bacteria using biochemical, phenotypical, and morphological assays (35-37).

These limitations were overcome by the implementation of the 16S rRNA gene as a molecular marker; a concept first introduced by Carl Woese and colleagues (38, 39). The 16S rRNA gene is approximately 1,542 base pairs (bp) long but can vary substantially across different taxonomic groups due to single nucleotide polymorphisms (SNPs) and insertions/deletions within segments of the gene known as hyper-variable regions (1). These hyper-variable sites are bound by highly conserved regions, which serve as binding sites for universal primers, making the 16S rRNA gene an ideal marker to establish evolutionary relationships between organisms, and to distinguish microbial taxa at various phylogenetic levels (40). Recent developments in Next Generation Sequencing (NGS) have allowed for rapid identification of the majority of bacteria and archaea present in an environmental sample (1, 41-43). A limitation of using the 16S rRNA gene for molecular studies is the inability to detect viruses.

1.2 Current Approaches to Gut Microbiome Studies

The National Institutes of Health (NIH) launched the Human Microbiome Project (HMP) in 2008 with the stated objective to characterize the microbiome of 300 healthy individuals from the USA in order to determine if there was a core healthy

microbiome (50). High-throughput 16S rRNA gene sequencing data from archaea and bacteria were generated from a variety of body sites including the oral cavity, skin, distal GI, and urogenital system (50). In tandem with data from the Metagenomics of the Human Intestinal Tract consortium (MetaHIT), a comprehensive view of human-associated microorganisms is now available (51, 52). Collectively, these studies have identified 2,172 microbial species inhabiting the human body, of which 386 are strict anaerobes (51).

The term "culturomics" was coined by Lagier and colleagues who have described a large number of novel organisms recovered from the human GI (53, 54). Briefly, samples are divided into multiple culture conditions to target taxa of interest. Bacteria were isolated in pure cultures and then rapidly screened by comparing the crude extract of total cellular proteins to a database using a mass spectrometer (for example, MALDI-TOF-MS) (55, 56). Those deemed to be novel are then subjected to screening in a 16S rRNA gene database such as EzTaxon (57) using BLAST (58).

Alongside the recovery of taxa to establish "who's there" using 16S rRNA gene profiles, determining the function of individual microorganisms via the implementation of NGS methods is now becoming common. These studies are now gaining importance in helping to understand host-microbe and microbe-microbe interactions in human health and disease states (59). One important question raised over the past decade is whether or not a core set of microbial species are shared between all humans. An important consideration into the investigation of this core-microbiome is the inclusion of individuals from non-industrial, traditional societies.

This dissertation explores microbial diversity using both culture dependent and independent approaches within the human GI of a traditional Peruvian community. In chapter 2, novel taxa within the *Peptoniphilaceae* family were recovered from an enrichment using Xylan, a substrate commonly present in the diet of these individuals. Using a polyphasic approach, three bacteria were taxonomically assigned into two novel genera (*Ezakiella* & *Citroniella*) and a novel species in the *Peptoniphilus* genus. In chapter 3, the respective genomes of these taxa were assembled, and phylogenetic trees constructed using nearest neighbors to further justify relationships. Data from these analyses suggest the reclassification of *Peptoniphilus catoniae* into a novel genus. In chapter 4, a photoreactive dye Propidium monoazide (PMA) was applied to human fecal samples and 16S rRNA gene libraries generated to discern only viable taxa in human fecal samples. These analyses suggest while no differences in species richness and phylogenetic diversity were observed, there was a shift in species evenness in samples treated with PMA.

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Chapter 2: Recovery of Novel Taxa Belonging to the *Peptoniphilaceae* family from a Fecal Sample of a Traditional Peruvian Community Member[1]

[1] Portions of this dissertation have been published as the following articles in peer-reviewed journals:

Ezakiella peruensis gen. nov., sp. nov. isolated from human fecal sample from a coastal traditional community in Peru. Anaerobe, April 2015 32C: 43-48.

Peptoniphilus catoniae sp. nov. isolated from human fecal sample from a coastal traditional community in Peru. Int J Syst Evol Microbiol, May 2016 66: 2019-2024.

Citroniella saccharovorans gen nov. sp. nov. isolated from human fecal sample from a coastal traditional community in Peru. Int J Syst Evol Microbiol, (In revision)

Abstract

The fundamental goal of cultivation-based studies is to recover organisms in pure culture. Methods used to describe microorganisms includes the determination of physiological, morphological, chemotaxonomic, and genetic properties. An enrichment of a fecal sample belonging to a member from a traditional Peruvian community was prepared using Xylan as the primary substrate. Amongst the recovered isolates, novel taxa belonging to the *Peptoniphilaceae* family were identified and given strain names as: M6.X2^T, M6.X9^T, and M6.X2D^T. Analysis based on 16S rRNA gene sequencing demonstrated that two isolates (M6.X2^T and M6.X9^T) were phylogenetically distinct from other taxa in the family *Peptoniphilaceae* prompting the characterization of two new genera (*Ezakiella peruensis* and *Citroniella saachavorans*). The third isolate M6.X2D^T was taxonomically placed within the genus *Peptoniphilus* but revealed no particular relationship with any other species demonstrating less than 90% 16S rRNA gene sequence similarity with all members of the genus, resulting in the designation *Peptoniphilus catoniae*.

2.0 Introduction

The characterization of the human gut microbiome using 16S rRNA gene surveys has demonstrated that while these studies have predominantly focused on populations from urbanized societies (20, 60-64). It is acknowledged by the literature that non-industrialized population groups with distinctive lifestyles and diets should be included to (65-69) better characterize global gut microbial diversity. Due to a presence

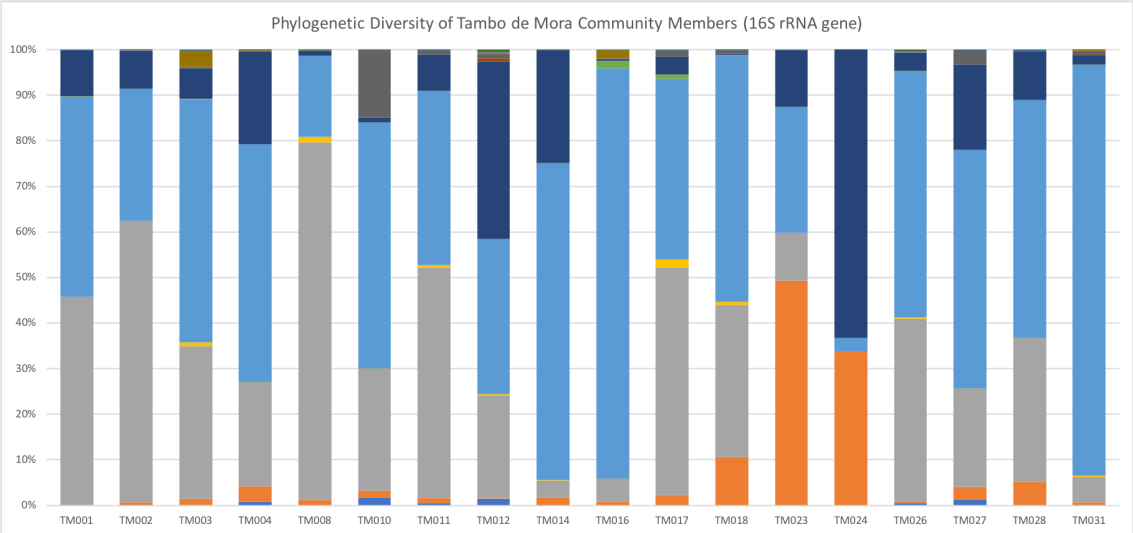


Figure 1: Phylogenetic Diversity of Tambo de Mora Community Members Based on 16S rRNA gene sequencing; TM=Tambo de Mora.

of *Treponema* and higher abundance of *Prevotella* in rural gut communities compared to urbanized societies, there has been a renewed interest in applying cultivation-based strategies to recover microorganisms.

Preliminary data employing molecular methods to characterize traditional populations from Peru (Matses (66) and Tambo de Mora (**Figure 1**) (unpublished (70)) suggest that members of the phylum Firmicutes comprised a large proportion of the fecal microbiome. In this study, a cultivation–based approach using an enrichment with Xylan was used to examine microbial diversity of a fecal sample (TM06). In addition to known xylanolytic bacteria, several isolates belonging to the Gram-stain Positive

Anaerobic Cocci (GPAC) group were recovered. Three were determined to be novel based on 16S rRNA gene similarities to nearest neighbors. A polyphasic approach was used to further characterize these novel taxa for proper taxonomic assignment.

2.1 Materials & Methods

2.1.1 Fecal Sample Collection

Samples (n=18) were collected from a traditional Peruvian community in Ica, Peru in the district of Tambo de Mora. The diet of community members consists of a high proportion of fish, fruits, and vegetables including corn, beans, cereal grains, chickpeas, rice, potatoes, and fried food (prepared at home). The community is agriculturally based where members raise chickens and ducks.

A sampling protocol was prepared by the Lawson Microbial Systematics Laboratory (Norman, Oklahoma) where members of the Peruvian team were trained in sample collection and processing as follows: freshly voided fecal samples were deposited into disposable plastic containers and immediately processed anaerobically in three separate methods; solid fecal samples were collected in microcentrifuge tubes, inoculated into pre-reduced anaerobically sterile transport media (PRAS) (Anaerobe Systems), and a fecal slurry prepared in anaerobic transport media with 20% glycerol; all samples were then transported in anaerobic jars with a 100% nitrogen headspace and kept on ice until they arrived at the Lawson Microbial Systematics Laboratory.

2.1.2 Enrichment Components

Medium two (71) was anaerobically prepared with the recipe modified to include xylan as the sole sugar source as follows (per 100 ml distilled water): Casitone

(1.0 g), yeast extract (0.25 g), minerals solution (A) (15 ml), minerals solution (B) (15 ml), clarified sterile rumen fluid (20 ml), resazurin (0.0001 g), sodium lactate (70% w/v) (1.0 g), xylan (0.2 g), cysteine HCl (0.05 g), sodium bicarbonate (0.4 g), distilled water (to 100 ml). Minerals solution (A) contains (per 1000 ml), K_2HPO_4 (3.0 g). Minerals solution (B) contains (per 1000 ml), KH_2PO_4 (3.0 g), $(NH_4)_2SO_4$ (6.0 g), NaCl (6.0 g), $MgSO_4*7H_20$ (0.6 g), $CaCl_2$ (0.6 g). The 50 ml medium was inoculated with 0.1 ml of fecal slurry and incubated for 14 days at 37°C with a gas mix of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen pressurized at 20 psi. After incubation, a sample of the enrichment was inoculated onto Medium two plus 1.5% agar. Isolates were then sub-cultured onto BD Bacto™ Brain Heart Infusion agar (Sparks, MD, USA) supplemented with 5% defibrinated sheep's blood until pure colonies were obtained. Isolates were incubated anaerobically with a gas mix of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen at 37°C.

2.1.3 DNA Isolation and 16S rRNA Gene Sequencing of Recovered Isolates

DNA was extracted from individual colonies using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) following manufacturer's instructions. Universal primers 8F (5’-AGAGTTTGATCCTGGCTCAG) (72) and 1492R (5’ GGTTACCTTGTTACGACTT) were used to amplify regions V1-V9 of the 16S rRNA gene (73). The resulting amplicon was purified (Exo-SapIT, USB Corporation) and sequenced (Big Dye Terminator v 3.1, model 3100 Avant, Applied Biosystems).

2.1.4 Phylogenetic Analysis of Recovered Isolates via 16S rRNA Gene Sequencing

Bidirectional sequencing allowed for ~1400 bp of the 16S rRNA gene to be sequenced for each isolate. Sequences were manually curated against the respective chromatograms and concatenated using Sequencher 5.4 (Gene Code Corporation) (74). Recovered sequences were tested for chimeras using USearch v8.0. (75). The closest known relatives of all isolates were determined by performing BLAST searches of their 16S rRNA gene sequence against the EzTaxon database (http://eztaxon-e.ezbiocloud.net) (57). The 16S rRNA gene sequences for nearest neighbors were then retrieved from the database, aligned, and trimmed (ClustalW (76)). A neighbor-joining phylogenetic tree was constructed using MEGA (Kimura two-parameter model, 1000 bootstrap replicates) (77).

2.1.5 Polyphasic Approaches to Characterizing Novel Taxa

Currently, a polyphasic approach is required for justifying the description of a novel bacterium (78). Before the routine use of 16S rRNA gene sequencing, isolates underwent time-consuming and labor-intensive methods to determine an exhaustive list of phenotypic and biochemical traits. However, the application of 16S rRNA gene sequencing and phylogenetic analysis allows for isolates to be first screened, and preliminary taxonomic classifications to be assigned.

For nearest relatives, literature searches can help determine the appropriate physiological, biochemical, and chemotaxonomic methods to be applied to each individual taxon (

Figure 2). These criteria will vary depending on the taxonomic group the novel bacterium is assigned to and must include methods that have been published and described for individual taxa. For example, members of the *Corynebacterineae* family require mycolic acid analysis, while polyamine distribution can serve as a discriminatory chemotaxonomic marker (78) for *Proteobacteria* and *Cyanobacteria*.

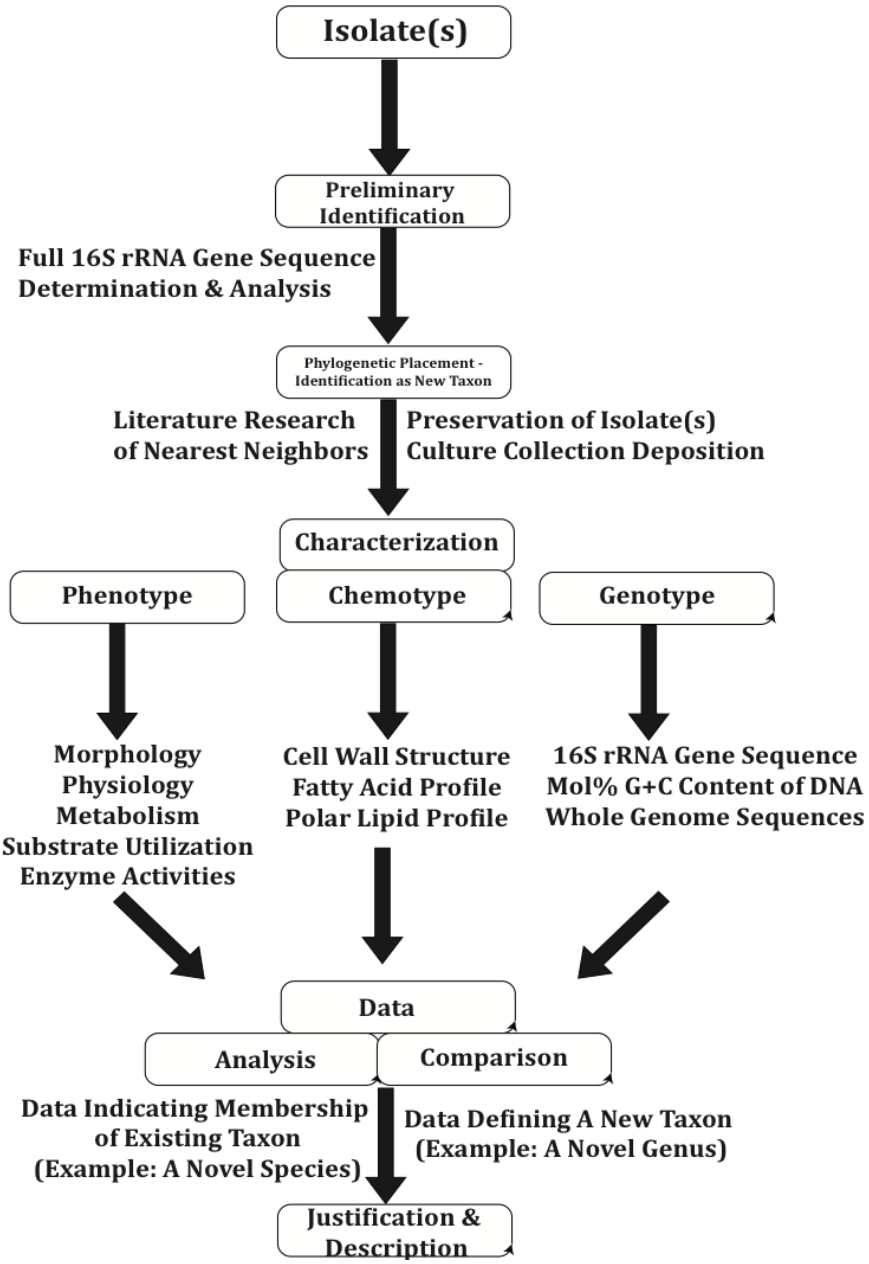


Figure 2: Road Map for the Description of a Novel Bacterium. Modified from Taxonomy of Prokaryotes (79).

2.1.6 Morphological, Physiological, and Biochemical Characterization

Cells were examined with an Olympus CX41 microscope using phase contrast 1000X magnification. Additional physiological characteristics included (under anaerobic conditions): temperature growth ranges (4-60 °C, in increments of ~5 °C) and

pH values (5.0-9.5, in increments of 0.5 pH units). Salt tolerance was examined using different concentrations of NaCl (0.0-0.5 % (w/v), and 1.0-9.0% (w/v), in increments of 1.0%). Optimum growth conditions were determined by monitoring the optical density (OD) using a spectrophotometer at 600 nm (Spectronic20D, Milton Roy, DE). An increase in OD >0.1 was considered an indicator of growth. All tests were performed in duplicate.

Metabolic end products were determined from cultures grown under anaerobic conditions in Peptone-Yeast-Glucose (PYG) broth (37°C, pH). Sample analyses were carried out in duplicate on an Aminex HPX-87H organic acid analysis column (Bio-Rad), using ion-exclusion HPLC with 0.015 HCl running buffer at a flow rate of 0.9 mL/min. Retention times and peak areas of fermentation products were compared to standards of acetate, butyrate, lactate, methyl-succinate, formate, and propionate.

API test strips (BioMérieux) involves the use of miniaturized enzymatic tests in the form of cupules containing dehydrated substrates to identify the enzymatic activity or the fermentation of sugars by the inoculated microbes. Upon incubation, color changes are observed or revealed by the addition of manufacturer provided reagents, thereby demonstrating enzymatic activity of the substrate of interest. Numerical profiles are then generated from the test results (**Figure 3**), which are then compared to data obtained from nearest relatives. The BIOLOG AN MicroPlate System (Hayward, CA) employs the use of redox chemistry to detect reactions within bacterial cells where each well contains a different carbon source. If cells are metabolically active (substrates are oxidized), tetrazolium violet dye is reduced and a purple color serves as an indicator for

a positive result. Both test systems were used in duplicate, according to manufacturer's instructions.

Positive reactions are coded into a numerical profile. Tests are separated into groups of three and the values corresponding to positive reactions are added together within each group. Image adapted from BioMérieux product insert.

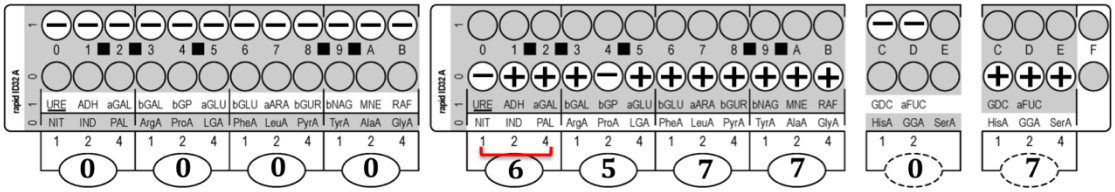


Figure 3: Example of API Test Cupule Interpretation

2.1.7 Chemotaxonomic Characterization

Fatty acids

The plasma membrane forms one of the major structural units of the bacterial cell envelope and is based on a bilayer system composed of polar lipids in intimate association with specific membrane proteins (80). Most chemotaxonomic procedures involve, to varying degrees, the extraction, fractionation, purification, and resolution of target compounds, and many of these chemotaxonomic procedures make use of the discontinuous distribution of chemical components (i.e. amino acids, sugars, lipids and proteins) found in the cell envelopes of prokaryotes (80). This variability of membrane composition has been used extensively in microbial systematics.

