COMMUNITY ANALYSIS OF SOUTHERN APPALACHIAN FENS, AND CHARACTERIZATION AND ISOLATION OF A NOVEL BACTERIUM AND ORDER

A Thesis
by
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Abstract

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Peatlands of all latitudes play an integral role in global climate change by serving as a carbon sink and a primary source of atmospheric methane; however, the microbial ecology of mid-latitude peatlands is vastly understudied. Herein, next generation Illumina amplicon sequencing of small subunit rRNA genes was utilized to elucidate the microbial communities in three southern Appalachian peatlands. In contrast to northern peatlands, *Proteobacteria* dominated over *Acidobacteria* in all three sites. Members of the *Proteobacteria*, including *Alphaproteobacteria* are known to utilize simple sugars and methane among other substrates. However, results described here and in previous studies, indicate that bacteria of the candidate order, Ellin 329, may also be involved in poly- and di-saccharide hydrolysis. Additionally, a novel isolate strain CS4 is proposed as a novel genus and species, *Micropepsia pineolensis* within the proposed novel family and order *Micropepsiaceae* and *Micropepsiales.*

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Dedication

This work is dedicated to my beloved grandparents, parents, partner and friends who have consistently supported my education, development and success. I love you all dearly.

Table of Contents

List of Figures

List of Tables

Foreword

Chapters 2 and 3 are prepared for publication and are formatted as such. Chapter 2 will be submitted to the International Journal of Systematic and Evolutionary Biology as part of the Microbiology Society and has been formatted for proper submission to this journal. Chapter 3 was submitted for review to the Federation of European Microbiological Societies Microbiology Letters on March 2, 2016, and has been formatted for this journal.

Chapter 1: Introduction

Wetlands contain approximately one-third of the world's organic carbon, serving an important ecological role as a natural filtration system (Gorham, 1991) and are considered to be the largest source of natural methane (CH_4) accounting for around 23% of the total methane produced annually (Aronsen *et al.*, 2013). CH_4 is an effective greenhouse gas, holding 2-3 times more heat than CO_2 (Schneider, 1989). Despite the ecological importance of wetlands, wetlands are understudied leading to an unclear classification system, improper use and treatment of the land, and most notably a large gap of information regarding the microbial ecology. The research conducted within this thesis aimed to elucidate the microbial assemblages involved in anaerobic respiration in three southern Appalachian peatlands through the use of Illumina sequencing. Herein, a novel alphaproteobacterium, strain CS4, is also characterized as a proposed novel genus and species, *Micropepsia pineolensis*, within the proposed novel family, *Micropepsiaceae*, and order, *Micropepsiales*.

Defining wetlands

Despite the aforementioned importance of wetlands, their contribution to global climate change was not always apparent. Prior to 1954, U.S. surveys were completed to determine which wetlands could be removed for agricultural usage (Messina & Conner, 1997). In 1954, the first inventory was completed to determine which lands needed to be conserved as water fowl habitat, which was followed by a treaty created in 1971 (ratified in 1986) during the Ramsar Convention, the first international wetlands conservation

initiative (Messina & Conner, 1997). In 1979, after many revisions, the U.S. Fish and Wildlife Service released a widely respected definition that reads:

> 'Wetlands are lands transitional between terrestrial and aquatic systems where the water table is usually at or near the surface or the land is covered by shallow water. For the purposes of this classification wetlands must have one or more of the following three attributes: (1) at least periodically, the land supports predominantly hydrophytes; (2) the substrate is predominantly undrained hydric soil; and (3) the substrate is non-soil and is saturated with water or covered by shallow water at some time during the growing season of each year' (Mitsch & Gosselink, 2000; Cowardin & Golet, 1995).

This broad definition of wetlands is utilized in modern literature to identify wetlands. Further confusion can stem from the improper usage of wetland sub-classifications of peatlands such as bog and fen. These terms have historically been used interchangeably and to describe a variety of different ecosystems internationally, leading to confusion as to what each of the specific terms refer.

Peatlands: Bogs & Fens

Peatlands can be defined as any wetland that has a naturally accumulated layer of peat on the surface (Joosten & Clark, 2002; Mitsch & Gosselink, 2000). Peatlands are also characterized by high water tables and low nutrient availability (Aerts *et al.,* 1999). In these environments, peat formation is regulated by the rate of litter decomposition (Aerts *et al.,* 1999) which is controlled by a number of biotic and abiotic factors (see section "Factors controlling anaerobic decomposition").

A bog is a subset of a peatland that is commonly raised above the upland elevation due to peat accumulation and contains interstitial water with low pH (~ <4.0) and low alkalinity due to its ombrotrophic qualities, i.e., a bog's nutrient supply is derived from precipitation only (Batzer & Sharitz, 2014; Joosten & Clark, 2002; Mitsch & Gosselink, 2000; Aerts *et al.,* 1999). Metallic cations such as Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}

are in low abundance in peatlands due to the fact that they are fed by precipitation. Bogs support the growth of acidophilic mosses, particularly *Sphagnum spp.* (Mitsch & Gosselink, 2000; Aerts *et al.,* 1999; Verhoeven *et al.,* 1990), ericaceous shrubs, and in less abundance evergreens, deciduous shrubs and trees (Mitsch & Gosselink, 2000; Aerts *et al.,* 1999) and are commonly found in the temperate and subarctic regions of the northern hemisphere (Aerts *et al.,* 1999). As nutrient limited environments, peatlands are efficient in nutrient recycling, and Sphagnum mosses are known to relocate nutrients such as N and P from the older, dying portions of the plant to the younger and growing portions in the capitulum (Rydin & Clymo, 1989). NPP values can vary, generally lower in bogs and higher in fens, higher with higher water tables, and are also dependent upon geographic location with northern bogs ranging between 129-303 g/m^2 and southern bogs ranging between 365-1045 g/m^2 (Wieder *et al.,* 1985). Bogs are known to have very low rates of decomposition due to the low nutrient content, acidic soils, and lack of metallic ions mentioned earlier. Additionally, the prevalence of *Sphagnum* species are inherently resistant to decomposition (Aerts *et al.,* 1999) contributing to the difficult decomposition conditions. Low rates of decomposition lead to greater peat formation, as the NPP rate is higher than the decomposition rate. Peat formation rates in bogs have been estimated to be between 100 to 200 cm/ 1,000 years (Mitsch & Gosselink, 2000).

Fens are another subset of peatlands. The higher nutrient content of fens can be attributed to not only precipitation, but surface water and ground water as well (Aerts *et al.,* 1999). Wetland environments that receive nutrients in this manner are referred to as minerotrophic (Sjors, 1959). The input of slow flowing water (Verhoeven *et al.,* 1990) and addition of greater levels of metallic ions promotes more neutral soils (Mitsch &

Gosselink, 2000). Specifically, fens typically have high concentrations of Ca^{2+} and HCO_3^- ions (Wieder, 1985). Like bogs, fens are commonly found in the temperate and subarctic regions of the northern hemisphere as well (Aerts *et al.,* 1999). Fens are commonly dominated by marsh-like vegetation, such as graminoids (grasses), *Carex* and *Cladium* species (Aerts *et al.,* 1999; Sjors, 1959), deciduous shrubs and trees. Plant species growing in fens also typically have higher N and P levels in their leaf litter (Aerts *et al.,* 1999; Verhoeven *et al.,* 1990). Despite higher levels of P in plant litter, fens are overall constrained by P levels (Chapin *et al.,* 2004). Due to richer plant species, greater numbers of labile nutrients and a higher pH, fens have higher rates of decomposition and lower rates of peat production (Verhoeven *et al.,* 1990). Fens have a peat formation rate that is significantly slower than bogs at approximately 27 cm/1,000 years (Ovenden, 1990).

Anaerobic decomposition

Anaerobic microbes utilize a cycle of four phases during anaerobic decomposition: hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis (both hydrogenotrophic and acetoclastic). Hydrolysis is a rate-limiting step during which insoluble macro-molecules such as proteins, carbohydrates, and fats are decomposed by bacteria utilizing exo-enzymes into simpler water soluble molecules, such as monosaccharides, amino acids and fatty acids (Ali Shah *et al.,* 2014; Van Haandel & van der Lubbe, 2007) Hydrolysis is meant to break down the molecules to a size that can penetrate the cell wall and be utilized by the bacteria (Kothari *et al.,* 2014). Following hydrolysis, fermentation of the recently produced monomers converts them to short chain organic acids (fatty acids) in order to produce ATP and recycle NAD^+

(Conrad, 2002). Fermentable monomers include sugars, polyols, organic acids, amino acids, purines and pyrimidines, which are all utilized as substrates by fast growing fermentative bacteria (Kothari *et al.,* 2014). The same fermentative bacteria that secrete the enzymes for hydrolysis then use the monomeric products for fermentation to fatty acids, acetate, CO_2 and H_2 (Conrad, 2002; Muller, 2001). From the acids produced in the fermentation reactions, the third step of anaerobic digestion occurs: acetogenesis (Conrad, 2002; Liesak *et al*., 2000; Leschine, 1995). During this step, acetogenic and syntrophic bacteria utilize acid byproducts to produce acetate and hydrogen (Ali Shah *et al.,* 2014).

The fourth and final step of the anaerobic degradation pathway is methanogenesis. During this step, anaerobic archaea convert most of the products from the acid fermentation steps, such as acetate (acetic acid), ethanol, methanol, H_2, and CO_2 into CH_4 (Kothari *et al.,* 2014; Ali Shah *et al.,* 2014; Conrad, 2002; Leschine, 1995). This step maintains balance because the acetogenic and syntrophic bacteria from the previous processes are limited by a hydrogen saturated environment, thus, methanogenic archaea inadvertently allow for the bacteria to continue growth by utilization of the di-hydrogen gas for methanogenesis. The produced CH_4 subsequently diffuses through the deep layers of peat towards the surface where it is released totaling approximately 0.4 Gt per year (Thauer *et al.,* 2008). The methane production of wetlands contributes significantly to atmospheric destruction, making wetlands an important ecological niche. Substantial research is focusing on wetlands because the methane production potential is unknown as global temperatures rise.

Factors controlling anaerobic decomposition

The carbon cycling processes of peatlands are tightly controlled by a number of factors, such as soil pH, temperature, and the microbial communities involved in the carbon cycling processes. The soil pH significantly affects the microbial composition, productivity and key metabolic processes. The correlation between aerobic metabolism and pH is either neutral or slightly negative (Fisk *et al.,* 2003). Several studies have also shown that as a whole, anaerobic metabolism can be limited by low soil pH (Faulwetter *et al.,* 2009; Fisk *et al.,* 2003) partly because phenol oxidase, an enzyme produced by bacteria to decompose complex high molecular weight compounds, is reduced by low pH (Kayranli *et al.,* 2010). Thus, the complex polymers of plant litter cannot be effectively broken down. Additionally, the pH of wetland and terrestrial soils has been shown to have a positive correlation with microbial diversity, thus, in low pH environments, a group of specialized acidophilic bacteria will dominate (Glime, 2007; Fierer & Jackson, 2006).

Soil temperature contributes greatly to the microbial processes as well. There is an overarching decrease in microbial activity and metabolic rates with decreasing temperatures (Faulwetter *et al.,* 2009). Temperature effects are clearly seen in the thawing of permafrost in the northern regions, and seasonal effects on peatlands with a substantial release of methane as temperatures rise (Hodgkins *et al.,* 2014; Sun *et al.,* 2012; Kayranli *et al.,* 2010). This is likely due to the fact that hydrolysis and fermentative reactions of complex molecules and monomers are not constrained by low temperature in the same manner as CH_4 production. Thus, as temperatures rise there is an abundance of substrate available (Sun *et al.,* 2012). Similar to pH, temperature also has an effect on the

degradative enzyme phenol oxidase. The activity of the enzyme increases with increasing temperature (Kayranli *et al.,* 2010). Increased enzymatic activity in turn increases the level of useable substrate in the soil from primary plant litter.

Common bacterial communities and their specific functions in peatlands influence the rates and by-products of carbon cycling pathways. For instance, *Acidobacteria* are prevalent in many peatland environments, commonly in the highest relative abundance, (Urbanova & Barta, 2014; Basiliko *et al.,* 2013; Serkebaeva *et al.,* 2013*;* Dedysh, 2009; Dedysh *et al.,* 2006; Morales *et al.,* 2006). *Acidobacteria* are thought to be involved in the degradation of cellulose and aromatic compounds, such as lignin and polyphenolic compounds (Tsitko *et al.,* 2014; Urbanova & Barta, 2014). This phylum is also linked to nitrogen cycling by having the genomic potential for nitrate and nitrite reduction (Tsitko *et al.,* 2014). Another highly characterized phyla in wetlands is the *Proteobacteria.* This phylum is divided into a number of classes, of which many are found in peatlands, including *Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria* (Tsitko *et al.,* 2014; Urbanova & Barta, 2014; Serkebaeva *et al.,* 2013; Dedysh, 2011; Hansel *et al.,* 2008*;* Morales *et al.,* 2006; Costello & Schmidt, 2006; Dedysh *et al.,* 2006). *Proteobacteria* are common in peatlands due to their diverse metabolic capabilities, which has a profound effect on bacterial density among different environments, but also has allowed their growth in almost all depths of a peatland, both anoxic and oxic (Tsitko *et al.,* 2014; Urbanova & Barta, 2014). Due to their diverse metabolic nature, *Proteobacteria* are also frequently correlated with more neutral pH and higher organic content (Urbanova & Barta, 2014). Of particular interest to this work is the *Alphaproteobacteria* and the functional diversity of the class in peatlands.