Fatty acids have been shown to be relatively stable taxonomic characters when extracted from cells grown under carefully standardized conditions. Taxonomic relationships can be resolved from detailed analysis of fatty acids, carboxylic acid derivatives of long-chain aliphatic molecules comprising the hydrophobic tail of polar lipids and usually varying between 12-24 carbon lengths (80, 81). Fatty acyl chains

present in bacteria include straight chain saturated/unsaturated fatty acids, iso and anteiso branched fatty acids, internally branched fatty acids, hydroxyl fatty acids, cyclopropane fatty acids among others (81) (**Figure 4**).

The Sherlock Microbial Identification Systems (MIS) (MIDI Inc) is a gas chromatography system used by microbial taxonomists to identify fatty acids and acyl compounds from bacterial and yeast cultures (81). As acyl chains can change with varying growth conditions, it is imperative that identical incubation temperature, growth phase, and medium be used for analysis between the novel taxa and previously described related species (81). Acyl chain identification is based on equivalent chain length (ECL), which correlates to a peak's retention time compared to standardized references.

Source Image modified from OpenStax College (https://openstax.org)

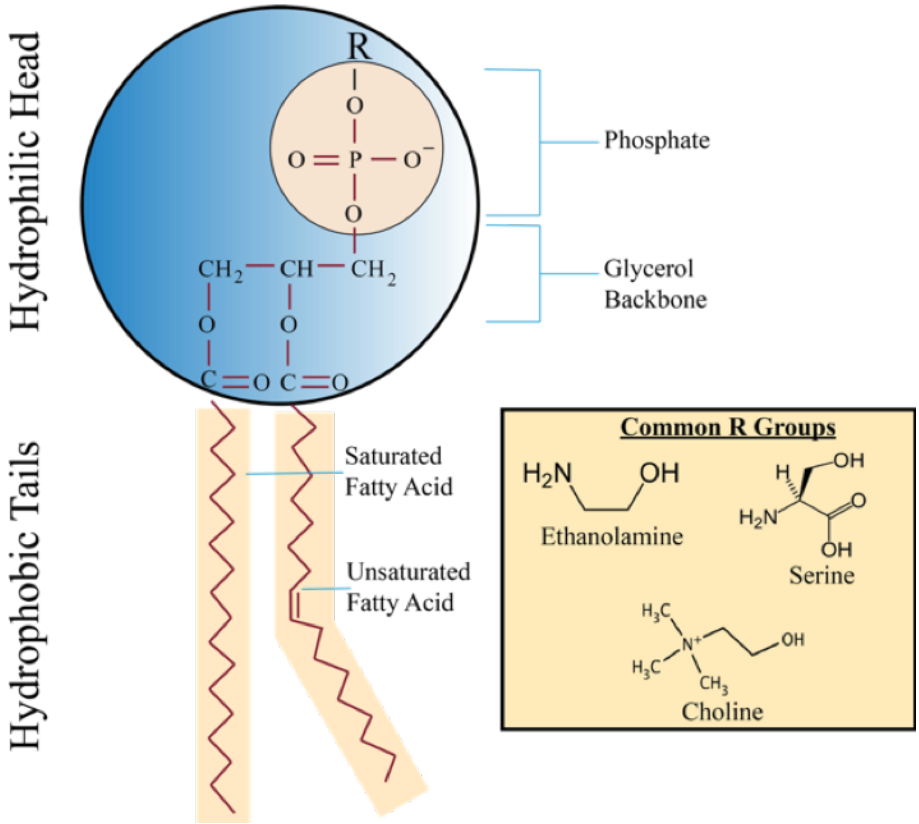


Figure 4: Phospholipids contain a hydrophobic head and hydrophobic tails.

Fatty Acid Methyl Ester (FAME) Identification

Biomass for fatty acid analysis was collected after a six-day incubation at 37 °C from a plate of BHI agar amended with 5% sheep's blood. Fatty acid methyl esters for all three isolates were extracted using the Sherlock Microbial Identification System (MIDI) version 6.1 as described previously (82, 83). Analysis was performed using an Agilent Technologies 6890N gas chromatograph equipped with a phenyl methyl silicone-fused silica capillary column (HP-2 25m 0.2 mm 0.33 mm film thickness) and a flame ionization detector with hydrogen used as the carrier gas. The temperature program was initiated at 170 °C and increased at 5 °C min^{-1} reaching a final temperature

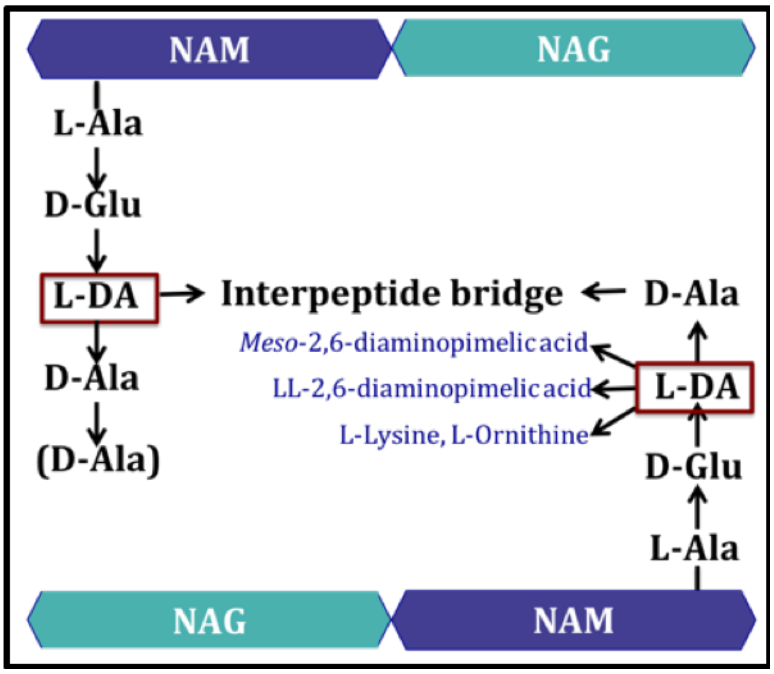
of 270 °C. Fatty acids were identified and expressed in the form of percentages using the peak naming database called QBA1.

Determination of Peptidoglycan Structure & Diagnostic Diamino Acid

Gram-negative bacteria have a remarkably uniform peptidoglycan structure, thus lacking resolution and providing limited taxonomic information for this group of organisms. However, Gram-positive bacteria have a large range of diversity in peptidoglycan structure that have been shown to be extremely useful in the assignment of taxonomic groups, especially in the pre-molecular era (84). The diamino acid at the third position of the stem peptide is recognized as an important diagnostic marker within the peptidoglycan (**Figure 5A**). The cross-linking peptide was determined using Protocol 1 (84). Briefly, three mg of lyophilized cells were incubated in 200 µL of 4.0 N HCl in a 2 ml glass ampoule. Upon incubation at 100 °C for 16 hours in a drying oven, the hydroxylate was filtered through charcoal to remove colored byproducts and dried down at 35°C. The residue was re-dissolved in 200 µL of distilled water and dried again. This procedure was repeated 5-6 times to remove all traces of acid. Two µL of sample dissolved in distilled water was spotted on the baseline of a 20 cm-long strip of a TLC plate (Cellulose; Merck 1.05577), along with the standards described by Schumann (84) and developed by spraying with ninhydrin reagent.

Gas chromatographic chiral analysis of the peptidoglycan amino acids and N-terminus of the interpeptide bridge (**Figure 5B**) were determined at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) according to Protocol 11 and 12 (84), or using the method of Hamada et al. (85) at the Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Japan.

A.



B.

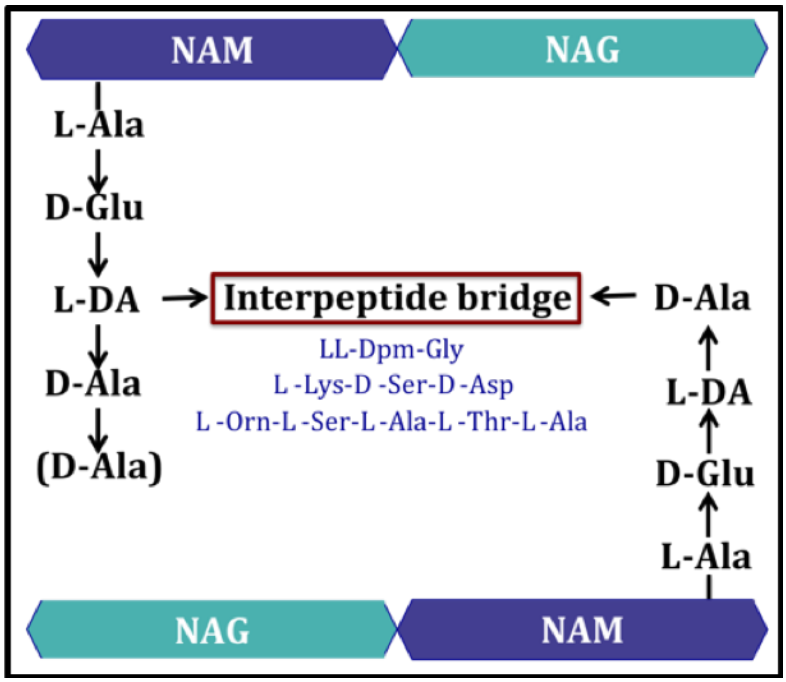


Figure 5: A). Examples of variations in the third position of the stem peptide (diagnostic diamino acid) and B). interpeptide bridge

Determination of Polar Lipids

A functional plasma membrane requires a balanced combination of the different structural types of polar lipids. Bacterial polar lipids typically consist of a glycerol

backbone attached to fatty acids via ester linkages with varying headgroups. The most common of these include phospholipids, glycolipids, glycophospholipids, aminolipids, and sulfur-containing lipids (78). While the polar lipids of Alpha, Beta, and Gammaproteobacteria have been well studied, those belonging to *Firmicutes* and *Actinobacteria* are not (78).

Polar lipids were extracted according to Tindall (1990) (86) via two-dimensional TLC run on silica gel plates (Macherey-Nagel). Two-dimensional TLC was run using chloroform/methanol/water (65:25:4, by vol.) as the solvent for the first phase, and chloroform/methanol/acetic acid/water (80:12:15:4, by vol) as the solvent for the second phase. Amino lipids were identified using ninhydrin, phospholipids were identified using molybdenum blue, and molybdophosphoric acid hydrate was used to obtain a total lipid profile (Sigma).

Determination of G+C Content

A standard description of bacterial taxa involves one the classic genotypic method of DNA base composition (mol% G+C) determination (87). Variations of G+C content are taxonomically-based, in that *Firmicutes* typically harbor low GC, compared to phyla (i.e., *Actinobacteria*) with higher GC values. For taxonomic purposes, the threshold for members of the same species and genus is 3% and 10% mol% G+C, respectively (87). Mol% G+ C was determined according to the method of Mesbah et al. (88).

Preservation of Novel Isolates in Culture Collection Depositions

In accordance with *International Code of Nomenclature of Prokaryotes* (89), all novel taxa must be deposited in two culture collections in two separate countries as part of the process to validly publish names of novel organisms proposed. Therefore, to comply with this rule, all three novel taxa were deposited with Culture Collection, University of Göteborg (CCUG) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), with isolate M6.X2^T being additionally deposited with Biological Resource Center, NITE (NBRC). Additionally, per an agreement with our Peruvian collaborators, the isolates were deposited with the Instituto Nacional de Salud (INS). Finally, the 16S rRNA gene sequence for M6.X2^T, M6.X9^T, and M6.X2D^T were deposited with the European Nucleotide Archive (https://www.ebi.ac.uk/ena/submit/sra/#home) and GenBank Sequence Databases (https://www.ncbi.nlm.nih.gov/genbank/).

2.2 Results & Discussion

2.2.1 Isolates Recovered from Xylan Enrichment

A total of 34 isolates were recovered and identified via full 16S rRNA gene sequencing (**Table 2**), with ~65% belonging to *Firmicutes* (*Enterococcus*, *Clostridium*, *Peptoniphilus*, and *Finegoldia*), ~32% to *Bacteroidetes* (*Bacteroides* and *Alistipes*), and ~3% to *Actinobacteria* (*Eggerthella*).

Eighteen (~53%) of the recovered clones including, *C. sporogenes*, *C. perfringens* (90, 91), *B. ovatus*, *B. intestinalis* (92-94) and *Alistipes* (95, 96) have been reported to degrade xylan or its derivatives.

Further, *Enterococcus* species have been associated with animals (GI tract) and humans (GI tract and clinical material) (97), and have not been observed to degrade xylan or its derivatives (98). Members of the *Peptoniphilaceae* family (*Peptoniphilus* & *Finegoldia*) also prefer to utilize nitrogenous compounds as opposed to carbohydrates (99). *Eggerthella* is a gut-associated bacterium that has been known to play a role in host lipid and xenobiotic metabolism (93), but also cause life-threatening infections in health-compromised patients (100). The type species for *E. lenta* has been shown to not utilize xylan or starch (101), so it is likely the isolate prefers the proteinaceous material (ie casitone or yeast extract) from the media as a source of energy.

In addition to isolates that matched previously described taxa, 18% of the recovered isolates did not match any known cultured representatives, demonstrating low 16S rRNA gene percent similarity (<94%). Isolates M6.X2^T, M6.X9^T, and M6.X2D^T were chosen for further taxonomic analysis, and a literature search of available characteristics for the nearest neighbors was performed to determine the appropriate methods for their taxonomic assignment.

Isolate	GenBank Accession #	DSM #	CCUG #	NBRC #
M6.X2^T *E. peruensis*	KJ469554	27367^T	4571^T	109957^T
M6.X9^T *C. saccharovorans*	MG496017	29873 ^{T}	66799 ^{T}	
M6.X2D^T *P. catoniae*	KR911910	29874^T	66798^T	

Table 1: Accession Numbers for Deposits into Culture Collections and GenBank

Strain Designation	Nearest Related Neighbor	Family;Genus	16S rRNA gene % Similiarity
Firmicutes			
M6.X2K M6.X2O M6.X2.V	*Enterococcus avium*	*Enterococcaceae;Enterococcus*	99.91
M6.X2.T	*Enterococcus raffinosus*	*Enterococcaceae;Enterococcus*	100.00
M6.X2L	*Enterococcus avium*	*Enterococcaceae;Enterococcus*	99.76
M6.X2G M6.X2H M6.X2I M6.X2J M6.X2M M6.3	*Clostridium sporogenes*	*Clostridiaceae;Clostridium*	99.73
M6.X2N	*Clostridium perfringens*	*Clostridiaceae;Clostridium*	99.83
M6.X6	***Negativicoccus succinicivorans***	***Veillonellaceae;Negativicoccus***	**98.81**
M6.X2^T	***Finegoldia magna***	***Peptoniphiliaceae;Finegoldia***	**86.70**
M6.X9^T	***Finegoldia magna***	***Peptoniphiliaceae;Finegoldia***	**88.76**
M6.X2D^T	***Peptoniphilus koenoeneniae***	***Peptoniphiliaceae;Peptoniphilus***	**90.95**
M6.X2S	***Peptoniphilus asaccharolyticus***	***Peptoniphiliaceae;Peptoniphilus***	**90.77**
M6.X2W	***Peptoniphilus methioninivorax***	***Peptoniphiliaceae;Peptoniphilus***	**90.95**
M6.X2U	*Peptoniphilus koenoeneniae*	*Peptoniphiliaceae;Peptoniphilus*	99.91
M6.X3	*Peptoniphilus harei*	*Peptoniphiliaceae;Peptoniphilus*	99.83
M6.X4	*Finegoldia magna*	*Peptoniphiliaceae;Finegoldia*	99.78
M6.X5	*Finegoldia magna*	*Peptoniphiliaceae;Finegoldia*	99.89
Bacteroidetes			
M6.X2-A M6.X2-B M6.X2-C M6.X2-D M6.X2.CP1 M6.X2.CP3 M6.X2Q M6.X2P M6.X2R M6.X7	*Bacteroides ovatus*	*Bacteroidaceae;Bacteroides*	99.70
M6.X2E	*Bacteroides intestinalis*	*Bacteroidaceae;Bacteroides*	99.91
Actinobacteria			
M6.X2.CP2	*Eggerthella lenta*	*Coriobacteriaceae;Eggerthella*	99.91

Table 2: Cultured Representatives from Xylan Enrichment.
Taxa designated by bold/underlining are those described in this chapter. Those in bold are novel taxa to be characterized in future work.

2.2.2 Phylogenetic Analysis of Novel Taxa

Justification of Delineation of Novel Taxa Within Peptoniphilaceae

Pairwise comparisons based on 16S rRNA gene sequence of isolates M6.X2^T, M6.X9^T, and M6.X2D^T showed their phylogenetic relationship to members of the Gram-Stain Positive Anaerobic Cocci (GPAC) group in the *Firmicutes* phylum. Gram-stain positive anaerobic cocci are part of the commensal flora of humans and animals, and are associated with a variety of human infections (102, 103). This group of organisms has undergone extensive taxonomic changes with many former members of the *Peptostreptococcaceae* being transferred to a number of novel genera that encompass *Peptoniphilus*, *Anaerococcus*, *Finegoldia*, *Gallicola*, and *Parvimonas* (103, 104). In addition, the genera *Anaerosphaera*, *Helcococcus,* and *Murdochiella* have also been described, and are phylogenetically related and phenotypically similar to the aforementioned genera (105-107). However, until recently, the precise relationship of these groups of organisms with other related members of *Firmicutes* was somewhat uncertain and was reflected in their placement in the Family XI *Incertae Sedis* (order *Clostridiales*, class *Clostridia*, phylum *Firmicutes*) in *Bergey's Manual of Systematic Bacteriology* (108, 109).

In order to provide some taxonomic structure, Johnson et al. (99) described the family *Peptoniphilaceae* to accommodate the genera *Peptoniphilus*, *Anaerococcus*, *Anaerosphaera*, *Finegoldia*, *Gallicola*, *Helcococcus*, *Murdochiella*, and *Parvimonas*. With the exception of *Helcococcus,* genera in this family do not ferment carbohydrates and prefer to metabolize amino acids and peptone for energy instead. Major end products as a result of fermentation processes are butyrate, acetate, and lactate.

Predominant fatty acids (>10%) include $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$. Within the peptidoglycan, the diagnostic diamino acids are alanine, aspartate, lysine, ornithine, or glumatic acid. Finally, the G+C content is between 27-35 mol%.

Pairwise comparisons demonstrated that isolates M6.X2^T and M6.X9^T shared the following 16S rRNA gene similarity values: *Finegoldia* (86.7% & 88.8% sequence similarity, respectively), *Gallicola* (83.5% & 87.1% sequence similarity, respectively) and *Parvimonas* (84.4% & 87.2% sequence similarity, respectively). These values are well below the 94.5% threshold currently recommended for delineation of genera based on 16S rRNA gene sequence analysis (110, 111).

Isolates M6.X2^T and M6.X9^T are Gram-stain positive, strictly anaerobic, non-motile, and non-sporeforming bacteria. Fatty acid profiles for both isolates are consistent with trends observed in this family with the presence of $C_{16:0}$ (31.6% & 21.7%) and $C_{18:1}\ \omega_{9C}$ (32.8 % 12.0) as major fatty acids (**Table 3**). Furthermore, cell-wall murein is consistent with the family description with the presence of Lysine (M6.X2^T) and Ornithine (M6.X9^T). Through these similarities, differences in enzymatic activities and substrate utilization show that on a physiological level, these isolates are distinct from their nearest neighbors. For example, M6.X2^T shows activity for Arginine arylamidase, while members of *Helococcus* and *Gallicola* do not. Additionally, M6.X9^T has the ability to ferment glucose and maltose. *Helococcus* is the only other genus in this family that also utilizes carbohydrates.