Other non-rare (>1%) bacterial phyla seen in peatlands include *Chloroflexi* (Tsitko *et al.,* 2014; Dedysh *et al.,* 2006; Costello & Schmidt, 2006), *Actinobacteria* (Urbanova & Barta, 2014; Serkebaeva *et al.,* 2013; Dedysh, 2011; Hansel *et al.,* 2008*;* Morales *et al.,* 2006; Costello & Schmidt, 2006; Dedysh *et al.,* 2006), *Verrucomicrobia,* and *Planctomycetes* (Tsitko *et al.,* 2014; Urbanova & Barta, 2014; Serkebaeva *et al.,* 2013; Dedysh, 2011; Hansel *et al.,* 2008*;* Morales *et al.,* 2006; Costello & Schmidt, 2006; Dedysh *et al.,* 2006). These phyla all contribute to the ecology of peatlands worldwide, though few conclusions can be drawn about each phylum's specific function due to the low number of cultured and characterized strains. Overall these phyla have representatives that have been shown to be involved in carbohydrate fermentation, nitrogen fixation, hydrolysis of cellulose/lignin, ANAMOX, methane oxidation, and aerobic decomposition (Tsitko *et al.,* 2014; Urbanova & Barta, 2014; Serkebaeva *et al.,* 2013; Dedysh, 2011; Dedysh, 2009; Dedysh *et al.,* 2006). Bacteria from these groups are located in a variety of niches within the peatland environment based on ambient pH, temperature, anoxic/oxic environment and amount of fresh carbon.

Archaea represented in peatland sites commonly include Euryarchaea, Crenarchaea and Thaumarchaea (Hawkins *et al.,* 2014). Of the Euryarchaea, Methanomicrobiales and Methanosarcinales have been shown to be the prevalent methanogenic archaea in the study sites (Hawkins *et al.,* 2014). Of the Crenarchaea and Thaumarchaea represented, terrestrial groups 1.1b and 1.1c, marine group 1.1a, SAGMCG-1, the deep peat lineage as well as groups 1.3, C3, pSL12, and AK59 have been observed in the study sites (Hawkins *et al.,* 2014). These groups are considerably less known but are thought to utilize ammonia oxidation as a metabolic function

(Könneke *et al.*, 2005) and possibly fermentation based on genome analyses (Lloyd *et al.*, 2013)

Alphaproteobacteria

Currently there are 11 recognized orders in *Alphaproteobacteria* including: *Caulobacterales, Kiloniellales, Kordiimonadales, Magnetococcales, Parularculales, Rhizobiales, Rhodobacterales, Rhodospirillales, Rickettsiales, Sneathiellales,* and *Sphingomonadales* (LPSN - bacterio.net). *Alphaproteobacteria* have been isolated from environments worldwide and carry out a large range of metabolic processes. Members of *Alphaproteobacteria* that have been isolated from peatlands are placed into three functional groups: Methanotrophs, chemo-organotrophs and phototrophs (Dedysh, 2011). Chemo-organotrophs are organisms that ferment organic carbon sources for energy, such as organic acids and sugars. The chemoorganotrophic bacteria are of high importance because they provide the substrate necessary for methane production and consumption.

The studies included in this thesis describe and propose a novel isolate, *Micropepsia pineolensis* strain CS4, as a representative of a novel family *Micropepsiaceae* and order *Micropepsiales.* Additionally, this work describes the microbial diversity of three southern Appalachian peatlands, highlighting the prevalence of the novel order (*Micropepsiales*) in all three sites. Results demonstrate a high level of diversity in southern Appalachian peatlands compared to more widely studied northern peatlands.

Chapter 2: *Micropepsia Pineolensis* gen. nov., sp. nov. an Acid-Tolerant Alphaproteobacterium Isolated from a Poor Fen, and Proposal of *Micropepsiaceae* fam. nov. within *Micropepsiales* ord. nov.

Summary

A novel, obligately anaerobic, acid-tolerant, fermentative alphaproteobacterium, designated strain CS4^T, was isolated from an acidic, oligotrophic (nutrient-poor) poor fen located near Pineola, NC, USA. Cultures contained Gram-negative, slightly curved, non-motile, non-spore forming, non-prosthecate rods (0.1-0.3 µm in diameter and 0.4-2.5 µm long, depending, in part, on the growth substrate). Growth optima were 35° C, pH 5.6, and with 0-50 mM NaCl. The culture fermented cellobiose, D-glucose, D-mannose, fructose, galactose, glycerol, lactose, maltose, peptone, sucrose, trehalose and xylose. Respiratory growth was not detected. Phylogenetic analysis of the SSU rRNA gene and physiological features, it is herein proposed that a novel order, family, genus and species be placed within the *Alphaproteobacteria: Micropepsiales* ord. nov., *Micropepsiaceae* fam. nov., *Micropepsia pineolensis* gen. nov. sp. nov. Strain CS4^T is the type strain CS4^T (=JCM 30711^T =ATCC BAA-2724^T).

Peatlands are unique environments that contain acidic soils, low redox potentials and associated low decomposition rates (Moore & Basiliko, 2006), and they serve as the largest natural source of atmospheric methane, releasing approximately 0.4 Gt of CH_4 annually (Thauer *et al*., 2008). Numerous studies have shown that *Proteobacteria*, specifically *Alphaproteobacteria*, are prevalent in peatlands, and thought to be involved in methane oxidation, as well as sugar fermentation, and anaerobic respiration (Lipson *et al.*, 2013; Tveit *et al.*, 2013; Dedysh, 2011). Herein, we describe the characteristics and phylogenetic placement of a novel alphaproteobacterium strain $CS4^T$ isolated from a poor fen, Pineola Bog, in the southern Appalachian mountains, Pineola, NC. Cultured and uncultured strains related to strain $CS4^T$ form a novel family and order within *Alphaproteobacteria*.

Peat samples were collected on September 12, 2013, from Pineola Bog, at three locations within the bog, at a depth of 25-30 cm in airtight jars and taken directly to an anaerobic glovebox. Inoculum was prepared by homogenization with DI water using an immersion blender and then pressed through a series of filters, with a final filter size of 0.45 µm. Enrichment was performed in Balch tubes, with 5 ml of a basal medium, Peat Medium 1 (PM1), prepared anaerobically as described in Bräuer *et al.* (2006). Prior to inoculation, the tubes were flushed with ultra-high purity N_2 gas and sterilized. Post-sterilization, the following additions were made (final concentrations in mg l^{-1} unless otherwise stated): ca. 0.5 mM Ti(III) Nitrilotriacetate (TiNTA) (preparation described in Bräuer *et al.,* 2006), 20 mM MES (pH 5.7), Vitamin Solution (0.2, 1.0, 0.5, or 0.01 of each vitamin as described in Bräuer *et al.,* 2006 and Balch *et al.,* 1979) 40 yeast extract, 1 g l^{-1} glycerol, and 10 rifampicin. Tubes inoculated with 0.5 ml of peat filtrate were

incubated in the dark at 28°C without shaking and growth was monitored by visual inspection and by use of a spectrophotometer at OD 600.

Colonies were isolated on 1.5% agar plates (PM1 plates) supplemented with the PM1 medium additions, excluding TiNTA, streaked aerobically, and incubated anaerobically in a mason jar with an N_2 headspace. After approximately one week of growth, single colonies were transferred to new plates. Colonies were re-streaked three times to ensure purity, and axenic colonies were transferred back to liquid media in the anaerobic glovebox. Purity was visually inspected using fluorescence microscopy on an Olympus BX51 using acridine orange with a FITC filter. The 16S SSU rRNA gene was extracted, PCR amplified, and sent to Beckman-Coulter Genomics (Danvers, MA) for Sanger sequencing. Once isolated, strain $CS4^T$ was also grown in PM1 medium containing the addition of 500 mg l^{-1} peptone and/or with 1 g l^{-1} of either sucrose or dextrose substituted for glycerol.

For physiological studies, the isolate was grown in PM1 media in triplicate trials, except for substrate utilization tests for substrates in limited supply that were conducted in duplicate trials. Effects of pH, salinity and temperature on growth were examined in PM1 medium supplemented with glucose (1 g l^{-1}). Aerobic growth was tested by addition of 25, 50 or 100% air in the N_2 headspace in Balch tubes with and without the reductant, TiNTA, or by incubating plates streaked with bacteria aerobically at 28 and 35°C. Gram-staining, oxidase and catalase tests were completed according to previously-described procedures (Brown, 2009). Presence or absence of cell motility was examined visually under a phase contrast microscope, and by culturing anaerobically in swarm plates (0.8% nutrient broth with 0.3% agar), supplemented with 1 g l^{-1} sucrose. Heat tolerance was

examined by submerging inoculated PM1 media in 80°C for ten minutes and determining subsequent bacterial viability. Cell morphology was observed by transmission electron microscopy of negatively stained (1% phosphotungsic acid) cells fixed on a 100 square formvar coated grid. Cells were imaged using a JEM-1400 Transmission Electron Microscope.

Fermentative growth was examined in PM1 media supplemented with substrates added at ca. 20 mM. Utilization of electron acceptors was tested in PM1 media supplemented with 1 g l^{-1} glucose as the sole carbon source and either Na_2SO_4, ferric ammonium citrate, sodium fumarate, sodium malate (each at 20 mM), or O_2 (headspace replaced with O_2) as the sole electron acceptor. The Fe (III) reducing capability of strain CS4^T was tested in PM1 media supplemented with 1g l^{-1} glucose (with and without TiNTA) and with ferric ammonium citrate added to a final concentration of 2, 4 and 8 mM. FerroZine Iron Reagent Solution Pillows (Hach, Loveland, CO) were used at pH 7.0 to determine the presence of Fe (II) colorimetrically. The Fe (III) reducing ability was additionally tested with the addition of acetate, ethanol, formate, glycerol, lactate, pyruvate, or succinate (20 mM) as the sole electron donor. Nitrate or sulfate reduction was determined using previously-described methods (Brown, 2009; Iizuka *et al.*, 1969). Phospholipid-derived fatty acids (PLFA) were analyzed via FAME analysis at MIDI Labs Inc. (Newark, DE, USA). High performance liquid chromatography (HPLC) was utilized to determine the production of organic acids on a Dionex platform operated isocratically at a flow rate of 0.4 ml/min. Separation was completed with a BioRad Aminex 87H ion exchange column, eluted compounds were detected with a refractive index detector, and UV detector, in tandem.

Sequences similar to the partial 16S rRNA gene sequence for strain CS4^T (KU738893) were collected from the GenBank database by use of the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were aligned using the SILVA aligner of the ARB program (http://www.arb-silva.de/aligner/). Aligned sequences were trimmed utilizing BioEdit software (Ibis Biosciences, Carlsbad, CA 92008). Phylogenetic trees were constructed with the neighbor-joining (Saitou & Nei, 1987) and maximum likelihood methods using the PHYLIP package (http://evolution.gs.washington.edu/phylip.html).

Results and Discussion

Colonies of strain CS4^T grown anaerobically on PM1 plates, that had been poured and stored aerobically prior to inoculation, were smooth, translucent and ca. 2 mm in diameter. Growth on the PM1 plates indicated that strain CS4^T can tolerate brief exposure to oxygen. However, bacterial growth was not observed with the addition of any percentage of air in the headspace, nor on the PM1 plates incubated aerobically; therefore strain CS4^T is considered an obligate anaerobe. Cells stained Gram-negative. When grown on glucose cells were curved rods 0.2-0.3 µm in diameter and 1-3 µm long (Fig. 1); when grown on sucrose cells were 0.2-0.4 µm in diameter and 2-4 µm long (Harbison *et al.,* in revision). Catalase and oxidase tests were negative. Division by binary fission was observed, but prosthecae and motility were not. Heat tolerant cells were not produced. Strain CS4^T tolerated up to 100 mM NaCl, and grew optimally with the addition of 0-50 mM NaCl to the medium. Optimal growth rates were observed at an initial pH of 5.6 (final pH 4.0) and at 35° C. Both members of *Rhizomicrobium* displayed

a higher NaCl tolerance of up to 1% (171 mM) and a pH optimum of 6.7-6.8 (Table 1). Strain CS4^T grew fermentatively on numerous substrates including plant disaccharides, such as cellobiose and maltose, as detailed below in the species description. Fermentation products from glucose (1g l^{-1}) were ethanol (ca. 1 mM), formate (ca. 3 mM), acetate (ca. 1.5 mM), H_2 (ca. 2 mmole l^{-1} culture), CO_2 (4 mmole l^{-1} culture) and trace amounts of lactate. Low fermentation yields were likely due to acidification of the medium, because cell growth ceased at pH 4.0. Unlike isolates of the closest related genus, *Rhizomicrobium*, strain CS4^T did not use Na_2SO_4, ferric ammonium citrate, sodium fumarate, sodium malate or O_2 as electron acceptors (Table 1). Fe (III) was not reduced with either acetate, ethanol, formate, glucose, lactate, pyruvate, or succinate as the sole electron donor.