Bootstrap values from the Neighbor-Joining Tree (**Figure 6)** indicate the phylogenetic placement for these two taxa are loosely supported within the

Peptoniphilaceae family and is likely to shift as novel taxa are added within this family. A maximum-likelihood tree showed the same overall tree topology. G+C values for M6.X2^T (38.4 mol%) indicate that the family description will need to be amended to include values belonging to this genus (current range is 27-35 mol%).

Based on differential characteristics within this family, the proposal for isolates M6.X2^T and M6.X9^T to represent novel genera within the *Peptoniphilaceae* family is supported with M6.X2^T being named as *Ezakiella peruensis* gen. nov. sp. nov, and M6.X9^T representing *Citroniella saccharovorans* gen. nov. sp. nov.

Isolate M6.X2D^T also demonstrated to be phylogenetically a member of the phylum *Firmicutes*, specifically placed within the genus *Peptoniphilus*, with 90.95% similarity to *Peptoniphilus koenoeneniae* (112). Pairwise comparisons of this novel organism in a phylogenetic tree demonstrates that the novel organism does not exhibit a close relationship with any other species within the *Peptoniphilus* genus with pairwise comparisons of the 16S rRNA gene giving values less than 90%. To date, the genus *Peptoniphilus* consists of 16 validly published named species- *Peptoniphilus asaccharolyticus* (104), *Peptoniphilus catoniae*, (113) *Peptoniphilus coxii* (114), *Peptoniphilus duerdenii* (112), *Peptoniphilus gorbachii* (115), *Peptoniphilus harei* (104), *Peptoniphilus indolicus* (104), *Peptoniphilus ivorii* (104), *Peptoniphilus koenoeneniae* (112), *Peptoniphilus lacrimalis* (112), *Peptoniphilus methioninivorax* (116), *Peptoniphilus olsenii* (115), *Peptoniphilus senegalensis* (117), *Peptoniphilus stercorisuis* (99), *Peptoniphilus timonensis* (118), *Peptoniphilus tyrrelliae* (114). While the majority of the species have been described from human clinical specimens, *P.*

indolicus was isolated from cattle, *P*. *methioninivorax* from retail ground beef, and *P*. *stercorisuis* from a swine manure storage tank.

A phylogenetic tree of M6.X2D^T with nearest relatives, yielded low bootstrap values, (~60%) indicating that while this isolated is related to the *Peptoniphilus* genus (**Figure 6**) the exact placement within this *Peptoniphilaceae* family cannot be conclusively resolved. However, the lack of carbohydrate fermentation and end products of acetate and butyrate are consistent with the genus description. Furthermore, the presence of ornithine in the cell wall murein also supports the inclusion of this organism within this genus coupled with the presence of $C_{16:0}$ as a major fatty acid. Justification for M6.X2D^T as a novel species can be made due to lack of enzymatic activity using the API test systems that resulted in only a positive observation for leucyl glycine arylamidase. Therefore, M6.X2D^T is presented as *Peptoniphilus catoniae* sp. nov.

Neighbor Joining Tree

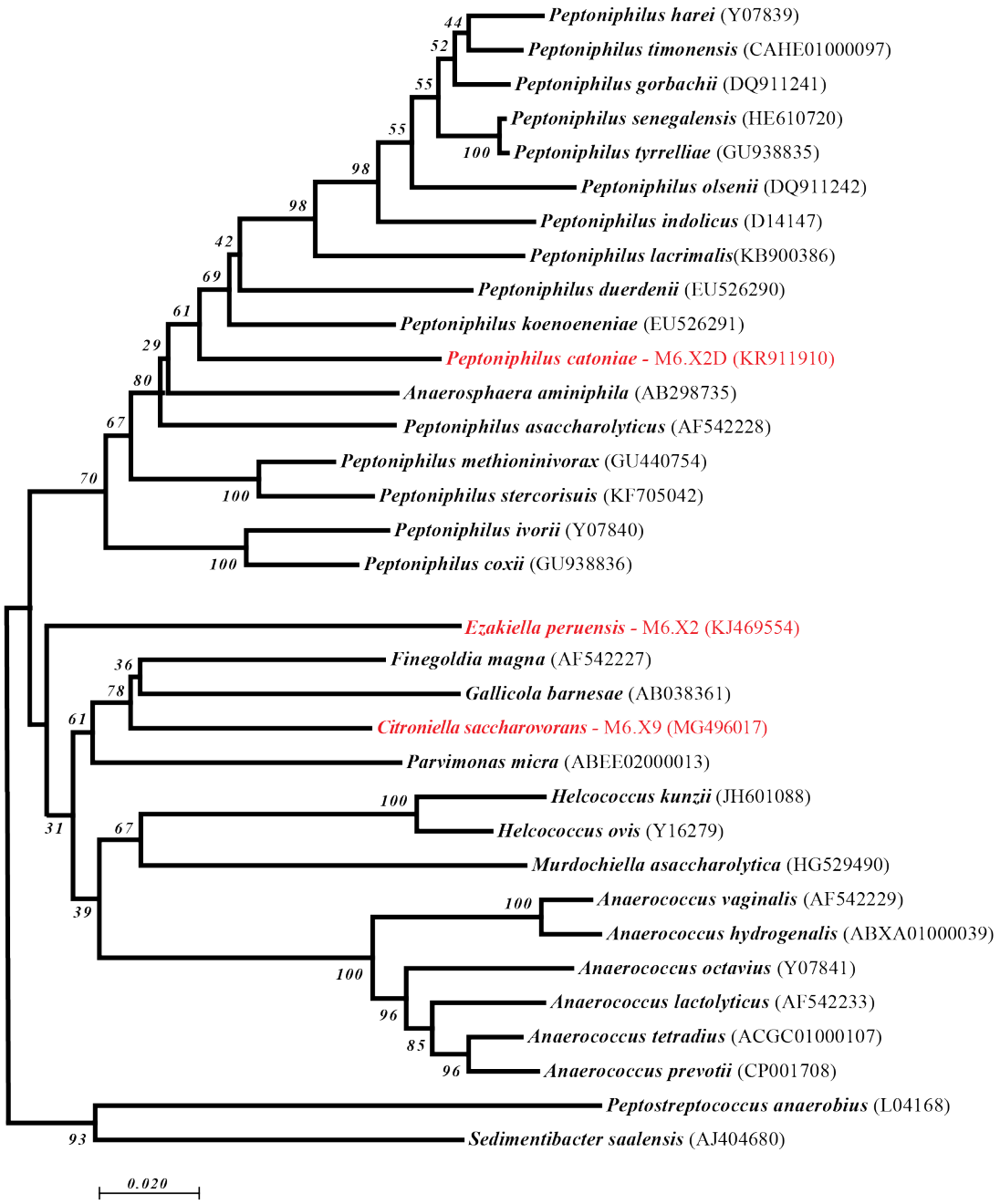


Figure 6: Phylogenetic Placement of Isolates M6.X2^T, M6.X2D^T, and M6.X9^T Within the *Peptoniphilaceae* Family Using the Neighbor Joining Treeing Method.

Peptostreptococcus anaerobius served as the outgroup. The tree was constructed using an alignment corresponding to *Escherichia coli* base-pair positions 100-1300 on the 16S rRNA gene. Bootstrap values (%) were obtained with 1000 replicates and are displayed on their relative branches.

2.2.3 Formal Description of *E. peruensis* gen. nov. sp. nov.

Ezakiella (E.za'kie.'lle N.L. fem. dim. named after the Japanese microbiologist Takayuki Ezaki who has contributed immensely to the taxonomy of the anaerobic Gram-positive cocci bacteria].

Ezakiella peruensis (pe.ru.en'sis. N.L. fem. adj. *peruensis* pe.ru.en'sis N.L. gen. n. pertaining to the country of Peru from where the organism was first isolated).

M6.X2^T is a non-motile, Grain-stain positive, diplococci that is strictly anaerobic, non-spore forming and non-hemolytic. Growth on BHI blood agar plates after six days at 37°C colonies are small (<1 mm in diameter), clear, circular, and convex. Cells are catalase negative, a characteristic that is common among anaerobes as the enzyme is typically found in microbes that have the ability to survive oxygen exposure. The isolate is also urease negative, indicating its inability to produce the enzyme to hydrolyze urea to ammonia and carbon dioxide. Nitrate is not reduced, meaning the isolate does not possess the enzyme nitrate reductase to reduce nitrate to nitrite. Finally, the isolate tested positive for its ability to convert tryptophan to form indole. The range for growth is from 30-37°C with an optimum temperature of 37°C. The pH range for growth is pH 7.0-8.5, with an optimum pH of 7.75. Growth occurs at NaCl concentrations of 0.5% (w/v) only.

Using the API Rapid 32A test system, positive reactions are observed for alkaline phosphatase, arginine arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, and glutamyl glutamic acid. Negative reactions were obtained for arginine dihydrolase, proline arylamidase, D-galactosidase, β-galactosidase, β-galactosidase-6 phosphate, D-glucosidase, bglucosidase, D-arabinosidase, β-glucuronidase, N-acetyl- β-glucosaminidase, glutamic

acid decarboxylase, D-fucosidase, mannose, raffinose, serine arylamidase and urease. The rapid ID 32A profile number is 0000 6577 07. Using the BIOLOG AN Microplate system, M6.X2^T showed utilization for L-malic acid, pyruvic acid, pyruvic acid methyl ester, L-serine, 2'-Deoxy adenosine, inosine, thymidine, uridine and uridine-5'Monophosphate. Weak reactions were observed for palatinose, L-rhamnose, D-fructose, and L-fucose. Negative reactions were observed for all other substrates in the BIOLOG AN MicroPlate. Fermentation end products from PYG are methyl succinate, acetate, and butyrate, but do not include formate. Fermentation end products from PY are methyl-succinate, acetate, propionate, and butyrate.

The fatty acid data of strain M6.X2^T represented the following major fatty acids (%) (**Table 3**): $C_{16:0}$ (18.3), $C_{18:1\omega9c}$ (39.8) and $C_{18:2\omega6,9C/C18:0\ ANTE}$ (13.2). The minor fatty acids were $C_{10:0}$ (7.3), $C_{13:0\ ANTEISO}$ (3.1), $C_{14:0}$ (3.0), $C_{17:1\ \omega8c}$ (1.6), $C_{18:0/17:0\ CYCLO}$ (5.2), $C_{18:1\ \omega7c}$ (4.2), $C_{15:0}$ (1.6), $C_{16:1\ \omega7c}$/$C_{16:1\ \omega6c}$ (1.4), and $C_{17:0}$ (5.2). The novel organism and its nearest relatives all produce $C_{16:0}$, $C_{18:1\omega9c}$ and $C_{18:2\omega6,9C/C18:0\ ANTE}$ as the major products. The peptidoglycan analysis revealed the presence of a A4∝ type (A11.35) with an interpeptide bridge comprising L-Lys-L-Ala-L-Glu. Isolate M6.X2^T possesses DPG (diphosphatidylglycerol), PG (phosphatidylglycerol), and PE (phosphatidylethanolamine) polar lipids in its membrane in addition to three unidentified phospholipids, two aminolipids, three unidentified lipids, and an unidentified cholesterol containing lipid. The type species is *E. peruensis*. M6.X2^T (DSM 27367^T = NBRC 109957^T = CCUG 64571^T). The DNA G+ C content of isolate M6.X2^T was determined to be 38.4 mol%.

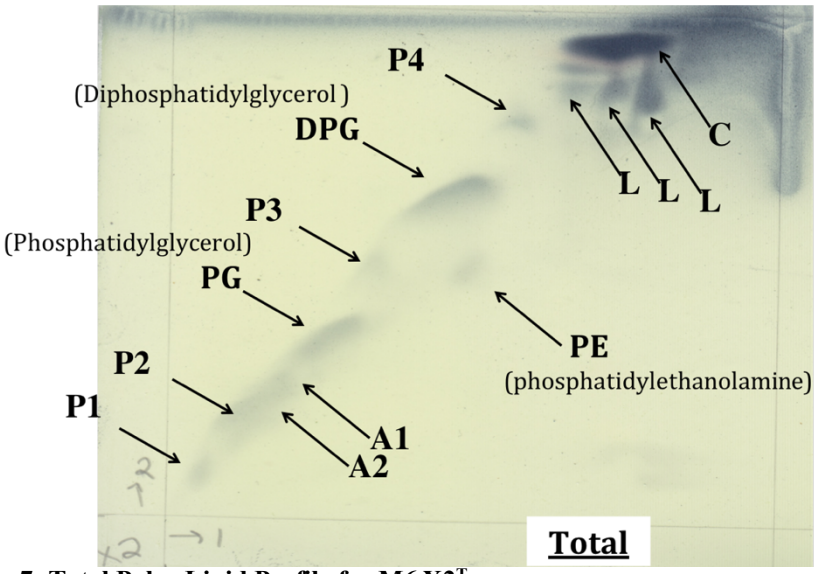


Figure 7: Total Polar Lipid Profile for M6.X2^T

Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; P, unidentified phospholipid; A, unidentified aminolipid; C, unidentified cholesterol lipid.

2.2.4 Formal Description of *C. saccharovorans* (M6.X9^T)

***Citroniella saccharovorans* gen. nov. sp. nov.**

Ci.tro.ni.el'la. N.L. fem. dim. n. *Citroniella* named in honor of the American microbiologist Diane Citron for her numerous contributions to the taxonomy of anaerobic bacteria.

(sac.cha.ro.vo'rans. Gr. n. sakchâr sugar; L. part. adj. *vorans* devouring; N.L. pres. part. *saccharovorans* sugar-devouring, pertaining to the utilization of sugars).

M6.X9^T is a strictly anaerobic, Gram-stain positive, non-motile, non-spore forming, coccus shaped bacterium. Growth on Brucella blood agar plates after six days at 37°C are observed to be non-hemolytic, small (<1 mm in diameter), white, smooth, circular, and convex. M6.X9^T is catalase and urease negative, but indole positive. Growth occurs between 25–37 °C (optimum at 30°C), at pH 6.5-9.0 (optimum between 6.5-7.3), and with 0.5-6.0% NaCl (w/v, optimum between 1-3%). Using the API Rapid ID 32A test systems, indole production, arginine arylamidase, leucine arylamidase are positive, with weak reactions for tyrosine arylamidase. The rapid ID 32A profile number is 0000 2120 00.

Using the BIOLOG AN Microplate system, α-D-glucose, D-cellobiose, dextrin, 3-methyl-D-glucose, maltose, maltotriose, palatinose, glyoxylic acid, turanose, pyurvic acid, pyruvic acid methyl ester, L-asparagine, glycerl-L-aspartic acid, glycyl-L-glutamine, glycyl-L-methionine, glycyl-L-proline, L-serine, L-threonine, inosine, and uridine demonstrated positive reactions. Weak reactions are observed for N-acetyl-D-glucosamine, D-fructose, L-fucose, D-galactose, lactulose, β-methyl-D-galactoside and thymidine. Negative reactions were observed for all other substrates in the BIOLOG AN MicroPlate. Fermentation end products from PYG and PY are acetate and methyl-succinate.

The FAME data of isolate M6.X9^T represented the following major fatty acids (%) (**Table 3**): $C_{16:0}$ (19.2), $C_{17:1\omega8c}$ (15.0), and $C_{18:1\omega9c}$ (12.0). The minor fatty acids were $C_{10:0}$ (4.6), $C_{12:0}$ (2.0), $C_{14:0}$ (4.30), $C_{15:0}$ (6.85), $C_{17:1\omega6c}$ (4.0), $C_{17:0}$ (7.6), $C_{18:2\omega6c,9c/18:0\ ante}$ (6.27), $C_{18:1\omega7c}$ (4.4), and $C_{18:0/17:0\ cyclo}$ (7.0). Given the typically high abundance of the straight-chain saturated fatty acid $C_{16:0}$ that is consistent between members in the *Peptoniphilaceae* family, the fatty acid profile of M6.X9^T supports the placement within this family.

The peptidoglycan analysis revealed the presence of glycine, glutamic acid, and ornithine with glutamic acid represented in the N-terminus of the interpeptide bridge. From the provided data, it was determined that the isolate displayed the peptidoglycan type A4β' type (L-Orn – D-Glu), similar to type A21.5, but with a substitution of the L-Ala residue that is normally found at position 1 of the peptide subunit in cross-linkage type A peptidoglycan by Glycine. Polar lipids in the cellular membrane consist of Diphosphatidylglycerol (DPG) and Phosphatidylglycerol (PG). Phosphatidylethanolamine is absent. Additional lipids include unidentified phospholipids, amino lipids, and glycolipids. The type species is *Citroniella saccharovorans* M6.X9^T (DSM 29873 = CCUG 66799). The DNA G+ C content of the type strain of the type species is 29.9 mol%.

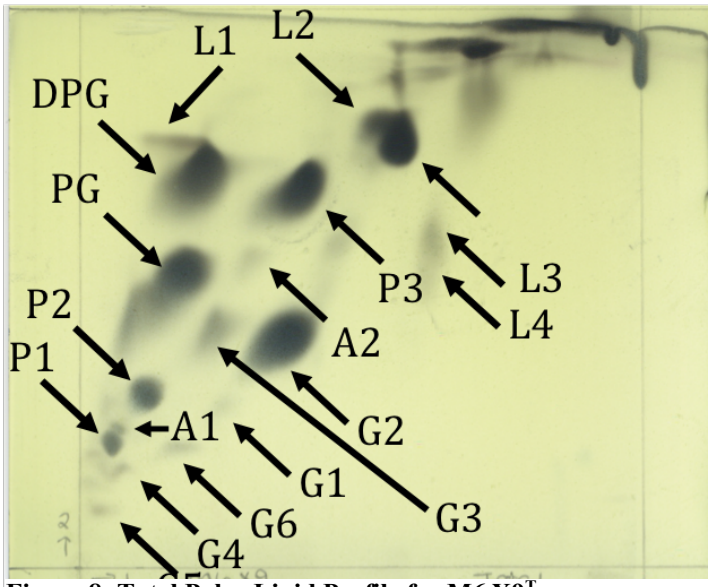


Figure 8: Total Polar Lipid Profile for M6.X9^T

Diphosphatidylglycerol; PG, phosphatidylglycerol; P, unidentified phospholipid; A, unidentified aminolipid; L, unidentified lipid; G, unidentified glycolipid

2.2.5 Formal Description of *P. catoniae* (M6.X2D^T)

***Peptoniphilus catoniae* gen. nov. sp. nov.**

Peptoniphilus catoniae (ca.to'ni.ae. L. gen. n. *catoniae*, of Cato, in honor of Elizabeth P. Cato, a United States microbiologist for her many contributions to anaerobic microbiology).

M6.X2D^T is a Gram-stain-positive, coccus shaped, strictly anaerobic, and non-motile bacterium. When grown on Brucella blood agar, colonies are needlepoint in size, beige, circular, with a smooth surface and raised elevation and are non-hemolytic. The cells are typically 1.0-2.0 μm x 1.0-5.3 μm in size and look like twisted chains. The isolate is catalase and urease negative with nitrate not reduced and indole not produced. The growth temperature range is between 30°C and 43°C with optimal growth occurring at 37°C. The growth pH range is between 6.5-9.0 with optimal growth at pH 6.5. Salt tolerance is between 0.5%-2.0% with optimal growth at 0.5%.

Using the API Rapid 32A test system, acid is produced with leucyl glycine arylamidase but not with arginine dihydrolase, proline arylamidase, D-galactosidase, ß-galactosidase, ß-galactosidase-6 phosphate, D-glucosidase, ß-glucosidase, D-arabinosidase, ß-glucuronidase, N-acetyl-ß-glucosaminidase, glutamic acid decarboxylase, D-fucosidase, serine arylamidase, arylamidase, alkaline phosphatase, arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid, mannose, or raffinose. The rapid ID 32A profile number is 0000 0400 00. Using the Biolog AN MicroPlate system, positive reactions are produced from utilization of malic acid, pyruvic acid and pyruvic acid methyl ester, and weaker reactions are obtained from d-fructose, l-fucose, palatinose,

fumaric acid, α-ketobutyric acid, α-ketovaleric acid, l-methionine, l-valine plus l-aspartic acid, inosine, thymidine, uridine and 2′-deoxyadenosine.