The fatty acids (>0.1%) of strain CS4^T were: $C_{18:1}\omega7c$ (36.7%); $C_{19:0}$ cyclo $\omega8c$ (22%); $C_{14:0}$ (17%); $C_{16:0}$ (13.2%); $C_{16:0}$ 3-OH (4.6%); $C_{18:0}$ (2.2%); $C_{14:0}$ 3-OH and/or iso-$C_{14:0}$ I (1.2%); $C_{18:1}\omega7c$ 11-methyl (0.9%); $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (0.8%); $C_{20:2}\omega6,9c$ (0.6%); $C_{12:}$ (0.4%); $C_{19:1}\omega6c$ and/or $C_{19:1}\omega7c$ and/or $C_{19:0}$ cyclo (0.2%); $C_{15:0}$ (0.2%); $C_{15:0}$ 3-OH (0.1%). Notably, the PLFA profile demonstrates that strain CS4^T can adapt to low pH environments by the significant presence of the saturated, cyclopropane-containing fatty acid $C_{19:0}$ $\omega8c$ (Mykytczuk *et al.*, 2010) indicating a stiffer and more stress-tolerant membrane structure. Members of both the *Rhizomicrobium* and *Micropepsia* genera contain $C_{18:1}\omega7c$ as their most abundant fatty acid, a trait shared only by a strain in *Sphingomonadales* (Table 2). The genomic DNA G + C content of strain CS4^T was estimated at ca. 62 mol%.

A database search of sequences closely related to strain CS4^T revealed that it was most closely related to (99%) two environmental clones 3C003352 (EU801969) and MUP3F11 (HQ178872) (Fig. 2). Two sequences from the cultured strains, *Rhizomicrobium palustre* A48 (ABO81581) and *Rhizomicrobium electricum* Mfc52 (AB365487) shared 94% and 93% identity respectively, to that of strain CS4^T. *R. palustre* was isolated from the roots of rice plants in a flooded rice paddy and was originally phylogenetically placed in *Alphaproteobacteria* as a representative of a novel genus related to *Sphingomonadales* (Ueki *et al.*, 2010). *R. electricum* was later isolated from a cellulose fed microbial fuel cell (MFC) and phylogenetically placed the *Rhizomicrobium* genus as most closely related to the *Rhizobiales* order, but not fully affiliated with any established order (Kodama & Watanabe, 2011). Strain CS4^T also shared relatively high identity (91-92%) to a group of isolates obtained on aerobic plates supplemented with xylan: Ellin 362, Ellin 335, Ellin 332, among others. The related Ellin isolates have been described as representatives of a novel order within the class *Alphaproteobacteria* (Sait *et al.*, 2002), named Ellin 329, in the Greengenes database (DeSantiz *et al.* 2006). In addition to the two *Rhizomicrobium* species, strain CS4^T also shares high identity (ca. 88%) with several members of the *Rhizobiales* (*Sinorhizobium morelense*, *Devosia submarina* strain S174, and *Ochrobactrum tritici*, among others). However, it shares equal identity (85-86%) with other members of the *Rhizobiales* (85% ID to *Methylobacterium thiocyanatum* strain ALL/SCN-P and *Cucumibacter marinus* strain CL-GR60) and members of the *Sphingomonadales* order (85% ID to *Sphingomonas paucimobilis* strain ATCC 29837 and 86% ID to *Erythrobacter longus* strain DSM 6997). Furthermore, in several different phylogenetic trees, either built here

or presented in the literature, organisms related to strain CS4^T can form a larger cluster either with *Sphingomonadales* (Ueki *et al.,* 2010), *Caulobacterales* (Sait *et al.,* 2002), or with *Rhizobiales* (Kodama & Watanabe, 2011), albeit with low bootstrap support (<50%), thus demonstrating a lack of strong affiliation with any of the identified orders. Similar to strain CS4^T, *R. electricum* shared 85% identity to both *S. paucimobilis* and *E. longus* and 87% identity to *S. morelense, D. submarina* and *O. tritici*. According to analyses by Yarza *et al.* (2014), of 85 orders analyzed the median SSU rRNA gene sequence identity is 89.2% (95% CI of 88.25-90.1%). Although Yarza *et al.* (2014) recommend a more stringent cutoff of 82% or lower for identifying a novel order based on sequence information alone, the values of 85-88% or lower identity between members of *Micropepsiales* and members of other orders are in line with those for distinct orders within the *Alphaproteobacteria*.

Several lines of evidence implicate members of the Ellin 329 order, herein proposed as *Micropepsiales*, in the utilization of both simple and more complex plant sugars such as cellobiose, maltose and/or xylan (Verastegui *et al.,* 2014; Ueki *et al.,* 2010; Sait *et al.,* 2002). At least one study has previously demonstrated the abundance of sequences affiliating with the *Micropepsiales* in the top layers of soil where plant litter and root exudates are abundant (Harbison *et al.,* in revision). Additionally, *R. electricum* was isolated from a cellulose-fed microbial fuel cell suggesting at least indirect involvement in polysaccharide degradation (Kodama & Watanabe, 2011). Finally, at least two known studies have classified members of Ellin 329 as having the capability to hydrolyze glycosidic bonds of complex carbohydrates including cellulose, cellobiose, xylose, arabinose (Verastrgui *et al.,* 2014), and xylan (Sait *et al.,* 2002).

Overall, strain CS4^T displays characteristics that clearly define it from previously characterized strains of the genus of closest relation, *Rhizomicrobium* (Table 1). The G + C content, growth optima (pH, salinity tolerance, and O_2 requirement), and utilization of growth substrates (Table 1) support our consideration that strain CS4^T is a member of a novel genus, *Micropepsia,* for which the proposed name is *Micropepsia pineolensis.* Additionally, based on phylogenetic analyses of the SSU rRNA gene, comparisons of metabolic capabilities (Table 2), and assertions from previous work (Kodama & Watanabe, 2010; Sait *et al.*, 2002) we propose that the genera *Micropepsia, Rhizomicrobium* and the isolates identified by Sait *et al.* (2002) form a new family, *Micropepsiaceae* within the new order *Micropepsiales.*

Description of *Micropepsiales* ord. nov.

Micropepsiales ord. nov. (Mi'cro.pep.si'ales. N.L. adj. *Micro* from Gr. n *mikrós* small; N.L. v *pepsi* from Gr. n *peptos* digest; N.L. *–ales* ending denoting an order; N.L. neut. n *Micropepsiales* small digesters, the order of *Micropepsia*). Gram negative, curved rod shaped bacteria that reproduce by binary fission or budding from prosthecate mother cells. Obligately anaerobic, facultative or aerobic. Neutrophilic or acid-tolerant. Mesophilic. Grow fermentatively on mono-, di- and poly-saccharides such as sucrose, dextrose, xylose, maltose, cellobiose and/or xylan. Ferric iron, nitrate, oxygen and/or fumarate may serve as electron acceptors. DNA G + C of 55-64 mol%.

Description of *Micropepsiaceae* fam. nov.

Micropepsiaceae fam. nov. (Mi'cro.pep.si'a.ce.ae. N.L. adj. *Micro* from Gr. n *mikrós* small; N.L. v *pepsi* from Gr. n *peptos* digest; N.L. –*aceae* ending denoting a family; N.L. neut. n *Micropepsiaceae* small digesters, the family of *Micropepsia*). Description of the family is the same as that for the order.

Description of *Micropepsia pineolensis* gen. nov.

Micropepsia gen. nov. (Mi'cro.pep.si'a N.L. adj. *Micro* from Gr. n *mikrós* small; N.L. v *pepsi* from Gr. n *peptos* digest; *Micropepsia* a small digester)

Cells are gram-negative, curved rods without prosthecae the reproduce by binary fission. Anaerobic, mesophilic, acid-tolerant and grows fermentatively on a variety of mono- and di-saccharides including sucrose, xylose, maltose, glucose, galactose, fructose, glycerol, D-mannose and cellobiose. The type species is *Micropepsia pineolensis*.

Description of *Micropepsia pineolensis* sp. nov. strain CS4^{T}

Micropepsia pineolensis (pine.ol.en.sis N.L. adj referring to the strain origin from Pineola, NC; N.L. neut. n *Micropepsia pineolensis* small digester from Pineola).

Cells are anaerobic, Gram-negative, non-spore forming and non-motile, slightly curved rods (0.2-0.3 µm diameter and 2.0-4.0 µm long) that reproduce by binary fission. The optimum growth temperature is 35°C (range of 15 - 35°C), the optimum pH is 5.6 (range 3.9 – 6.8). The optimum salinity concentration for growth is 0 mM NaCl (growth observed $\leq$ 100 mM NaCl). Grows fermentatively on cellobiose, D-glucose, D-mannose,

fructose, galactose, glycerol, lactose, maltose, peptone, sucrose, trehalose and xylose. Does not utilize 2-butanol, acetate, alanine, arabinose, aspartic acid, cellulose, citrate, cysteine, esculin, ethanol, fumarate, glycine, inulin, lactate, mannitol, methanol, propanol, proteose, pyruvate, rhamnose, ribose, serine, sodium acetate, sodium formate, soluble starch, sorbitol, sorbose, succinate, or xylan. Major fermentation products on glucose (1g l^{-1}) are: E (ca. 1 mM), F (ca. 3 mM), A (ca. 1.5 mM), H_2 (ca. 2 mmoles l^{-1} culture) and CO_2 (ca. 4 mmoles l^{-1} culture). Does not utilize acetate, ethanol, formate, lactate, pyruvate, or succinate as an electron donor. Does not utilize Fe (III), Na_2SO_4, sodium fumarate, sodium malate or O_2 as an electron acceptor. The major cellular fatty acids (>5%) are $C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$, $C_{14:0}$, and $C_{16:0}$.

The type strain CS4^T (=JCM 30711^T =ATCC BAA-2724^T) was isolated from anoxic peat soil in a southern Appalachian peatland known as Pineola Bog, Pineola, NC.

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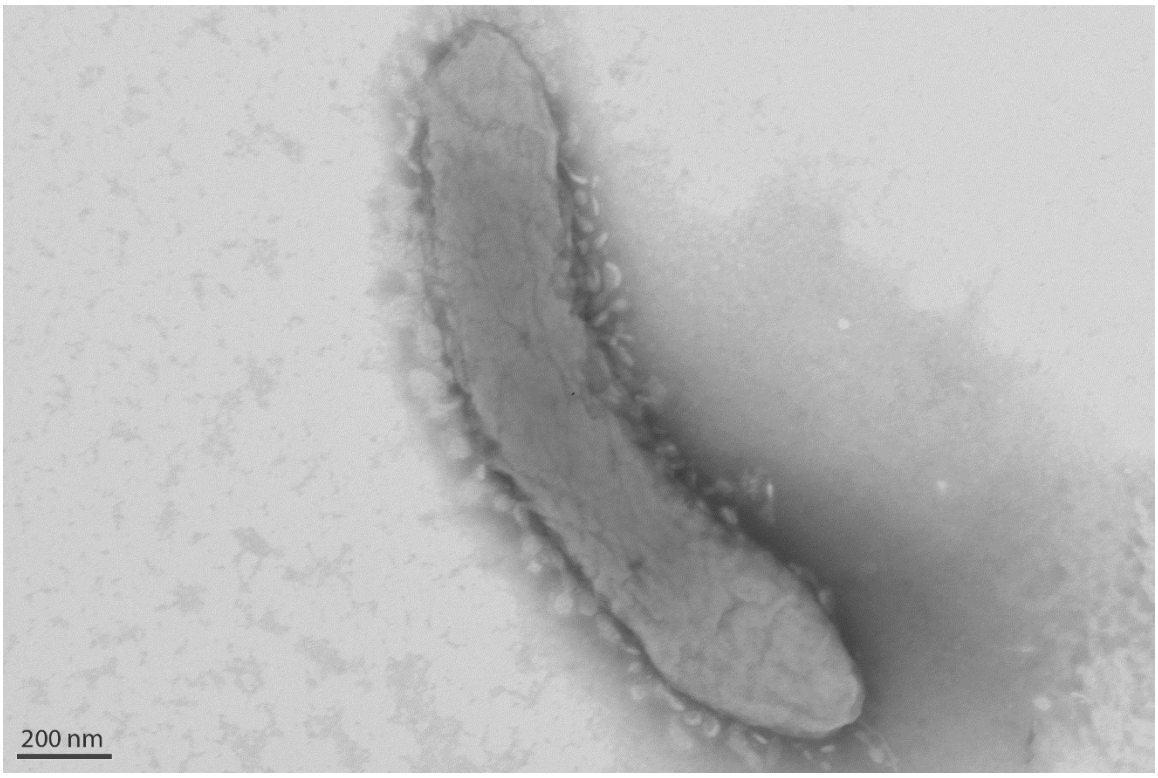

Figure. 2.1. Transmission electron micrograph of strain CS4^{T}. Cells were grown in PM1 media supplemented with glucose, negatively stained using phosphotungsic acid, and showing curved rods.