The FAME data of isolate M6.X2D^T represented the following major fatty acids (%): ($C_{16:0}$ (16.4) and $C_{18:1}\omega$9c (44.9). The minor fatty acids were $C_{10:0}$ (1.6), $C_{12:0}$ (1.4), $C_{13:0\ ANTEISO}$ (2.6), $C_{14:0}$ (7.9), $C_{15:0\ ANTEISO}$ (1.4), $C_{15:0}$ (3.4), $C_{17:1}$ ω8c (3.3), and $C_{19:0\ CYCLO}$ ω8c $_{/20:0}$ (1.7). $C_{15:0\ ISO}$ and $C_{17:0}$ were present in trace amounts. The fatty acid composition of strain M6.X2D^T as well as some other type strains of the genus *Peptoniphilus* are illustrated in Table 3. The major products of $C_{16:0}$ and $C_{18:1}\omega$9c are consistent with most species of the genus. The end products from PYG are acetate and butyrate, which are consistent with observations reported in other studies regarding volatile short-chain fatty acid metabolic end products generated by members of the genus *Peptoniphilus* (99, 112). Polar lipid determination was not performed for M6.X2D^T since this particular chemotaxonomic method has not been applied to this genus and therefore would not be useful for differential purposes for describing a new species. The type strain M6.X2D^T (DSM 29874^T = CCUG 66798^T). The DNA G+ C content of the type species is 34.4 mol%.

Fatty Acid[a]	1	2	3	4	5	6	7	8	9	10
$C_{10:0}$		4.6	**12.1**			9.4				
$C_{12:0}$		2.0	6.2			4.8				**12.0**
$C_{14:0}$	7.7	4.3	**12.8**	2.0	2.0	5.9	**11.0**	2.5	1.6	5.4
$C_{15:0}$	2.7	6.9	2.1							
iso-$C_{15:0}$										2.6
anteiso-$C_{15:0}$	1.5									
$C_{16:0}$	**31.6**	**21.7**	**19.1**	**17.1**	9.4	**17.6**	**34.0**	**30.0**	**13.4**	**14.4**
$C_{16:0\ alde}$					6.8	6.4				
$C_{16:0\ DMA}$					6.5					
$C_{16:1}$ w7c / $C_{16:1}$ w_{6c}	1.4		1.9							
$C_{16:1}$ w7c	2.4				2.0			2.8	2.0	3.9
$C_{16:1}$ w9c					7.4					
$C_{17:1w}$8c		**15.0**	1.6		**14.3**					
$C_{17:1w}$6c		4.0								
$C_{17:0}$		7.6								
$C_{17:1w}$9c					7.1					
iso-$C_{17:1w}$5c				3.9						3.0
anteiso-$C_{17:0}$	1.5			1.7		4.5				1.6
iso-$C_{17:1}$/ $C_{16:0\ DMA}$						**18.2**				
Cyclo $C_{17:0}$ / $C_{18:0}$	5.9	7.0								
$C_{18:0}$				**11.5**				**16.0**	6.8	9.4
$C_{18:0\ ald}$					4.4					
$C_{18:0\ DMA}$					2.0					
$C_{18:1}$ w_{7c}	4.9	4.4			6.9					
$C_{18:1}$ w_7 DMA					**12.2**					
$C_{18:1}$ w9c	**32.8**	**12.0**	**18.3**	**19.3**	2.0	3.6	**54.0**	**19.3**	**15.5**	**20.2**
$C_{18:2}$ $w_{6,9c}$ / $C_{18:0\ ANTE}$	10	6.3	**10.8**	**27.0**		5.6		**29.4**	**58.3**	**22.0**
$C_{18:1}$ w9c DMA					6.4	**11.1**				6.6
iso-$C_{19:1}$				2.0						1.5
Unknown $C_{17.045}$		1.4								
Unknown $C_{18.177}$				6.9		**13.1**				5.1
Unknown $C_{18.465}$		2.9								

Table 3: Fatty acid profiles of isolate M6.X2^T, M6.X9^T, and M6.X2D^T and close relatives. (>10%) are shown in bold, values below 1% are not shown**.**

1., *Ezakiella peruensis* M6.X2^T (119); 2., *Citroniella saccharovorans* M6.X9^T (120); 3., *Peptoniphilus catoniae* M6.X2D^T (113); 4. *Anaerococcus prevotii* CCUG 41932^T (104); 5., *Anaerosphaera aminiphila* JCM 15094^T (105); 6., *Finegoldia magna* CCUG 17636^T (121); 7., *Murdochiella asaccharolytica* CCUG 55976^T (107); 8., *Helcococcus kunzii* CCUG 32213^T (122); 9., *Parvimonas micra* CCUG 46357^T (123); 10., *Peptoniphilus asaccharolyticus* CCUG 9988^T (104). Unless stated, data was taken from www.CCUG.se. [a] Predominant products.

ACTIVE INGREDIENTS	REACTIONS	M6.X2^{T}	M6.X9^{T}	M6.X2D^{T}
Urea	Urease	-	-	-
L-arginine	Arginine Dihydrolase	-	-	-
4-nitrophenyl-DD-galactopyranoside	D-Galactosidase	-	-	-
4-nitrophenyl-ßD-galactopyranoside	ß-Galactosidase	-	-	-
4-nitrophenyl-ßD-galactopyrano-side-6-phosphate-2CHA	ß-Galactosidase 6-Phosphate	-	-	-
4-nitrophenyl-DD-glucopyranoside	D-Glucosidase	-	-	-
4-nitrophenyl-ßD-glucopyranoside	ß-Glucosidase	-	-	-
4-nitrophenyl-DL-arabinofuropyranoside	D-Arabinosidase	-	-	-
4-nitrophenyl-ßD-glucuronide	ß-Glucuronidase	-	-	-
4-nitrophenyl-N-acetyl-ßD-glucosaminide	N-Acetyl-ß-Glucosaminidase	-	-	-
D-mannose	Mannose fermentation	-	-	-
D-raffinose	Raffinose fermentation	-	-	-
Glutamic acid	Glutamic acid Decarboxylase	-	-	-
4-nitrophenyl-DL-fucopyranoside	D-Fucosidase	-	-	-
Potassium nitrate	Reduction of Nitrates	-	-	-
L-tryptophan	Indole production	+	+	-
2-naphthyl-phosphate	Alkaline Phosphatase	+	-	-
L-arginine-ß-naphthylamide	Arginine Arylamidase	+	+	-
L-proline-ß-naphthylamide	Proline Arylamidase	-	-	-
L-leucyl-L-glycine-ß-naphthylamide	Leucyl Glycine Arylamidase	+	-	+
L-phenylalanine-ß-naphthylamide	Phenylalanine Arylamidase	+	-	-
L-leucine-ß-naphthylamide	Leucine Arylamidase	+	+	-
Pyroglutamic acid ß-naphthylamide	Pyroglutamic acid Arylamidase	+	-	-
L-tyrosine-ß-naphthylamide	Tyrosine Arylamidase	+	-	-
L-alanyl-L-alanine-ß-naphthylamide	Alanine Arylamidase	+	-	-
L-glycine-ß-naphthylamide	Glycine Arylamidase	+	-	-
L-histidine-ß-naphthylamide	Histidine Arylamidase	+	-	-
L-glutamyl-L-glutamic acid ß naphthylamide	Glutamyl Glutamic acid Arylamidase	-	-	-
L-serine-ß-naphthylamide	Serine Arylamidase	+	-	-

Table 4: APIRapid ID 32A Results of Distinguishing Characteristics Comparing M6.X2^{T}, M6.X9^{T}, and M6.X2D^{T}.

	Ezakiella peruensis M6.X2^{T}	*Citroniella saccharovorans* M6.X9^{T}	*Peptoniphilus catoniae* M6.X2D^{T}
D-Galactose	-	+	-
D-Cellobiose	-	+	-
Dextrin	-	+	-
D-Glucose	-	+	-
Maltose	-	+	-
Maltotriose	-	+	-
3-Methyl-D-Glucose	-	+	-
Palatinose	w	+	w
Turanose	-	+	-
Fumaric Acid	-	-	+
Glyoxylic Acid	-	+	-
L-Malic Acid	+	-	+
Pyruvic Acid	+	+	+
Pyruvic Acid Methyl Ester	+	+	+
L-Serine	+	+	-
L-Threonine	-	+	-
L-valine plus L-aspartic acid	-	-	w
2'-Deoxy Adenosine	+	-	w
Inosine	+	+	w
Thymidine	+	w	w
Uridine	+	+	w
Uridine-5'-Monophosphate	w	-	-

Table 5: BIOLOG Results of Distinguishing Characteristics Comparing M6.X2^{T}, M6.X9^{T}, and M6.X2D^{T}.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Spore formation or thermotolerant cell	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metabolism	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Production of Indole	-	V	-	+	V	V	+	-	+	-	-	V	+		+	+
Fermentation of:																
Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Activity of:																
Alkaline phosphatase	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-
Arginine arylamidase	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Histidine arylamidase	-	V	-	-	+	V	-	-	W	+		+	+	+	+	+
Leucine arylamidase	-	V	-	+	+	V	-	-	-	+	+	+	-	+	W	+
Proline arylamidase	-	-	+	-	-	-	-	+	W	-	+		-	-	-	-
Phenylalanine arylamidase	-	-	-	-	+	-	-	-	+	+	W	+	-	+	W	-
Pyroglutamic acid arylamidase	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Leucyl Glycine arylamidase	+	N/A	-	-	-	N/A	N/A	N/A	-	N/A	N/A	+	w	w	-	-
End Products from PYG	Acetate, Butyrate	Acetate, Butyrate Propionate	Acetate, Butyrate	Propionate Acetate, Butyrate	Acetate, Butyrate	Butyrate	Butyrate	Butyrate	Butyrate, Propionate	Acetate, Butyrate	Acetate, Butyrate, Propionate	Acetate, Butyrate	Acetate, Butyrate	Acetate, Butyrate	Acetate, Butyrate	Acetate, Butyrate
Major Fatty Acids	$C_{10:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:1\omega9c}$	$C_{14:0}$, $C_{16:0}$, $C_{16:0ALDE}$, $C_{16:1\omega7c}$	N/A	$C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{16:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{16:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{14:0}$, $C_{16:0}$, $C_{17:1\omega8c}$, $C_{18:2\omega6,9c}$	$C_{16:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	$C_{14:0}$, $C_{16:0}$, $C_{16:1\omega7c}$	$C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$, $C_{18:2\omega6,9c}$	N/A	$C_{14:0}$, $C_{16:0}$, $C_{16:1\omega7c}$, $C_{16:0ALDE}$	N/A	N/A
Cell-wall murein	Ala Orn, D-Glu	Orn, D-Glu	N/A	Ala, Orn, D-Glu	N/A	Ala, Orn D-Glu,	Orn, D-Asp	Ala, Orn, D-Glu	Ala, Orn, D-Glu	Ala, Orn, D-Glu	Ala, Orn, D-Glu	N/A	Ala, D-Glu	Orn, D-Glu	Ala, D-Glu	N/A
DNA G+C Content (mol%)	34.4	31-32	44.6	33.4	44.0	25.0	32-34	29.0	32.4	30-31	32.0	30.0	32.2	31.8	30.7	30.0
Source	HF	CM, V, OV, AB	CM	CM	AB	C	AB	CM	CM	GB	CM	HF	SW	HF	CM	

*HF, human feces; CM, clinical material, V, vaginal discharge; OV, ovarian; AB, bodily abcesses; C, cattle mastitis; SW, swine manure; GB, retail ground beef

Table 6: Morphological, biochemical and chemotaxonomic properties of related genera in the *Peptoniphilus* genus.

1., *P. catoniae* (113); 2., *P. asaccharolyticus (104)*; 3., *P. coxii* (114); 4., *P. duerdenii* (112); 5., *P. gorbachii (115)*; 6., *P. harei* (104); 7., *P. indolicus* (104); 8., *P. ivorii* (104); 9., *P. koenoeneniae* (112); 10., *P. lacrimalis* (112); 11., *P. methioninivorax* (116); 12., *P. olsenii (115)*; 13., *P. senegalensis* (117); 14., *P. stercorisuis* (99); 15., *P. timonensis* (118); 16., *Peptoniphilus tyrrelliae* (114).

acteristic	*Citroniella*	*Ezakiella*	*Anaerococcus*	*Anaerosphaera*	*Finegoldia*	*Gallicola*	*Helcococcus*	*Murdochiella*	*Parvimonas*	*Peptoniphilus*
e formation or notolerent cell	-	-	-	+	-	-	-	-	-	-
bolism	OA	OA	OA	OA	OA	OA	FA	OA	OA	OA
e Production	+	+	-	-	-	w	-	+	-	+
entation of:										
se	+	-	v	-	-/w	-	+	-	-	-
ose	-	-	-	-	-	-	+	w	-	-
ose	-	-	w	-	-	-	+	w	-	-
se	+	-	-	-	ND	ND	+	ND	ND	-
ity of :										
ine phosphatase	-	+	-	ND	+	-	-	-	+	-
ine arylamidase	+	+	+	ND	+	-	-	+	+	+
ne arylamidase	-	+	+	ND	w	-	-	+	+	+
ne arylamidase	+	+	w	ND	+	-	-	+	+	-
ne arylamidase	-	-	-	ND	-	-	-	+	+	-
ylalanine midase	-	+	w	ND	-	-	+	+	+	-
glutamic acid midase	-	+	+	ND	+	-	-	-	+	-
products - PYG	A, MS	A, B, MS	B	A, B	A	A, B	A, L	L	A	B, A
r Fatty Acids	$C_{16:0}$, $C_{17:1\ \omega8c}$, $C_{18:1\ \omega9c}$	$C_{16:0}$, $C_{18:1\ \omega9c}$, $C_{18:2\ \omega\ 6,9C/18:0\ ante}$	$C_{16:0}$, $C_{18:1}$, $C_{16:1}$, $C_{18:0}$	$C_{16:0}$, $C_{18:1\ \omega7\ DMA}$, $C_{17:1\ \omega8}$	$C_{16:0}$, $C_{18:1}$ $C_{16:1}$, $C_{18:0}$	$C_{16:0}$, $C_{18:1}$ $C_{16:1}$, $C_{18:0}$	ND	$C_{14:0}$, $C_{16:0}$, $C_{18:1}$	$C_{16:0}$, $C_{18:1}$ $C_{16:1}$, $C_{18:0}$/$C_{18:2}$	$C_{16:0}$, $C_{18:1}$ $C_{16:1}$, $C_{18:0}$
Wall Murein	Orn	Lys	Lys, D-Glu	Lys	Lys, D-Asp	D-Asp	D-Asp	ND	Lys	Orn, D-Glu
G + C Content %)	29.9	38.4	30-35	32.5	32-34	27-34	ND	ND	27-28	30-34
ce	HF	HF	HF, CM, V, OV, AB, N	C	V, OR, AB	CF	SK, CM, SH	CM, AB	OR, AB	HF, CM, V, OV, AB, C, SW

bligate anaerobe; FA, facultative anaerobe | A, acetate; B, butyrate; L, lactate; MS, Methyl-Succinate |HF, human feces; CM, clinical material, V, vaginal discharge; OV, n; AB, bodily abcesses; N, nasal passage; C, methanogenic cattle manure reactor; OR, oral cavity; CF, chicken feces; SK, skin; SH, sheep; SW, swine manure

Table 7: Morphological, biochemical and chemotaxonomic properties of related genera in the *Peptoniphilaceae* family.

1., *Citroniella saccharovorans* M6.X9^T *(120)*; 2., *Ezakiella peruensis* M6.X2^T (119); 3., *Anaerococcus (104);* 4., *Anaerosphaera (105);* 5., *Finegoldia (121)*; 6., *Gallicola (104); 7., Helcococcus (106)*; 8., *Murdochiella (107)*; 9., *Parvimonas (123)*; 10., *Peptoniphilus (104);* Additional data obtained from de Vos et al., (109) and the CCUG web site. (www.ccug.se).

2.3 Conclusion & Future Directions

In addition to isolates representing previously uncultured organisms, our xylan enrichment yielded isolates belonging to taxa that have been previously described to be xylanolytic (53%). Future studies could include (i) establishment of a defined medium with xylan as the sole substrate and enumeration of xylan utilizing bacteria using the MPN method (most probable number estimation), (ii) determination of xylanase activity in an agar plate (124) and then identification of recovered isolates using 16S rRNA gene sequencing.

Growth was not observed upon inoculation of *Ezakiella peruensis* (M6.X2^T), *Citroniella saccharovorans (*M6.X9^T*),* and *Peptoniphilus catoniae* (M6.X2D^T) into minimal media with xylan as the sole substrate. It is hypothesized that substrates in the original enrichment were made available by a number of different processes (i) the breakdown of xylan, (ii) nutritional components within the rumen fluid (ie SCFAs) and (iii) presence of nitrogenous compounds (amino acids & peptides). These hypotheses are supported by data generated using API and BIOLOG assays that show utilization of proteinaceous compounds. As the recovered taxa have been shown to produce butyrate, acetate, and propionate, it is plausible they are part of the microbial community contributing to the health of the human host.

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Chapter 3: Genome Sequencing and Annotation of *Ezakiella peruensis*, *Citroniella saccharovorans*, and *Peptoniphilus catoniae*

Abstract

The genomes of three novel isolates (*Ezakiella peruensis*, *Citroniella saccharovorans*, and *Peptoniphilus catoniae*) were sequenced from human fecal samples of a traditional community member and analyzed to examine in more detail the physiological potential of these organisms. Furthermore, the concordance between four phenotypic traits observed from the API 32A test kits was investigated against their respective genes. The genomes of the novel taxa ranged between 1.6-1.8 mb with *E. peruensis* and *P. catoniae* assembled into 64 contigs and *C. saccharovorans* assembled into 362 contigs. The number of predicted ORFs ranged between 1,553-1,659. While concordance (95%) was observed for indole production and proline arylamidase, for the novel taxa and nearest neighbors, discordance was observed for alkaline phosphatase, in that taxa with positive reactions in the lab were lacking the required enzymes within the genome; likely due to errors in annotation. *C. saccharovorans* showed the greatest potential for carbohydrate metabolism with the presence of starch, maltose, galactose breakdown genes.

3.0 Introduction

The sequencing of prokaryotic genomes was initially focused on microbes with medical or biotechnological applications (125), with the first bacterium, *Haemophilus influenzae*, sequenced in 1995. Advances in Next Generation Sequencing (NGS) technologies, lowered sequencing costs, and initiatives such as the Human Microbiome Project (HMP) led to an exponential increase of available genomic data over time (126) (**Figure 9**). The incorporation of genomic analysis for microbial systematics has provided valuable insights into identification and phylogeny, and the potential for evaluating metabolic capabilities for the microbe of interest. Furthermore, the substitution of traditional microbiology assays, specifically, DNA-DNA hybridization with genome-based programs such as Average Nucleotide Identity, (ANI), have proved to be reliable when determining the precise relationships of closely related organisms.

The inference of phenotypic traits from genomic information is of interest to help circumvent challenges from laboratory-based assays. The potential for the use of genomic data was first reported by Amaral et al (127) wherein 14 routinely used diagnostic features (Voges–Proskauer reaction, indole production, arginine dihydrolase, ornithine decarboxylase, utilization of myo-inositol, sucrose and L-leucine, and fermentation of D-mannitol, D-sorbitol, L-arabinose, trehalose, cellobiose, D-mannose and D-galactose) were examined across 26 *Vibrio* species. Overall, the authors found that a majority of the genes involved in the metabolic pathways were observed in the genome.