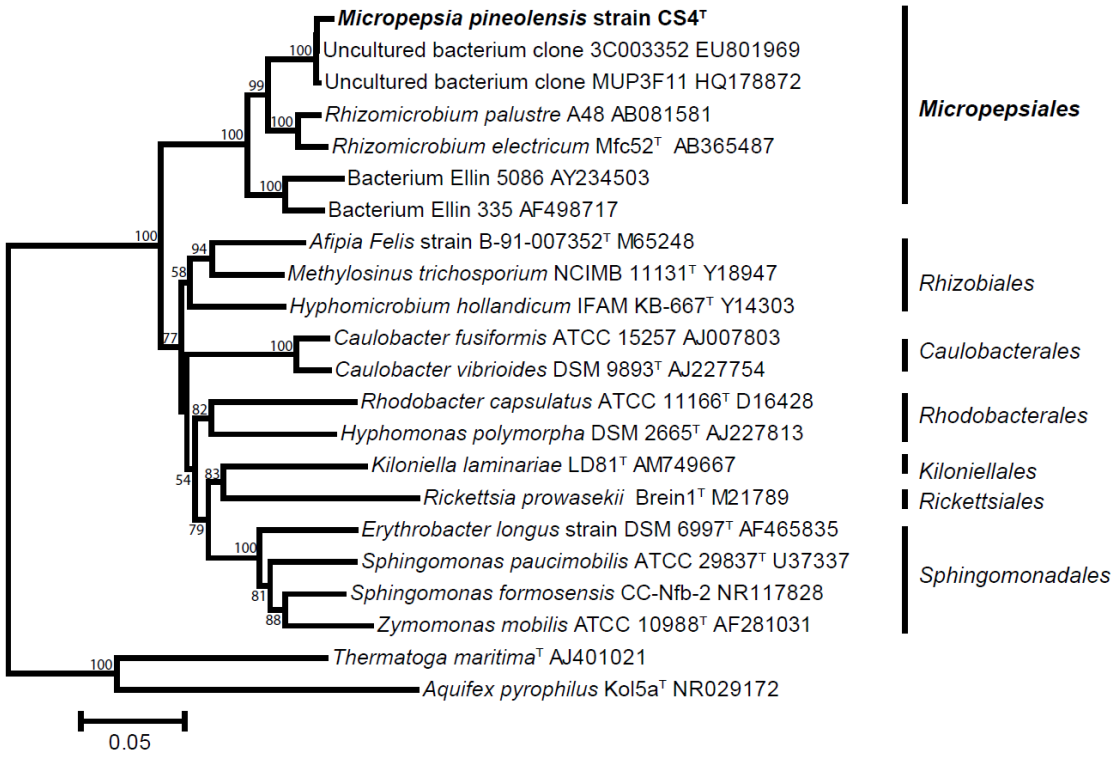

Figure. 2.2. Neighbor-joining dendrogram showing the phylogenetic position of strain CS4^T (KU738893) in relation to the *Rhizomicrobium* genus. Members of *Micropepsiales* ord. nov. are shown in comparison to other Alphaproteobacteria orders including *Rhizobiales* and *Sphingomonadales*. Nodal support was determined using bootstrapping values of 100 replicates, in neighbor-joining, and values are displayed only for those nodes with support values >50% that were also supported by maximum likelihood analyses.

Table 2.1. Differential characteristics of Strain CS4^T, *R. electricum* Mfc52^T and *R. palustre* A48

Characteristic	**CS4^T**	***R. electricum* Mfc52^T***	***R. palustre* A48†**
Prosthecae	-	+	+
pH optimum (range)	5.6 (5.0-6.8)	6.7 (5.0-7.5)	6.8 (5.5-7.3)
NaCl range (mM)	0-0.6%	0-1%	0-1%
O_2 requirement	Anaerobic	Facultatively-anaerobic	Facultatively-anaerobic
Anaerobic reduction of			
Nitrate	-	+	-
Fumarate	-	+	-
Fe (III)	-	+	+
Fermentive growth on			
Cellobiose	+	+	+
Glycerol	+	-	-
Trehalose	+	+	-
Mannose	+	+	-
Ribose	-	+	-
Arabinose	-	+	+
Xylan	-	-	+

+, Positive; -, negative
*Data collected by Kodama & Watanabe, 2011
†Data collected by Ueki *et al.*, 2010

Table 2.2. Characteristics of isolates within the *Micropepsiales* ord. nov. lineage compared to those within related families of *Alphaproteobacteria*. Order, family (genus): **1**, *Micropepsiales, Micropepsiaceae* (*Micropepsia* CS4^{T}); **2**, *Micropepsiales, Micropepsiaceae* (*Rhizomicrobium*); **3**, *Rhizobiales, Hyphomicrobiaceae* (*Hyphomicrobium*); **4**, *Rhizobiales, Hyphomicrobiaceae* (*Rhodoplanes*); **5**, *Rhizobiales, Methylocystaceae* (*Methylocystis*); **6**, *Rhizobiales, Bradyrhizobiaceae* (*Afipia*); **7**, *Sphingomonadales, Sphingomonadaceae* (*Sphingomonas*); **8**, *Sphingomonadales, Erythrobacteraceae* (*Erythrobacter*); **9**, *Caulobacterales, Hyphomonadaceae* (*Hellea*)

	Micropepsiales		*Rhizobiales*				*Sphingomonadales*		*Caulobacterales*
Characteristic	1	2*	3†	4†	5†	6†	7†	8‡	9ϕ
Cell division	Binary division	Binary Division	Polar growth, budding	Budding	Binary division	Binary division	Binary division	Binary division	Budding
Prosthecae	-	Stalk	Hypha	Division tube	-	-	-	Stalk-like	One, polar
Growth	Obligate anaerobe	Facultative anaerobic	Aerobic, nitrate reduction	Phototrophic (anoxic), chemotrophic (oxic), nitrate reduction	Aerobic, obligately methanotrophic	Aerobic	Aerobic	Aerobic, chemo-heterotrophic	Aerobic, heterotrophic
Utilization of C1 compounds	-	-	+	-	+	-	-	-	-
Major CFAs	C18:1ω7c, C19:0 cyclo ω8c, C14:0, C16:0	C18:1ω7c, anteiso-C15:0, C16:0	C18:1	C18:1, C16:0	C18:1	C18:1, branched-C19:1, C17:0 cyclo	C18:1, C14:0 2-OH, C16:0	C18:1ω7c, C17:1ω6c,	3-OH C10:0, 3-OH C12:1, 3-OH C11:0
DNA G+C content (%mol)	61.9	55-64	66-71	67-70	61-67	61.5-69	59-68	60-64	47

(+) Positive, (-) Negative, (ND) Not determined

* Kodama & Watanabe (2011)
† Ueki *et al.* (2010)
‡ Xue-Wei *et al.* (2009)
ϕ Alain *et al.* (2008)

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Chapter 3: A Novel Isolate and Widespread Abundance of the Candidate Alphaproteobacterial Order (Ellin 329), in Southern Appalachian Peatlands

Summary

Peatlands of all latitudes play an integral role in global climate change by serving as a carbon sink and a primary source of atmospheric methane; however, the microbial ecology of mid-latitude peatlands is vastly understudied. Herein, next generation Illumina amplicon sequencing of small subunit rRNA genes were utilized to elucidate the microbial communities in three southern Appalachian peatlands. In contrast to northern peatlands, *Proteobacteria* dominated over *Acidobacteria* in all three sites. An average of 11 bacterial phyla was detected at relative abundance values >1%, with three candidate divisions (OP3, WS3 and NC10) represented, indicating high phylogenetic diversity. Physiological traits of isolates within the candidate alphaproteobacterial order, Ellin 329, obtained here and in previous studies, indicate that bacteria of this order may be involved in hydrolysis of poly-, di-, and mono-saccharides. Community analyses indicate that Ellin 329 is the third most abundant order and is most abundant near the surface layers where plant litter decomposition should be primarily occurring. In sum, members of Ellin 329 likely play important roles in organic matter decomposition, in southern Appalachian peatlands and should be investigated further in other peatlands and ecosystem types.