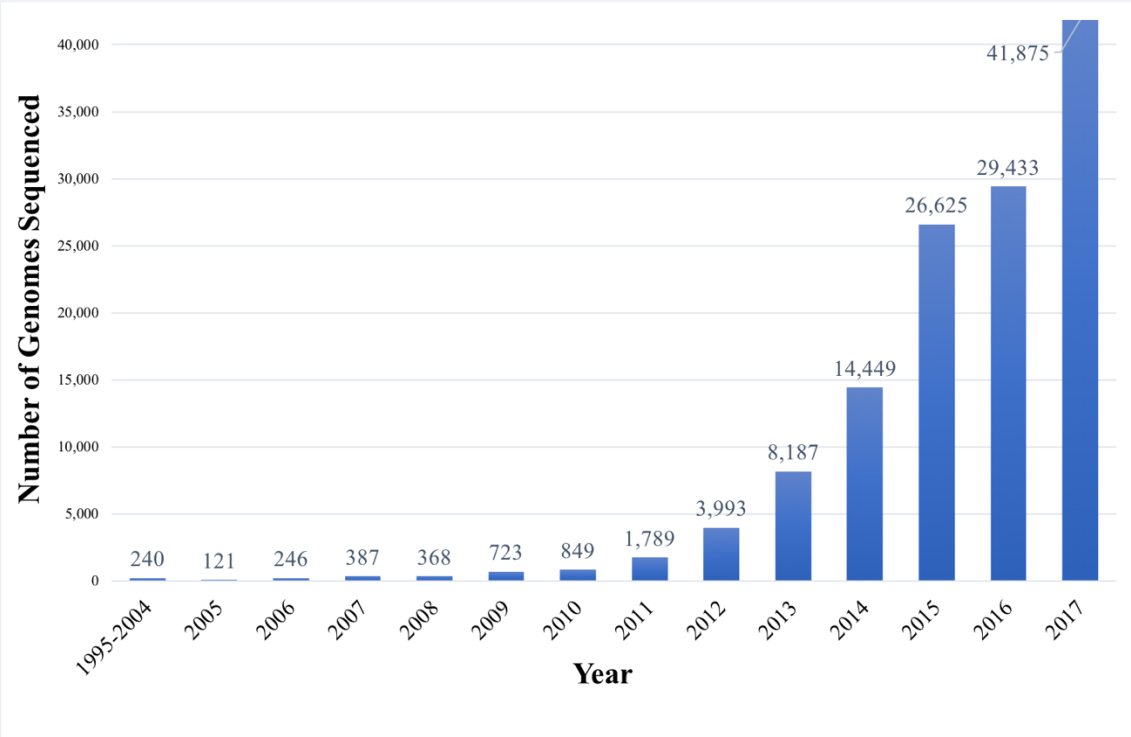


Figure 9: The number bacterial and archaeal genomes submitted to NCBI between 1995-2017. Data collated from: GenBank prokaryotes.txt (July 2018)

The goal of this study was to reconstruct the genomes of three novel gut bacteria *Ezakiella peruensis*, *Citroniella saccharovorans*, and *Peptoniphilus catoniae* to clarify their phylogenetic relationship to known members of the GPAC group as well as to explore enzymatic reactions commonly observed using the commercial enzymatic characterization kit API 32A (BioMérieux). The use of these assays are standard when justifying the description of novel taxa belonging to the *Peptoniphilaceae* family. After collating information obtained from laboratory derived results of the three novel isolates and its nearest neighbors, only four reactions had associated Enzyme Commission (E.C) numbers that were relevant to common phenotypes associated with these taxa. The continued genome sequencing of members belonging to taxa in the GPAC group is

important to not only improve annotation models, but also to better predict phenotypic traits commonly observed for these taxa.

3.1 Materials & Methods

3.1.1 Construction of Shotgun Libraries

Genomic DNA was extracted from a culture grown in Peptone-Yeast (PY) broth using the DNeasy UltraClean Microbial Kit (Qiagen, Germany) and quantified using the Qubit 2.0 fluorometer, dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Each sample was normalized to contain 100 ng of DNA per 100 μL and fragmented using a sonicator (QSonica USA). To prepare the ends of the fragmented DNA for Illumina adapter ligation, the KAPA Hyper Prep Kit (Kapa Biosystems, USA) was used to form the 5'-phosphorylated, 3'-dA-tailed dsDNA fragments (End repair and A-tailing). Reactions were prepared according to manufacturer instructions with the exception that adapter ligation time was set to 30 minutes at 20 °C instead of 15 minutes. Concentrations of libraries were estimated using quantitative polymerase chain reaction (qPCR) (FastStart Essential Green) and the resulting Cq values used to normalize libraries. Unique dual indexed primers (Phusion Buffer) were used for library amplification. Finally, libraries were pooled in equimolar concentrations and sequenced on an Illumina MiSeq platform (V2 chemistry, 2 X 250bp).

3.1.2 Data Analysis & Processing

Raw reads were obtained from the sequencing platform. Adapters were trimmed, and the resulting reads were merged and quality filtered using AdapterRemoval v2 (3). Key parameters included; “trimns” to trim ambiguous bases (N) at 5'/3' termini and

"trimqualities" to trim 5'/3' termini bases with quality scores less than 30 (1:1,000 chance of incorrect base call). Reads less than 25 bp were discarded. These analysis ready reads were then assembled into contigs using Newbler v2.9 (454 Life Sciences). To verify the quality of the genome assembly, merged and quality filtered reads were re-mapped to the assembled contigs using Bowtie2 (v 2.2.6) (128). Finally, G+C values were predicted using the "infoseq" package within EMBOSS (v6.6) (129, 130).

3.1.3 Checks for Genome Completeness & Contamination

Three quality checks for contamination were implemented: (i) to screen for environmental contamination (i.e., during sample/library preparation or DNA extraction) Metagenomic Intra-Species Diversity Analysis System (MIDAS) v1.3 (131) and CheckM (132) were run; (ii) to check for cross contamination between the respective genomes that were sequenced, a pairwise BLAST search was used between each of the three genomes; (iii) confirmation of identity by comparing the 16S rRNA gene sequence from the respective cultured clones to the predicted 16S rRNA genes (RNAmmer) of the assembled genome.

CheckM allows for assessment of genome completeness and contamination using single-copy housekeeping genes. Additionally, CheckM (132), estimates the completeness and contamination of a genome by using lineage-specific, single copy, marker genes that are extracted and aligned to reference genomes of varying quality. The program can distinguish between data sets containing genomic fragments that belong to multiple taxa and those fragments belonging to more divergent taxa via the implementation of Amino Acid Identity (AAI) (132, 133).

MIDAS contains 31,007 bacterial reference genomes clustered into 5,952 species groups based on 96.5% sequence identity across 30 universal single copy marker genes encompassing 15 gene families, including ribosomal proteins, DNA polymerase, 30S ribosomal protein S16 (*rpsP*), DNA primase (*dnaG*), leucyl-tRNA synthetase (*leuS*), and holliday junction DNA helicase (*ruvA*).

3.1.4 Gene Prediction

Gene prediction of the draft genome sequence for all three organisms was accomplished using Prokka v1.11 (134) which includes the following tools: Prodigal v2.6 (135) for Open Reading Frame (ORF) prediction, RNAmmer (136) for Ribosomal RNA prediction. Aragorn (137) and tRNA-Scan (138) for transfer RNA prediction, and Infernal (139) for inference of RNA secondary structure alignments.

3.1.5 Gene Annotation

Ortholog assignment of predicted ORFs and subsequent mapping to metabolic pathways were performed using RAST (Rapid Annotation using Subsystem Technology) (140) with the number of ORFs annotated to functional groups compiled using SEED (141). Enzyme commission numbers corresponding to enzymes responsible for indole, urease, alkaline phosphatase, and proline arylamidase activity were determined using KEGG (Kyoto Encyclopedia of Genes and Genomes) (142). The KEGG database (143) contains a collection of manually curated metabolic pathways based on experimental evidence in order to link genes to gene products in their respective pathways. The genomes of the nearest neighbors were examined in KEGG for the presence or absence of the enzymes of interest. In cases where the genome was not already part of the KEGG database, predicted ORFs were submitted through Kegg's

annotation pipeline (KAAS) and then examined for the presence or absence of the enzyme of interest.

3.1.6 ANI, AAI, and UBCG Metrics

Traditionally, the gold standard for separate species status was DNA-DNA hybridization (DDH), which had a 70% demarcation cut-off for species. Currently, this value can be equated with 98.8% 16S rRNA gene similarity (111). It is now accepted that the use of average nucleotide identity (ANI) analysis between two species can be used as a substitute for experimental DDH (110, 111). ANI is calculated using two genome sequences: one as a subject, the second as a query. The genome belonging to the latter is divided into 1,020-bp long fragments. A BLASTn (BLAST) (144) search of the query genome against the subject genome calculates nucleotide identity values between both genomes; the mean of the two resulting reciprocal values are used for taxonomic purposes. To solve the problems of reciprocal inconsistency using this algorithm, OrthoANI was developed as an improved, faster tool to measure ANI values. Briefly, both genomes are fragmented, with fragments. less than 1020 bp discarded. Using BLASTn (Usearch), a reciprocal search between fragments of both genomes results in a single value that represents a measure of nucleotide based genomic similarity between the coding regions of two genomes.

The demarcation cut-off for species falls between 95-96%, with the caveat that other taxonomically useful criteria are incorporated to support their findings. Values are not used to compare genomes belonging to different genera as this method is intended to replace the traditional DNA-DNA hybridization methods used to establish relationships between members of the same species. However, should ANI values

between members of different genera fall below this cut-off, a polyphasic approach should be used in conjunction with this data to determine taxonomic placement.

A second measure of genetic relatedness between bacteria employs the calculation of Amino Acid Identity (AAI) of shared genomes. The algorithm is based on two-way BLAST searches between genes (at least 50 and typically >500 genes in total) to determine phylogenetic relationships between taxa of interest. The species cut-off is similar to ANI (~95 to 96% AAI).

Finally, a set of single-copy homologous genes can be concatenated and aligned to nearest neighbors, resulting in phylogenomic trees to infer taxonomic relationships. First, Universal Bacterial Core Genes (UBCGs) are extracted from targeted genomes using Prodigal (135) and hmmsearch is used to identify genes with significantly similar sequence matches using hidden Markov Models (HMM) (145) from assembled whole genomes. The resulting JSON file is used to create multiple alignments of all 92 genes using MAFFT (146). A phylogenetic tree is then constructed using the concatenated sequence of all UBCG genes using FastTree (147). This final UBCG tree represents a valid evolutionary history using whole genomes. In order to estimate the robustness of each UBCG tree branch, Gene Support Index values are calculated and designated on each branch. A GSI value of 92 indicates the branch is supported by all UBCG genes. Therefore, the higher the GSI value, the greater confidence can be placed on the positions of the branch in the tree.

3.2 Results & Discussion

3.2.1 Genome Descriptions

A total of 64 contigs were assembled for *E. peruensis* and *P. catoniae* representing an average predicted coverage of ~130X of the genome. In addition, 362 contigs were assembled for *C. saccharovorans* with a predicted 81X coverage (**Table 8**). From these results, the genome length for all three taxa (approximately 1.7 to 1.8 mb) and G+C (30-38 mol%) is consistent with other members of the *Peptoniphilaceae* family (1.6 to 2.0 mb). Read mapping using Bowtie2 resulted in a 99.4-99.9% overall alignment rate for all three taxa. No taxonomic hits indicative of contamination was identified using MIDAS and CheckM for any of the three genomes. Genome completeness was between 97-99%.

Ribosomal RNAs for *E. peruensis* were predicted as follows: (5S n=1, 16S n=2, and 23S n=0 rRNAs). A blast search of assembled nucleotide contigs against the 23S genes (*RlmN*, *RlmB*, *RlmH* for nearest neighbor *E. massiliensis* showed 100% coverage for three contigs that had 95%, 98%, and 99% identity respectively. This indicates that the 23S rRNA gene was split among three different contigs, resulting in the inability for rnammer to predict the molecule. Examining regions with higher coverage for *E. peruensis* were determined to belong to genes coding a hypothetical protein (96% coverage; 54% identity; 60X) related to *Ndongobacter massiliensis*, an organism belonging to the *Peptoniphilaceae* family that has not been validly published.

Ribosomal RNAs for *C. saccharovorans* were predicted as follows (5S n=3, 16S n=0, and 23S n=3 rRNAs). A BLAST search was performed using the 16S rRNA gene

sequence of the recovered clone against assembled nucleotide contigs in order to identity the 16S rRNA gene. A 100% identity and coverage showed a match with one contig. An explanation for why the gene was not detected by rnammer could be the result of improper annotation. Contigs with greater depths of coverage were the following: an 18 bp sequence (~311X; no BLAST matches), a hypothetical protein matched to *Finegoldia magna* (98% coverage; 47% identity; ~312X) and *Murdochiella massiliensis* (44% coverage; 68% identity), a terminase large subunit matched to *Domibacillus antri* (96% coverage; 78% identity; ~310X), N-acetylmuramoyl-L-alanine amidase matched to *Finegoldia magna* (98% coverage; 47% identity 362X), cell wall-binding repeat-containing protein matched to *Peptostreptococcus russellii* (99% coverage, 41% identity 362X). Ribosomal RNAs for *P. catoniae* were predicted as follows (5S n=4, 16S n=3, and 23S n=4 rRNAs). Contigs with greater depths of coverage were the following: NCS2 (Nucleobase cation symporter-2) family permease

matched to *Anaerosphaera aminiphila* and *Peptoniphilus* sp. BV3C26 (97% coverage; 71% identity; ~177X) and (99%; 68% identity; ~177X).

Genome Statistics	*Ezakiella peruensis*	*Citroniella saccharovorans*	*Peptoniphilus catoniae*
# of Reads	875,244	942,752	1,135,138
# of Contigs	64	362	64
Genome Length	1,662,391 bp	1,845,895 bp	1,666,445 bp
Average Contig Length	29,653 bp	5,531 bp	29,720 bp
Maximum Contig Length	190, 789 bp	54,118 bp	168,963 bp
N50 Value	53, 396	9,262 bp	75,107 bp
Bowtie Alignment Rate	99.38%	99.41%	99.91%
G+C Content	37.6%	29.8%	34.6%
Mean Genome Coverage	130X	81X	139X
Median Genome Coverage	84X	77X	118X
MIDAS	No Taxonomic Hits	No Taxonomic Hits	No Taxonomic Hits
Coding DNA Sequences	1,553	1,659	1,553
Ribosomal RNAs	5S n=1 16S n=2 23S n=0	5S n=3 16S n=0 23S n=3	5S n=4 16S n=3 23S n=4
tRNAs	48 loci	34 loci	46
CRISPRs	2	2	0

Table 8: Genomic Information for *Ezakiella peruensis*, *Citroniella saccharovorans*, and *Peptoniphilus catoniae*

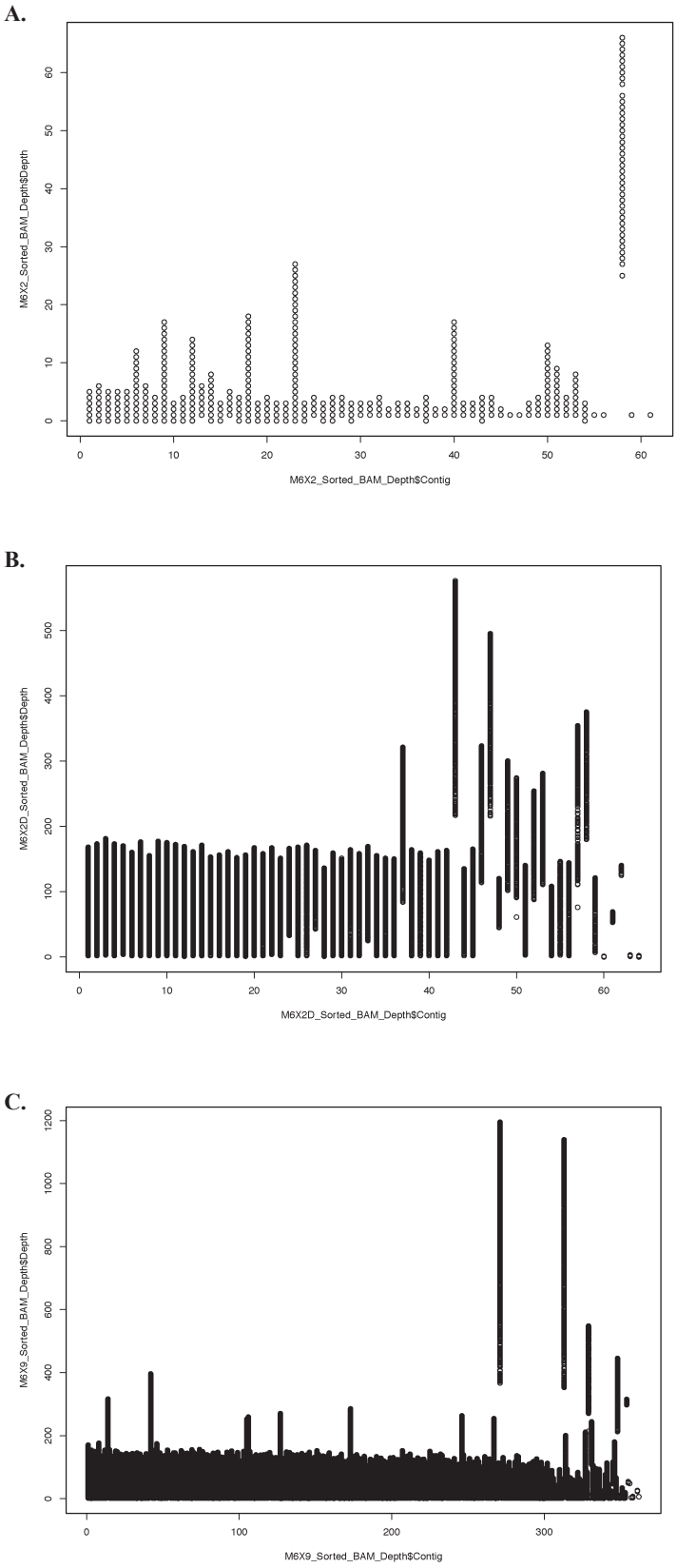
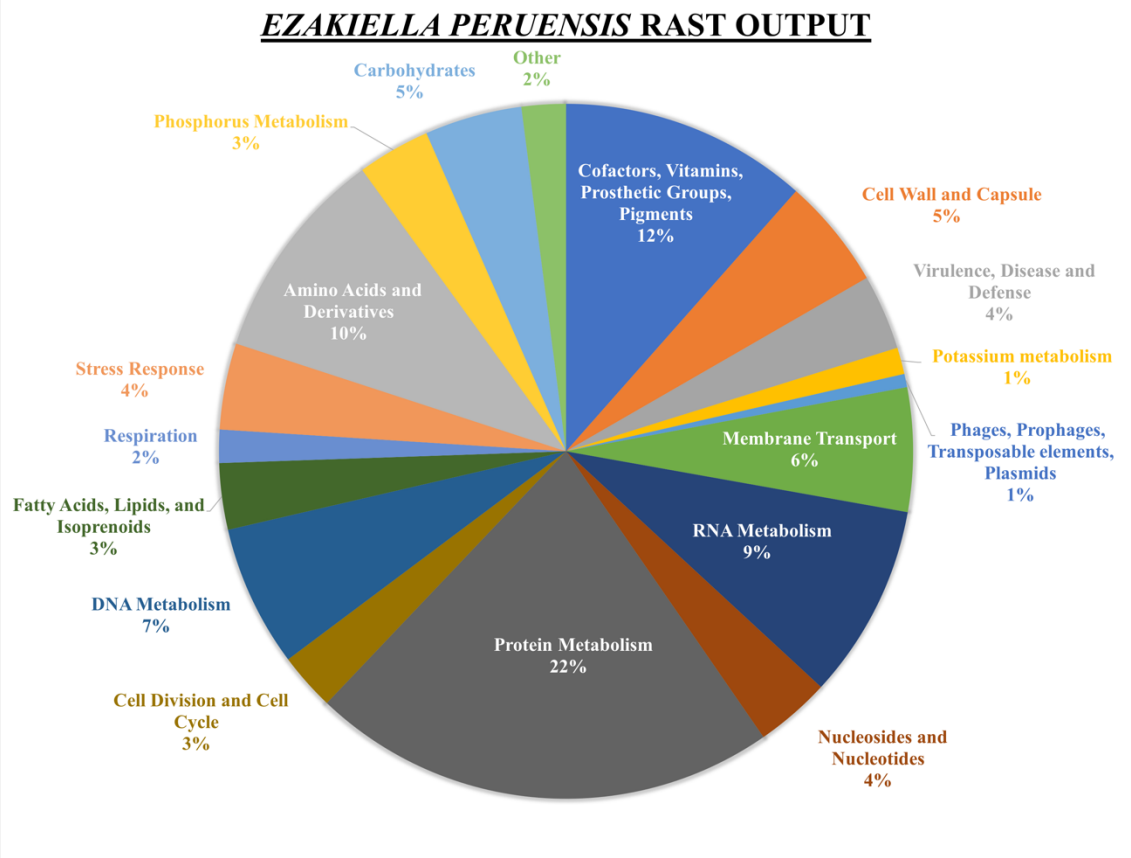
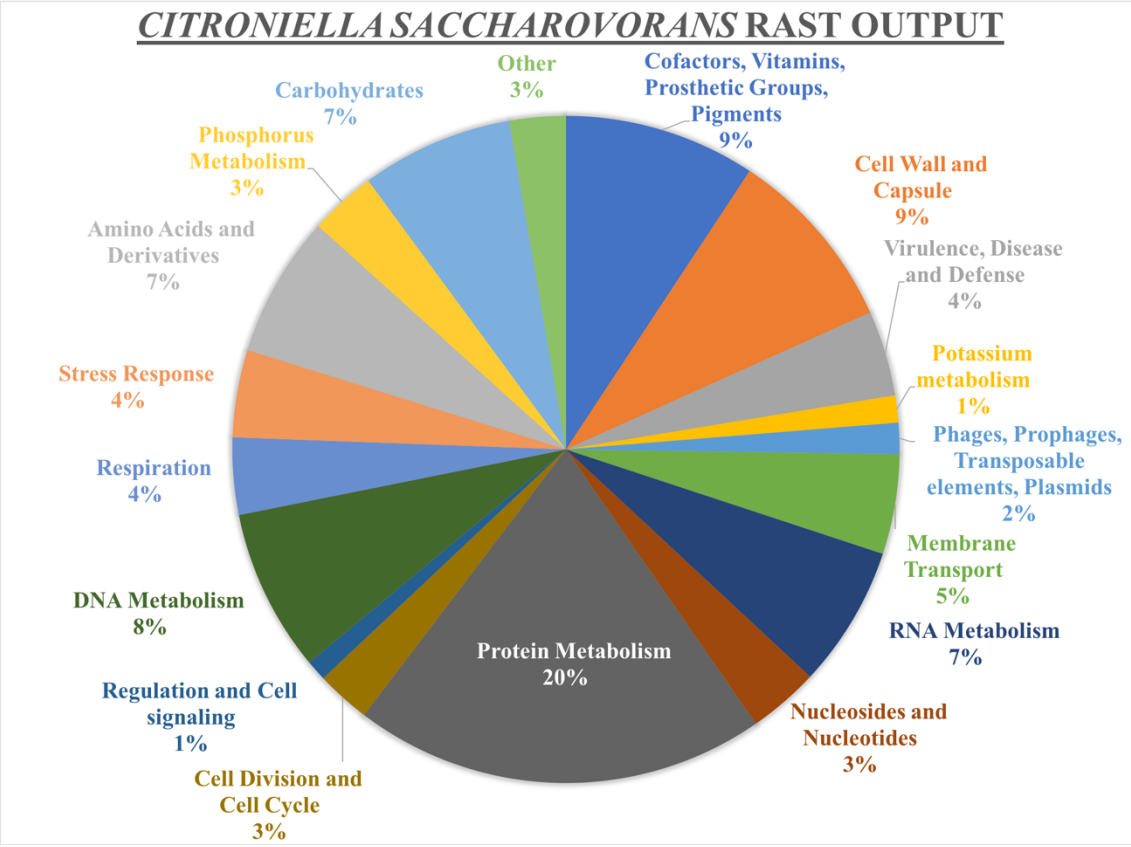


Figure 10: Depth of Coverage for Novel Taxa
A). *E. peruensis*, B). *P. catoniae*, C). *C. saccharovorans*

3.2.2 Gene Prediction & Annotation

The number of predicted proteins assigned to known functions ranged from 60-72% with *P. catoniae* showing the highest values (Figure 11). Enzymes for beta-lactamase, and fluoroquinolone resistance were detected in all three taxa, a common characteristic among members in the GPAC group. Enzymes involved in the metabolism of major carbohydrates were not observed for *E. peruensis* and *P. catoniae* although both possessed genes for alanine, methionine & serine biosynthesis. The genome for *C. saccharovorans* showed greater potential for carbohydrate metabolism as demonstrated in laboratory tests. Genes for galactose, maltose, and starch uptake and utilization were observed. In addition, the presence of choloylglycine hydrolase (EC 3.5.1.24), which is involved in bile hydrolysis was also detected.


EZAKIELLA PERUENSIS RAST OUTPUT
Other 2%
Carbohydrates 5%
Phosphorus Metabolism 3%
Cofactors, Vitamins, Prosthetic Groups, Pigments 12%
Cell Wall and Capsule 5%
Virulence, Disease and Defense 4%
Potassium metabolism 1%
Phages, Prophages, Transposable elements, Plasmids 1%
Membrane Transport 6%
RNA Metabolism 9%
Nucleosides and Nucleotides 4%
Protein Metabolism 22%
Cell Division and Cell Cycle 3%
DNA Metabolism 7%
Fatty Acids, Lipids, and Isoprenoids 3%
Respiration 2%
Stress Response 4%
Amino Acids and Derivatives 10%


CITRONIELLA SACCHAROVORANS RAST OUTPUT
Other 3%
Carbohydrates 7%
Cofactors, Vitamins, Prosthetic Groups, Pigments 9%
Phosphorus Metabolism 3%
Cell Wall and Capsule 9%
Amino Acids and Derivatives 7%
Virulence, Disease and Defense 4%
Stress Response 4%
Potassium metabolism 1%
Respiration 4%
Phages, Prophages, Transposable elements, Plasmids 2%
Membrane Transport 5%
DNA Metabolism 8%
RNA Metabolism 7%
Protein Metabolism 20%
Regulation and Cell signaling 1%
Nucleosides and Nucleotides 3%
Cell Division and Cell Cycle 3%

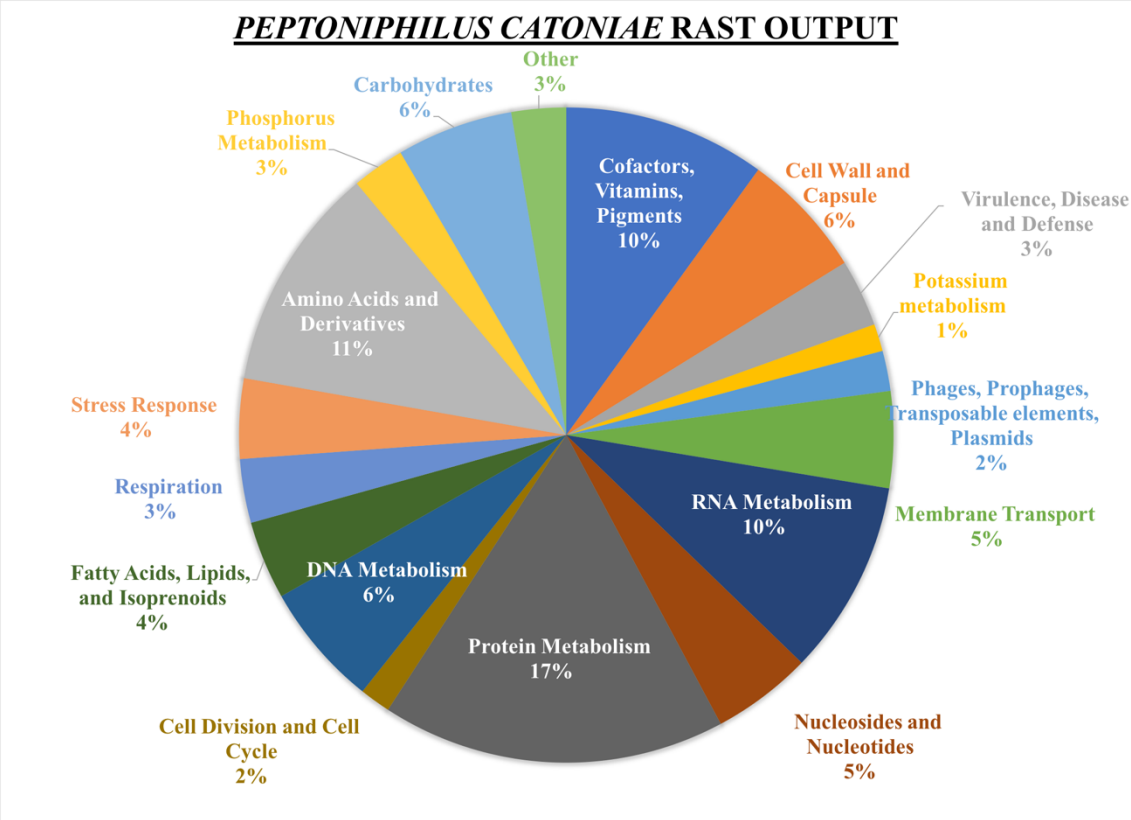


Figure 11: Breakdown of Open Reading Frames (ORFs) By Functional Categories as provided by RAST's Seed Viewer

3.2.3 ANI, AAI, and UBCG Analysis

OrthoANI analysis comparing *P. catoniae* against nearest neighbors in the genus do not show values in the range for species demarcation (**Table 9**). This further justifies its placement as a separate species within this group. Furthermore, both OrthoANI and AAI values of *E. peruensis* against *E. massiliensis* resulted in a value of 94% just below the delineation for species cut off. OrthoANI values of *C. saccharovorans* did not show relationships between nearest neighbors (Table 10).

	Peptoniphilus catoniae	*Peptoniphilus timonensis*	*Peptoniphilus duerdenii*	*Peptoniphilus coxii*	*Peptoniphilus asaccharolyticus*	*Peptoniphilus lacrimalis*	*Peptoniphilus indolicus*	*Peptostreptococcus anaerobius*
Peptoniphilus catoniae	100	72	73	70	71	72	70	69
Peptoniphilus timonensis	72	100	72	70	71	72	70	66
Peptoniphilus duerdenii	73	72	100	76	74	74	70	75
Peptoniphilus coxii	70	70	76	100	73	74	73	77
Peptoniphilus asaccharolyticus	71	71	74	73	100	72	84	72
Peptoniphilus lacrimalis	72	72	74	74	72	100	70	70
Peptoniphilus indolicus	70	70	70	73	84	70	100	68
Peptostreptococcus anaerobius	69	66	75	77	72	70	68	100

Table 9: Ortho ANI Values for *P. catoniae against Peptoniphilus*.

	Eubacterium pernanse	*"Ezakiella massiliensis"*	*"Murdochiella vaginalis"*	*Peptoniphilus coxii*	*Peptoniphilus duerdenii*	*Peptoniphilus harei*	*Peptoniphilus lacrimalis*	*Peptoniphilus manuensis*	*Peptoniphilus asaccharolyticus*	*Peptoniphilus indolicus*	*Peptoniphilus ovalis*	*Anaerosphaera multiphila*	*Cloacibacillus saccharovorans*	*Finegoldia magna*	*Parvimonas micra*	*Anaerococcus cerealiphilus*	*Anaerococcus provencii*	*Anaerococcus hydrogenalis*	*Anaerococcus vaginalis*	*Anaerococcus lactolyticus*	*Helcococcus kunzii*	*Helcococcus sueciensis*	*Peptostreptococcus anaerobius*
Eubacterium pernanse	**100**	94	66	67	71	70	72	72	69	67	73	70	71	70	67	72	72	72	74	73	66	69	65
"Ezakiella massiliensis"	94	**100**	67	69	79	75	73	73	73	68	74	71	75	72	68	80	75	76	77	78	69	68	66
"Murdochiella vaginalis"	66	67	**100**	65	69	67	66	68	73	73	66	66	67	67	71	66	66	76	66	73	67	67	69
Peptoniphilus coxii	67	69	65	**100**	76	74	74	70	73	73	70	66	78	73	68	73	71	83	76	78	73	76	77
Peptoniphilus duerdenii	71	79	69	76	**100**	76	74	72	74	70	73	70	76	75	68	78	74	81	79	81	70	70	75
Peptoniphilus harei	70	75	67	74	76	**100**	73	83	73	70	71	70	75	73	68	74	69	75	74	75	67	68	69
Peptoniphilus lacrimalis	72	73	66	74	74	73	**100**	72	72	70	72	70	72	73	68	73	73	72	71	76	69	69	70
Peptoniphilus manuensis	72	73	68	70	72	83	72	**100**	71	70	72	71	68	71	68	73	72	71	70	70	67	67	66
Peptoniphilus asaccharolyticus	69	73	73	73	74	73	72	71	**100**	84	71	70	73	69	69	74	69	75	73	77	68	69	72
Peptoniphilus indolicus	67	68	73	73	70	70	70	70	84	**100**	70	71	69	69	69	68	68	72	69	72	68	69	68
Peptoniphilus ovalis	73	74	66	70	73	71	72	72	71	70	**100**	71	71	72	68	73	71	74	73	76	67	68	69
Anaerosphaera multiphila	70	71	66	66	70	70	70	71	70	70	71	**100**	67	69	67	71	70	70	70	70	66	66	65
Cloacibacillus saccharovorans	71	75	67	78	76	75	72	68	73	69	71	67	**100**	70	68	74	72	74	72	77	69	68	69
Finegoldia magna	70	72	67	73	75	73	73	71	69	69	72	69	70	**100**	68	72	72	73	71	75	69	68	68
Parvimonas micra	67	68	71	68	68	68	68	68	69	69	68	67	69	68	**100**	68	67	69	68	69	68	68	69
Anaerococcus cerealiphilus	72	80	66	73	78	74	73	71	74	68	73	71	74	72	68	**100**	79	74	74	75	68	69	69
Anaerococcus provencii	72	75	66	71	74	69	72	72	69	68	71	70	72	72	67	79	**100**	73	73	73	69	69	66
Anaerococcus hydrogenalis	72	76	76	83	81	75	72	75	72	72	74	70	74	73	69	74	73	**100**	88	76	69	69	75
Anaerococcus vaginalis	74	77	66	76	79	74	71	70	73	69	73	70	72	71	68	74	73	88	**100**	74	68	68	70
Anaerococcus lactolyticus	73	78	73	78	81	75	76	70	77	72	76	70	77	75	69	75	73	76	74	**100**	70	69	73
Helcococcus kunzii	66	69	67	73	70	67	69	67	68	68	67	66	69	69	68	68	69	69	68	70	**100**	73	66
Helcococcus sueciensis	69	68	67	76	70	68	69	67	67	69	68	66	68	69	68	69	69	69	68	69	73	**100**	68
Peptostreptococcus anaerobius	65	66	69	77	75	69	70	66	72	68	69	65	69	68	69	69	66	75	70	73	66	68	**100**

Table 10: Ortho ANI Values for members in the *Peptoniphilaceae* Family

***E. peruensis* vs Nearest Neighbors**	**% AAI**	***P. catoniae* vs Nearest Neighbors**	**% AAI**	***C. saccharovorans* vs Nearest Neighbors**	**% AAI**
A. aminiphila	45.2%	*A. aminiphila*	61.0%	*A. aminiphila*	46.2%
A. hydrogenalis	44.1%	*A. hydrogenalis*	48.7%	*A. hydrogenalis*	49.8%
A. lactolyticus	44.8%	*A. lactolyticus*	48.8%	*A. lactolyticus*	50.6%
A. prevotii	45.1%	*A. prevotii*	47.7%	*A. prevotii*	48.0%
A. tetradius	45.1%	*A. tetradius*	48.7%	*A. tetradius*	48.4%
A. vaginalis	45.0%	*A. vaginalis*	49.2%	*A. vaginalis*	49.1%
E. massiliensis	94.7%	*E. massiliensis*	48.0%	*E. massiliensis*	49.0%
F. magna	46.4%	*F. magna*	48.8%	*F. magna*	51.4%
H. kunzii	42.6%	*H. kunzii*	44.4%	*H. kunzii*	46.2%
H. sueciensis	43.7%	*H. sueciensis*	45.5%	*H. sueciensis*	46.7%
E. peruensis	100.0%	*P. catoniae*	100.0%	*P. catoniae*	48.5%
P. catoniae	46.7%	*E. peruensis*	47.0%	*E. peruensis*	47.2%
C. saccharovorans	47.5%	*C. saccharovorans*	48.7%	*C. saccharovorans*	100.0%
M. vaginalis	42.2%	*M. vaginalis*	44.9%	*M. vaginalis*	44.9%
P. anaerobius	42.8%	*P. anaerobius*	45.1%	*P. anaerobius*	44.8%
P. asaccharolyticus	45.5%	*P. asaccharolyticus*	59.8%	*P. asaccharolyticus*	48.3%
P. coxii	44.4%	*P. coxii*	51.0%	*P. coxii*	46.4%
P. duerdenii	46.6%	*P. duerdenii*	56.0%	*P. duerdenii*	50.3%
P. harei	45.9%	*P. harei*	58.2%	*P. harei*	48.8%
P. indolicus	45.0%	*P. indolicus*	59.3%	*P. indolicus*	46.8%
P. lacrimalis	46.4%	*P. lacrimalis*	58.5%	*P. lacrimalis*	48.5%
P. micra	45.5%	*P. micra*	47.2%	*P. micra*	49.4%
P. timonensis	47.2%	*P. timonensis*	58.0%	*P. timonensis*	47.1%
Sedimentibacter sp.	38.9%	*Sedimentibacter sp.*	40.5%	*Sedimentibacter sp.*	39.1%

Table 11: AAI Values of *E. peruensis*, *P. catoniae*, and *C. saccharovorans* against nearest neighbors

Whole genome comparisons based on the UBCG marker genes set (**Figure 12**), show that the taxonomic placement between *E*. *peruensis* and nearest neighbors "*M*. *vaginalis"* and *P*. *coxii* shared a GSI value of 45. This indicates that the branch is only supported by 45 out of 92 UBCGs. Furthermore, *P*. *catoniae* and *A*. *aminiphila* share a GSI value of 92, indicating the robustness of their phylogenic relationship. These two taxa may warrant reclassification as two separate genera based on this data. This will only be possible after performing a comprehensive polyphasic taxonomic study as currently API32A data is not available for *A*. *aminiphila.* Finally, the relationship between *C*. *saccharovorans* and nearest neighbors *F*. *magna* and *P*. *micra* share a GSI value of 66.

In order to further establish tree stability, the UBCG tree was reconstructed in MEGA using the Neighbor-Joining Algorithm with 1000 bootstrap values (**Figure 13**). The results of the tree support those obtained from the UBCG tree in that *P*. *catoniae* will need to be reclassified with *A*. *aminiphila.* The determination of whether these will be all separate genera or one genera being reclassified into the other will be based on considering results from polyphasic characterization.

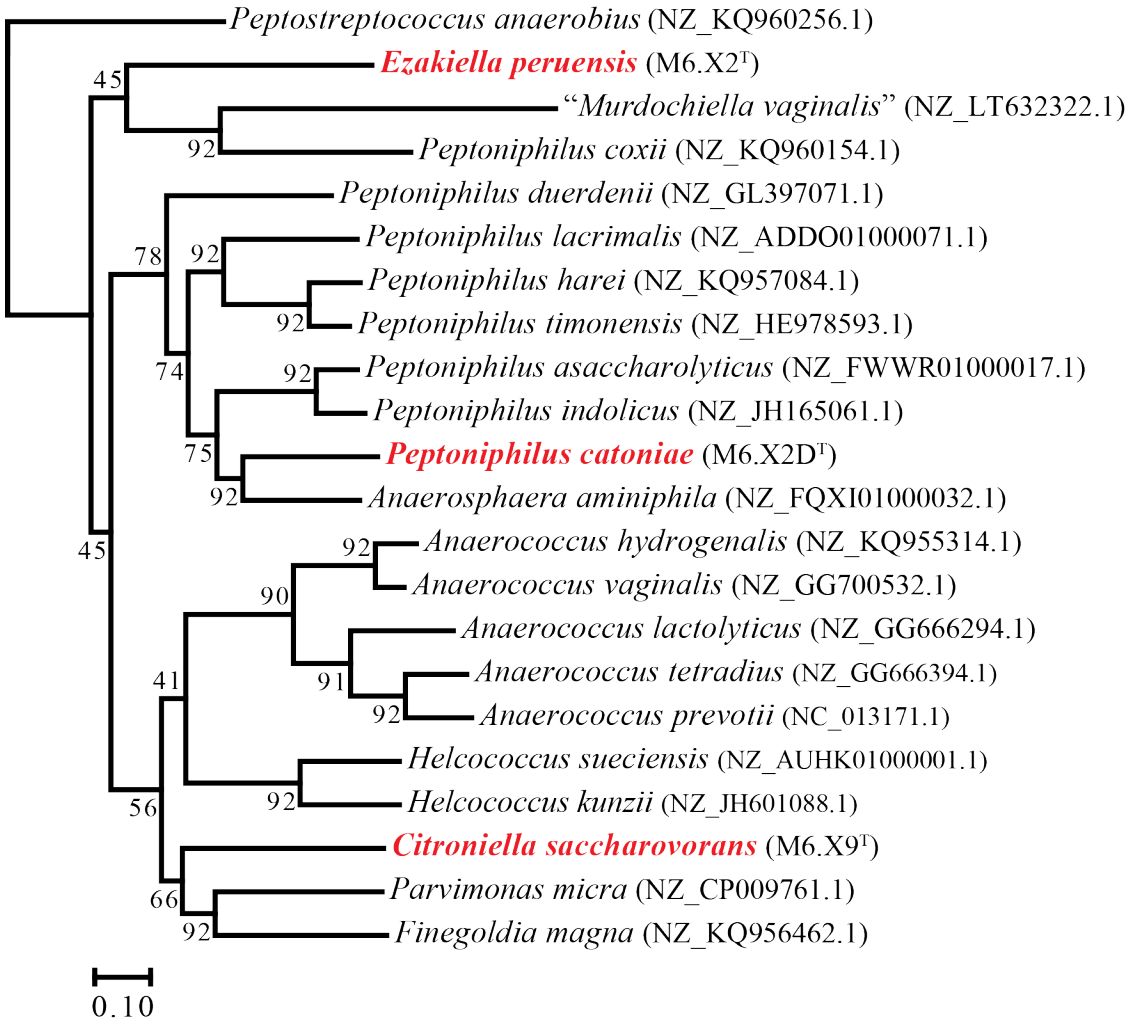


Figure 12: Phylogenomic tree inferred using a concatenated alignment of 92 core genes constructed using FastTree v 2.1.10. Values on the branches support Gene Support Index (GSI). Quotes represent an invalidly published name.

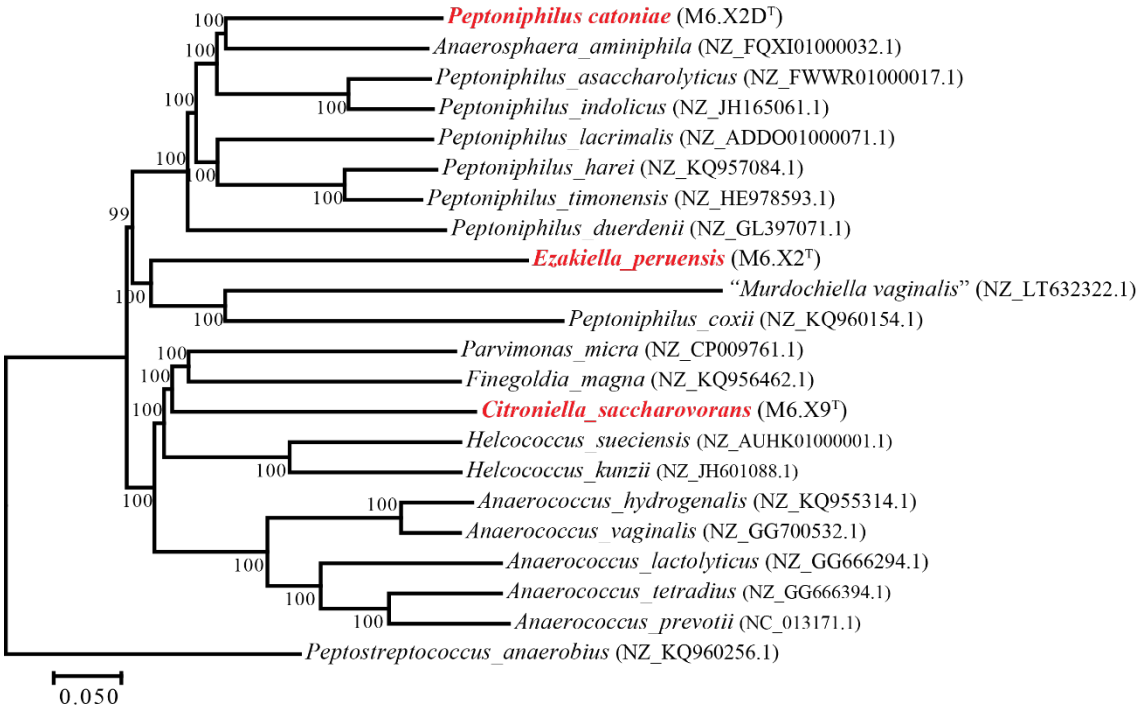


Figure 13: Neighbor-joining phylogenomic tree constructed from concatenated alignment of 92 core genes using MEGA. Bootstrap analysis was carried out using 1000 replications; percentage values are provided at branching points

3.2.4 Examination of Metabolic Pathways

Data available for *E. peruensis*, *C. saccharovorans*, and *P. catoniae* from API32A test kits were cross referenced against data available for nearest neighbors. The collated information was then further filtered to select for test assays with assigned EC numbers, resulting in only four assays; urease, indole, alkaline phosphatase, and proline arylamidase. Urease (urea amidohydrolase; EC 3.5.1.5) is an enzyme that releases ammonia upon hydrolysis of urea present in the human host. Concordance was observed for the *Helcococcus* species testing positive for urease activity.

Indole is produced by bacteria from tryptophan, through the tryptophanase enzyme encoded by the *tna*A gene. Overall, with the exception of *P. duerdenii*, the presence of tryptophanase showed strong concordance with genomic data. Detection of alkaline phosphatase, however, showed less concordance as four organisms *P. indolicus*, *A. vaginalis*, *P. micra*, and *H. sueciensis*, showed activity of the enzyme, but lacked the genes associated with this function, while *P. timonensis* did not show enzymatic activity for alkaline phosphatase in the laboratory even though the associated gene was detected in the genome. This could have been due to either user error in interpreting the data from the API test system, problems with annotation, or the expression of the enzyme was not possible under these lab conditions. Strong concordance was observed for proline arylamidase with the exception of *P. coxii* which lacked the required enzyme, but exhibited enzymatic activity in the lab. Although the dataset was limited by combination of availability of data and EC commission numbers, the results from alkaline phosphatase activity show miniaturized tests such as API, can still be a

	Urease (EC 3.5.1.5)	Indole (EC 4.1.99.1)	Alkaline phosphatase (EC 3.1.3.1)	Proline arylamidase (EC 3.4.11.5)
Peptoniphilus coxii	-	-	-	+
Peptoniphilus timonensis	-	+	-	-
Peptoniphilus asaccharolyticus	-	+	-	-
Peptoniphilus indolicus	-	+	+	-
Peptoniphilus duerdenii	-	+	-	-
Peptoniphilus lacrimalis	-	-	-	-
Peptoniphilus harei	-	-	-	-
Peptoniphilus catoniae	-	-	-	-
Ezakii peruviensis	-	+	+	-
Citroniella saccharovorans	-	+	-	-
Finegoldia magna	-	-	-	-
Anaerococcus hydrogenalis	-	+	-	-
Anaerococcus lactolyticus	+	-	-	-
Anaerococcus vaginalis	-	-	+	-
Anaerococcus tetradius	+	-	-	-
Anaerococcus prevotii	+	-	-	-
Parvimonas micra	-	-	+	+
Peptostreptococcus anaerobius	-	-	-	+
Helcococcus sueciensis	-	-	+	-

-	Lab test was negative, but enzyme was present
(+)	Lab was positive, but enzyme was absent in the genome

valuable tool for characterization until these traits can be more accurately determined using genotypic information.

3.3 Concluding Remarks & Future Investigations

The genomes of three novel taxa recovered from fecal samples of a traditional Peruvian community member, *E. peruensis*, *P. catoniae*, and *C. saccharovorans* were sequenced to better determine phylogenetic relationships with nearest neighbors, especially those of clinical relevance. Future directions should include re-sequencing *C. saccharovorans*, as the genome was assembled into over 300 contigs. Using nanopore for the sequencing platform will result in longer reads and fewer contigs. Future directions should also include expanding in-silico exploration by including fermentation end products. Historically, the use of API test kits to characterize members of the GPAC has proved to be taxonomically meaningful to phenotypically distinguish taxa. However, the inclusion of additional test kits (i.e. BIOLOG) should be included to determine better markers to use for in-silico analysis. From a phylogenetic viewpoint, it is clear that the genus *Peptoniphilus* will require future revisions. The recent description of a number of novel species has added to the phylogenetic depth and diversity, suggesting that some *Peptoniphilus* species may represent novel genera.

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Chapter 4: High-Throughput Screening for Viable Bacteria In Fecal Material Using Propidium Monoazide

Abstract

Advances in DNA and RNA-based sequencing have allowed for methods that have provided insights into the microbial community diversity in any given ecosystem. However, a limitation of these methods is the inability to differentiate between viable and nonviable cells of different taxa in these samples to further direct cultivation efforts. Examination of microbial physiology and function are routinely determined from cultivation studies, so it is imperative to maximize the chance of their recovery from a sample in the laboratory. The photoreactive dye, Propidium Monoazide (PMA), has typically been employed for use in food and water safety pipelines for its ability to neutralize DNA from compromised bacterial cells, allowing for amplification of only viable cells during downstream applications. In this study, the use of PMA was applied to human fecal samples of a traditional Peruvian community. The molecular inventory data generated from subsequent 16S rRNA gene libraries could then serve as a predictor of the appropriate samples to select for targeted cultivation efforts. While the proportion of live cells in each sample varied, PMA treatment did not significantly reduce microbial community richness and phylogenetic diversity. However, differences in the relative abundance of gut microbes, *Faecalibacterium*, *Holdemanella*, *Catenibacterium*, *Dorea*, and *Senegalemassilia* were observed. The protocol proposed in this chapter would help inform microbiologists on selecting the appropriate sample for traditional cultivation approaches.

4.0 Introduction

Establishing microbial viability is important for all fields of microbiology, from food and water safety monitoring (148) to waste-water management programs. A common assay involves the use of Propidium Monoazide (PMA) which has been used in studies to quantify viable bacteria from piggery effluents (149), recovery of viable *Bacteroidales* bacteria from fecal samples (150), and human sputum samples (151).

Propidium Monoazide (Biotium), is an intercalating dye that is unable to diffuse through intact bacterial cell membranes. Cell death resulting in the loss of cell membrane integrity, allows for PMA to bind to DNA, thereby preventing its amplification during PCR (152). This process is the result of exposure to blue light (460 nm) which initiates the irreversible covalent binding of the reagent to DNA.

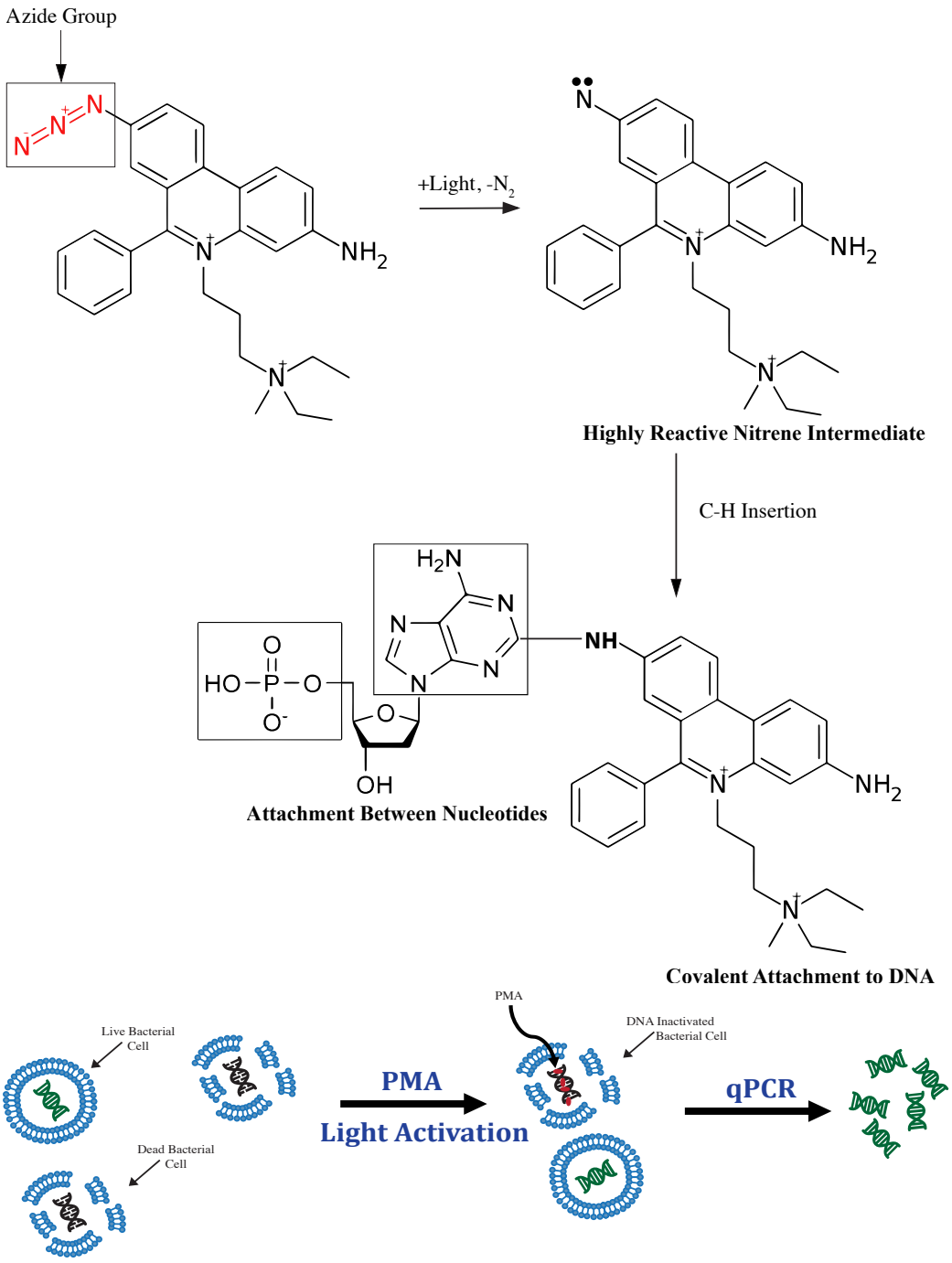

Figure 14: Mechanism of Action for PMA

The presence of an azide group allows for DNA crosslinking upon light exposure. Upon photoactivation, the DNA-PMA complex prevents subsequent PCR amplification of dead cells, as it intercalates between DNA base pairs.

The outcomes of viability PCR assays are determined by bacterial viability. A bacterial cell is considered to be non-viable when it loses the ability to replicate with or

without a loss in metabolic activity (153). Cell death could result from damage of vital cellular components via UV damage (154), inactivation of the respiratory chain by solar disinfection (155, 156), low temperature pasteurization (157), or inhibition of DNA, RNA, or protein synthesis (via antibiotics). However, a caveat to this approach is that the above circumstances result in bacterial cell membranes remaining intact and impermeable to reagents such as PMA even after viability is lost (153).

The use of PMA on multiple human fecal samples for community analysis and targeted cultivation approach has not been extensively studied. By adding PMA to samples to inhibit amplification of non-viable cells, a 16S rRNA gene profile can be generated providing a census of viable cells in a sample. Furthermore, this could be of particular importance where cultivation approaches will be the focus of an investigation. For example, members of *Treponema* are notoriously difficult to culture. It would be advantageous to know if the species of interest are viable when a sample is delivered to the laboratory. The goal of this study was to determine the efficacy of PMA in removing genomic signals from non-viable bacterial cells in human fecal samples.

4.1 Materials & Methods

4.1.1 Samples Used for Optimization

Pure Bacterial Cultures

Bacterial cultures *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6051^T, and *Staphylococcus epidermidis* ATCC 14990^T were inoculated in 100 mL of Tryptic Soy Broth (TSB) (BD Bacto) and incubated for 12 hours at 37 °C with shaking at 150 rpm. The cultures were serially diluted to generate stocks containing 1 x10^9 CFU/mL (CFU = Colony Forming Units).

Human Fecal Samples

A slurry of human fecal material and anoxic PBS (1X) (4g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 0.24g KH_2PO_4; pH=7.2; per 1L) was prepared for each sample (n=20) in an anaerobic chamber (Coy Laboratory Products). For every 1g of fecal sample, 10 mL of anoxic PBS was added to 15 mL falcon tubes. Samples were preserved in anoxic glycerol stocks (20%).

4.1.2 Treatment Conditions

Pure Bacterial Cultures & Human Fecal Samples

Bacterial cultures and human fecal samples were prepared as (250 µL) aliquots in 1.5 mL Eppendorf tubes with the former subjected to two different treatments at 1 x 10^9 CFU/mL. For cell lysis, cultures were placed in an autoclave on a 20-minute liquid cycle (121 °C at 21 psi). To increase cell membrane permeability, cultures were suspended in 70% ethanol for 20 minutes. Cell pellets were then harvested via centrifugation (10,000 xg; 5 minutes) and resuspended in ultrapure water (Milipore, Bedford, MA, USA). Treated and untreated (no autoclave or ethanol exposure) bacterial

cell suspensions were serially diluted and plated onto TSA to calculate Colony Forming Units (CFUs). Samples were treated with 2.5 µL of 100 µM PMA (20 mM, catalog 40019; Biotium, Hayward CA USA) for 15 minutes in the dark, followed by a 15-minute LED exposure (PMA-Lite™ LED Photolysis Device). Treatments without PMA were also included as controls.

4.1.3 DNA Extraction & qPCR

DNA was extracted from fecal samples collected from individuals living in a rural Peruvian population (n=40) and pure bacterial cultures using the AllPrep PowerViral DNA/RNA Kit (Qiagen) with the following modification. Upon adding reagent C1, samples were heated for 10 minutes at 60 °C, followed by a 10-minute bead-beating step. DNA extraction post bead-beating was performed according to the manufacturer's recommended protocol. Extracted samples were quantified via QPCR (LightCycler® 96 Instrument) using the FastStart Essential DNA Green Master reaction mix (Roche). Samples were then normalized based on obtained Cq values to ensure equimolar quantities of DNA were used for subsequent library preps.

4.1.4 Amplicon Library Preparation & Sequencing

For library preparation, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal bacterial/archaeal primers F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (66). Amplicon libraries were prepared using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific). Size selection for pooled libraries were performed using Pippin Prep (Sage Science). Barcoded libraries were pooled and sequenced on an Illumina MiSeq (V2 chemistry; 2 x 250 bp)

4.1.5 Read Filtering & OTU Picking

Fastq files obtained from Illumina's BaseSpace Sequence Hub were processed using Pear (v 0.9.6) using the following parameters: maximum assembly length, 270; minimum assembly length, 150; quality threshold, 30; minimum overlap, 30; and p value, 0.001. In total, 99.7% (7.6M) reads were quality filtered, merged, demultiplexed and 3232 chimeras filtered using ('usearch' v 10.0). Reads occurring less than five times reflected artifacts from sequencing error and were removed for OTU picking.

Reads were clustered de novo at 97% similarity into Operational Taxonomic Units (OTUs). Using UCLUST, taxonomy was assigned to the OTUs with the EzBioCloud16S database (v1.5) (158) serving as a reference database. Overall, 92.7 % of the total sequences were assigned to OTUs (singletons removed) using this approach from 7,614,911 merged and filtered reads. A phylogenetic tree was constructed using the FastTree (v2.1.3) (159) package in QIIME. Analysis of alpha, beta-diversity, and taxonomic composition were performed on a biom table with samples rarefied to 10,000 sequences each.

4.2 Results & Discussion

4.2.1 Differential Cell Lysis Effects on PMA Treatments

Compared to their non-PMA treated counterparts, *E. coli*, *S. epidermidis*, and *B. subtilis* showed a substantial decrease in DNA concentration after ethanol/autoclave treatment. The effect is larger in autoclave treated cells versus ethanol treatment. This indicates three things; i) that upon ethanol and autoclaving treatment, the latter has more free-floating DNA for PMA to bind to resulting in decreased amplifiable DNA (higher Ct values), ii) PMA is better able to permeate cells that are completely lysed as opposed

to those that are slightly permeable ii) when comparing cell death in ethanol vs autoclave, the latter has a larger effect as evidenced by the higher Ct values. If the Ct values from ethanol and autoclave treatments had been the same, this would indicate that PMA could penetrate slightly permeable and complete lysed equally. This data is supported by the fact that no colonies were observed on plates inoculated from ethanol and autoclaving treatments.

Bacterial Culture	**Ct**	**Δ Ct**	**Bacterial Culture**	**Ct**	**Δ Ct**	**Bacterial Culture**	**Ct**	**Δ Ct**
E. coli, Ethanol +PMA	14.5	7.1	*S. epi*, Ethanol +PMA	13.8	3.0	*B. sub* Ethanol +PMA	21.0	10.4
E. coli, Autoclave +PMA	19.2	11.8	*S. epi*, Autoclave +PMA	26.1	15.4	*B. sub* Autoclave +PMA	26.8	16.2
E. coli, Control +PMA	7.7	0.3	*S. epi*, Control +PMA	12.6	1.8	*B. sub* Control +PMA	10.3	-0.2
E. coli, Control -PMA	7.4	N/A	*S. epi*, Control -PMA	10.8	N/A	*B. sub* Control -PMA	10.6	N/A
E. coli, Control +PMA -Light	8.5	1.0	*S. epi*, Control +PMA -Light	11.5	0.7	*B. sub* Control +PMA -Light	10.8	0.2

Table 12: Ct values of *E. coli*, *S. epidermidis*, and *B. subtilis* upon exposure to PMA with ethanol and autoclave killing methods. ΔC_T ($Ct_{\text{sample w/ PMA}} - Ct_{\text{sample w/o PMA}}$)

Positive controls were also included to indicate the CFUs/mL of viable cells in each culture from a stock of 10^9 cells/mL. It is likely the dilutions that were used for CFU counts were too low to be detected. Additionally, pre and post PMA treatment controls yielded Ct values for *E. coli* and *B. subtilis* that were very close together, indicating that the cells in the culture were mostly viable, while the *S. epidermidis*

cultures had a slightly larger proportion of dead cells compared to *E. coli* and *B. subtilis*.

Cultures	Positive Control			Ct value
	10^{-4}	10^{-5}	10^{-6}	
E. coli	TNTC	TNTC	1.12E+09	28.9
S. epi	TNTC	5.96E+08	16	29.5
B. subtilis	1.06E+07	8	-	26.0

Figure 15: Calculated CFUs for positive controls. TNTC indicates "too numerous to count." Whole numbers represent the number of colonies.

4.2.2 Live/Dead Characterization of Matses Fecal Samples Using PMA Treatment

An average increase in Ct values was observed in all samples after PMA treatment. These results suggest that during sample collection, processing, or freeze-thaw cycles, there was a loss in viable cells ($p=<0.05$). The impact of post-collection processes on cell viability has been documented in several studies. While these results are suggestive of a moderate loss of cell viability, the relative contribution of these post collection impacts cannot be determined in the current study.

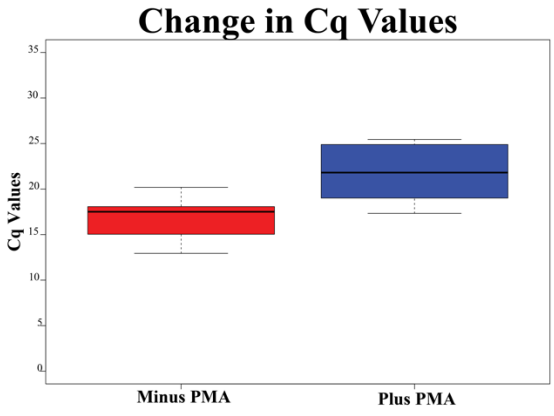

Figure 16: Collective Ct values for Matses fecal samples pre and post PMA treatment

Observations in Phylum Level Diversity

In order to assess differential preservation of taxa, phylum level diversity was investigated using the paired Wilcox test. Results showed statistical differences between pre and post PMA treatments for *Actinobacteria* and *Bacteroides* respectively ($P<0.05$), where a decrease in relative abundance was observed for *Actinobacteria*. Statistical differences between PMA treatments were not observed for *Firmicutes*, *Verrucomicrobia, Cyanobacteria, Euryarchaeota, Proteobacteria, Spirochetes, and Tenericutes* ($P >0.05$). Based on observations of samples post-PMA treatment, the recovery of members belonging to *Actinobacteria* is less likely compared to members belonging to other phyla.

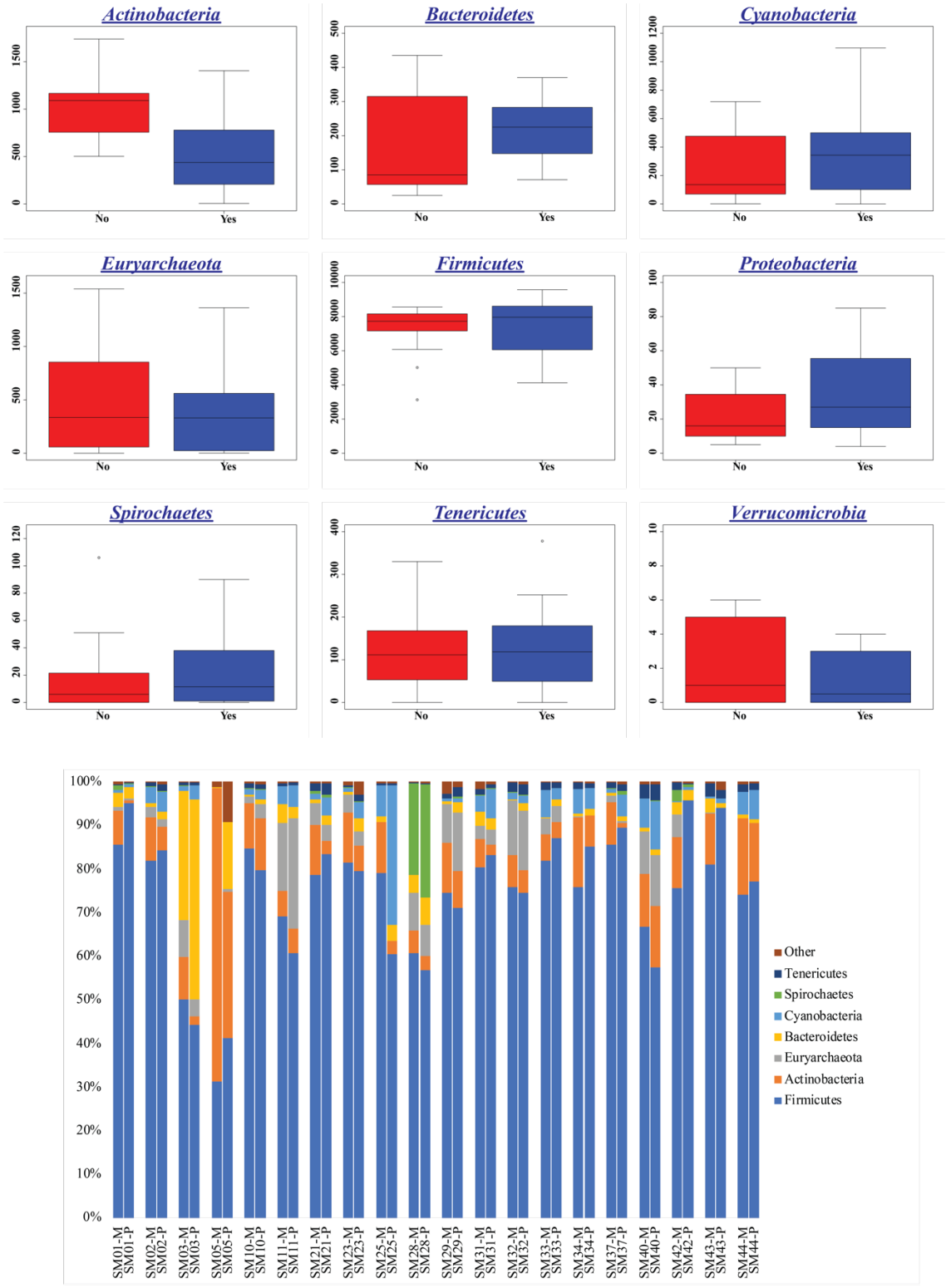

Figure 17: Relative abundance of Bacteria & Archaea for each sample, pre and post PMA treatment "M" and "P" indicates a sample without or with PMA treatment.

Differences in Relative Abundance of Discriminant Genera

The common taxonomic composition for PMA treated samples consisted of *Senegalemassilia*, *Faecalibacterium*, *Lachnospiraceae*, *Collinsella*, *Halomonas*, *Clostridium, Romboutsia*, and *Oscillibacter*. Pre-PMA treated samples had a higher abundance of *Subdoligranulum*, while abundance levels between both treatment types remained unchanged for *Prevotella*, *Blautia*, and *Akkermansia*. In order to determine which taxa showed statistical differences pre and post PMA treatments, the raw abundance table was merged with the metadata and the median abundance for each taxa was obtained for each treatment category. The significance of comparisons between taxa were corrected for multiple comparisons using False Discovery Rates (FDR), and taxa with P values <0.05 and FDR <0.1 were retained.

Out of the genera that showed a statistical significance between treatments (**Figure 18**), only *Faecalibacterium* showed an increase in abundance indicating their viability for cultivation efforts. These Gram-stain positive anaerobes are one of the most abundant and vital commensal bacteria for the human gut microbiota (160). Evidence of differential preservation is observed as a dramatic decrease is observed for all taxa listed in (**Figure 18**) with the exception of *Faecalibacterium* which is proportionally more abundant due to the loss of multiple other taxa. Additionally, the decrease in *Catenibacterium* combined with the stability of *Prevotella* indicates that pre-PMA samples better reflect the rural gut ecology compared to post PMA.

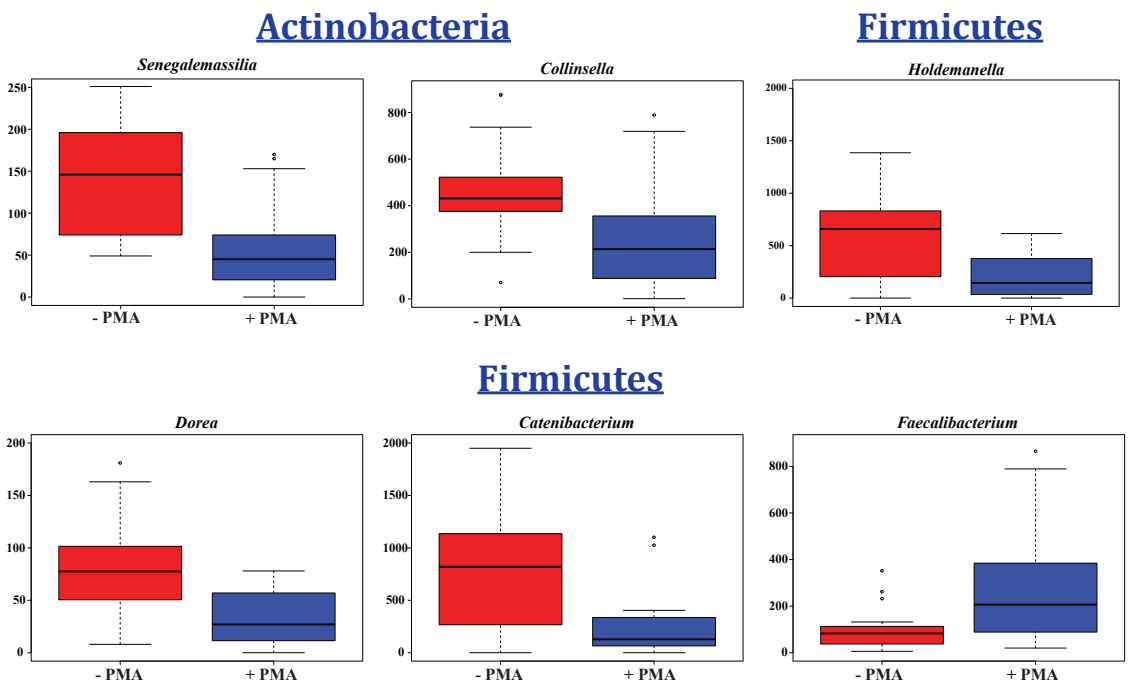

Figure 18: Boxplots showing specific differences in the relative abundance of discriminant phyla between Pre & Post PMA treatments.

Alpha & Phylogenetic Diversity of Human Fecal Samples Treated With & Without PMA

Paired Wilcox tests on alpha diversity metrics (species richness (observed OTUs), evenness (Berger Parker) and phylogenetic diversity (Faith's PD)) demonstrated no significant difference (**Figure 19** A & B). A slight, but insignificant, difference in species evenness was detected between the PMA treated and untreated samples, likely due to an increase in relative abundance of members of the *Clostridia* genus for a subset of the PMA treated samples. The presence of *Clostridia* is not unexpected as they are spore formers and more capable of tolerating environmental changes. Beta diversity was illustrated via Principal Coordinates Analysis where each square or circle represents the microbial community of a sample belong to a PMA treated (blue) or untreated (red) fecal sample. Ordination of weighted unifrac distance matrix, an analysis that accounts for both taxa presence/absence and abundance, showed differences between PMA treated and non-treated groups (**Figure 19**D) that were driven by *Bifidobacterium, Blautia, Catenibacterium, Clostridium, Holdemanella, Methanobrevibacter, Prevotella*, *Ruminococcus*. Unweighted unifrac distance ordination is qualitative, looking at the presence or absence of OTUs and is more sensitive in observing differences in low abundance OTUs (**Figure 19**E). Outliers within the dataset were also observed in that samples SM05, were clustered away from the majority of the dataset.

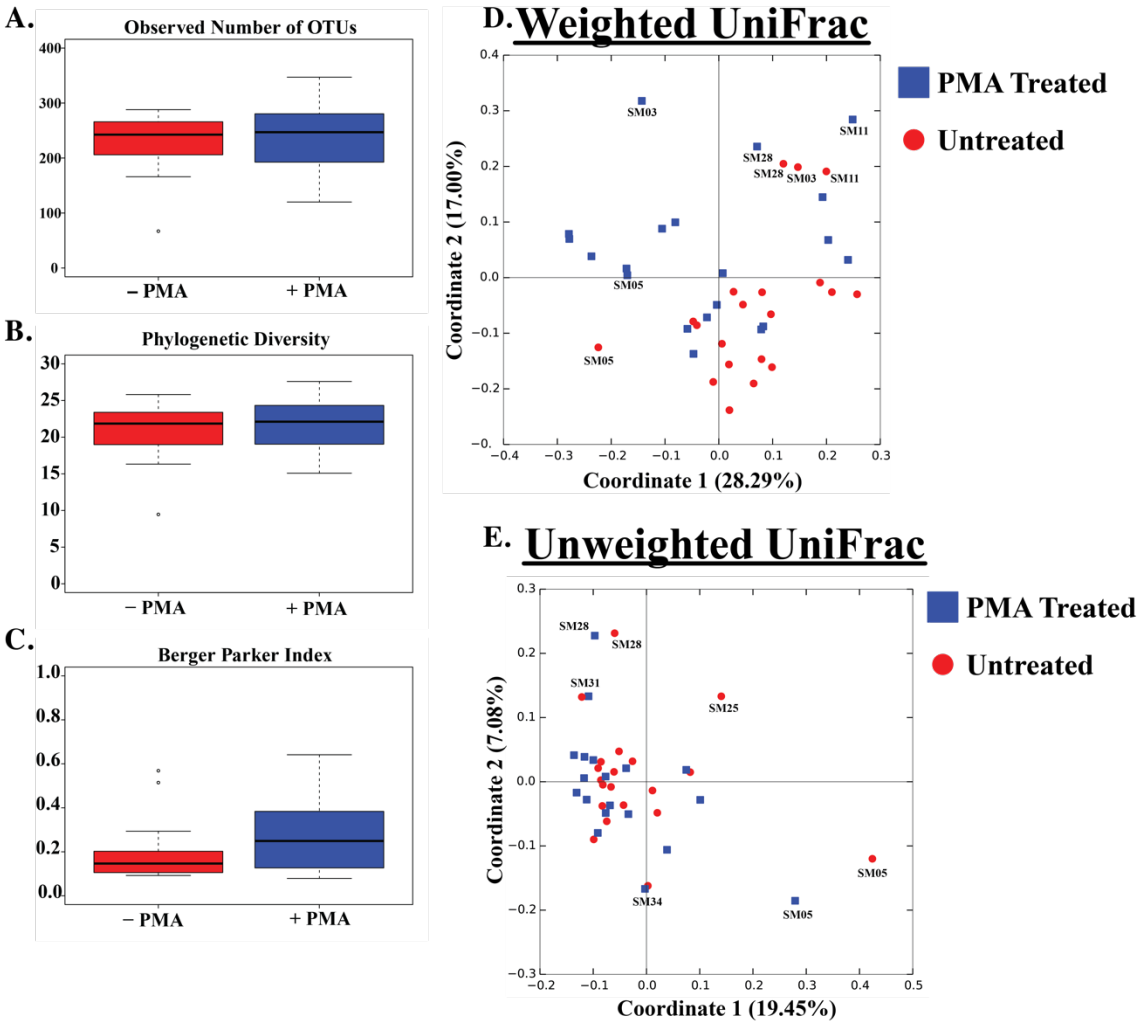

Figure 19: Alpha Diversity metrics (A & B), Berger Parker index to examine the proportional abundance of the most abundant OTUs. Unifrac distance matrices (D & E).

4.3 Conclusion & Future Directions

The rationale of this study was to examine if PMA-pretreatment of human fecal samples could accurately differentiate between viable and nonviable bacterial cells. PMA treatment would emphasize changes in the relative abundance of OTUs in the human fecal samples which would help in human gut microbiota analysis without overestimating the abundance of viable microorganisms. This technique could prove to be valuable for indicating which taxa are more likely to survive sample processing and transport and will help to better direct cultivation efforts for taxa of interest. Limitations

of this study are that certain taxa could be over-represented in molecular inventories (i.e. *Clostridia*). Although differences in species richness and phylogenetic diversity were not observed, it is clear that PMA treatment has an effect on species evenness.

The study was limited by not including additional methods of cell inactivation (ie UV exposure and surfactant), which should be incorporated into future studies in order to better evaluate the effect PMA could have on binding to DNA. Furthermore, in order to establish proof of concept, enrichments should be designed to culture and isolate taxa indicating viability post-PMA treatment. This type of assay would help to establish two things:- i) whether the taxa identified in the molecular inventory post-PMA treatment are a true indicator of viability; ii) the lowest detected abundance that will allow for isolation in pure culture.

Future studies should involve the consideration of a mock community, provided by a culture collection; American Type Culture Collection (ATCC) or prepared in-house in the laboratory using isolates of varying cell structures and sensitivities. Since the number of bacterial cells are strictly controlled, this approach would have given a better approximation of cell counts pre and post PMA treatment after applying the assays previously described in the dissertation. As ATCC is able to provide custom mock communities, fastidious microbes such as members of the *Treponema* genus could be introduced as a control to allow for better testing of PMA sensitivity to fastidious anaerobes.

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