Introduction

Wetlands account for approximately 23% of total atmospheric methane emissions (Aronsen *et al.* 2013; Mitsch & Gosselink 2000; Gorham 1991), affecting the global climate system and playing important roles in biosphere feedbacks to climate and environmental changes. Mid- and southern latitude wetlands have been identified as the source of approximately 70% of the total methane released by wetlands (Walter *et al.* 2001), due to longer and more productive growing seasons. As well as emitting methane, peat forming wetlands (peatlands) are also a substantial net carbon sink, having stored over one-third of global soil carbon over the Holocene as partially decomposed organic soil matter (Gorham 1991). A large portion of peatlands are *Sphagnum*-dominated, characterized by acidic soils, small pools of available macro and micro nutrients, low water flow and associated low redox potentials (Moore & Basiliko 2006; Aerts *et al.* 1999; Benoit *et al.* 1998; Wieder 1985), and are prevalent throughout the northern parts of North America, Europe and Asia. Southern North American peatlands are less abundant than boreal counterparts but share many common traits, leading to increased interest in their importance in global climate studies.

Complex microbial processes determine rates of C loss from peatlands; yet the microbial ecology (Dedysh 2011) and key anaerobic degradation processes are vastly understudied. Studies have mainly focused on the archaea and bacteria involved in methane production or consumption (Reviewed in Mandic-Mulec *et al.* 2014; Anderson *et al.* 2013; Bridgham *et al.* 2013). A few studies have examined overall microbial diversity in peatlands and found that the *Proteobacteria* and *Acidobacteria* are detected in the greatest abundance (Dedysh 2011). Other phyla almost always reported as non-rare (relative abundance values >1%) in peatlands include: *Verrucomicrobia, Actinobacteria* and *Planctomycetes;* and those

sometimes considered non-rare have thus far included *Firmicutes, Bacteroidetes, Chloroflexi, Spirochaetes,* (Puglisi *et al.* 2014; Tsitko *et al.* 2014; Serkebarva *et al.* 2013; Dedysh 2011) *Chlamydiae* (Urbanová & Bárta 2014) and *Nitrospirae* (Urbanová & Bárta 2016). Members of *Proteobacteria* are very diverse organisms that can adapt to a wide variety of lifestyles (Serkebaeva *et al.* 2013) and environments. *Alphaproteobacteria* in particular have been isolated from environments worldwide, carrying out a range of ecological processes. In peatlands, *Alphaproteobacteria* are thought to be primarily involved in methane oxidation (Tsitko *et al.* 2014; Dedysh 2011), sugar fermentation and anaerobic respiration (Lipson *et al.* 2013; Tveit *et al.* 2013).

Herein, we employed Illumina Miseq amplicon sequencing technology to elucidate the microbial assemblages involved in southern Appalachian peatlands. Additionally, using peat collected from Pineola Bog, we obtained a novel isolate, strain CS4, within the candidate alphaproteobacterial order, Ellin 329. The few known cultured (Kodama *et al.* 2011; Ueki *et al.* 2010; Sait *et al.* 2002; this publication) and uncultured members (Verastegui *et al.* 2014) of Ellin 329 have been implicated in degradation of sugars and more complex plant di- and poly-saccharides such as cellobiose, xylan, and/or other compounds. Culture independent Illumina data further demonstrated the prevalence, and potential importance, of members of the Ellin 329 order.

Materials and Methods

Site description:

The three peatland study sites in north-western North Carolina, USA, have been described in detail by Hawkins *et al.* (2014). The sites represent a nutrient gradient from

poor to intermediate fens, each having at least partial *Sphagnum* moss cover at each of three within-site sampling locations. Sugar Mountain Bog had an average pore water pH of 4.9 (range 4.6-5.4) and the water table depths at time of sampling ranged from 8-22 cm below the surface; Pineola Bog had an average pore water pH of 5.1 (range 4.9-5.2) and the water table depths at time of sampling ranged from 17-38 cm; Tater Hill Bog is a more minerotrophic site with numerous *Carex* species, an average pore water pH of 6.1 (range 6.0-6.2) and the water table depths at time of sampling ranged from 0-14 cm below the surface. All three sites are between 1080 and 1300 meters elevation.

Sampling method:

Peat samples were collected on September 12, 2013 from Pineola Bog at three locations within the bog at a depth of 25-30 cm (Pineola 2013, P.13. or P.25). Samples for a collaborative U.S. Department of Energy's Joint Genome Institute (JGI, Walnut California) study collected on June 19 and 20, 2014, from all three sites, denoted as 2014, were collected in triplicate at a depth of 10-20 cm, and 30-40 cm from all sites and additionally, at a depth of 60-70 cm from Pineola Bog (Pineola 2014), since this was the only site containing peat below 45 cm. Pineola, 2013 samples were collected in airtight jars, while the 2014 JGI samples were collected using an 11 cm diameter PVC corer and stored in Ziplock bags. Samples for molecular work were frozen at -80°C until further use and samples for culturing were taken directly to the glove box.

Community analysis:

Samples collected in 2013 were processed for community sequencing as follows. DNA was extracted from the peat material using the MoBio Laboratories PowerSoil® DNA Isolation Kit according to the manufacturer's instructions. Extracted DNA was quantified

using a nanodrop and stored at -4°C until further use. PCR amplification was performed in a 25 µl reaction containing 10 µl PCR master mix (Q5 Hot start High Fidelity 2X Master Mix, New England BioLabs), 1 µl of 50 µM primers modified with Illumina adapters (515F 5' GTGCCAGCMGCCGCGGTAA and 806R 5' GGACTACVSGGGTATCTAAT), forward and reverse respectively, PCR water to bring the volume to 25 µl and DNA to equal approximately 15 ng of DNA per reaction. The PCR protocol was executed using the following parameters: 94°C denaturation for three minutes, then 35 cycles of a 94°C denaturation for 45 seconds, 55°C primer annealing for 60 seconds, and 72°C extension for 90 seconds. The last step was a 72°C extension for 10 minutes followed by a hold at 10°C until storage. After PCR purification, samples were sent to West Virginia University's Genomic Core Facility (Morgantown, WV) for pair end sequencing on an Illumina MiSeq system. 2013 sequencing data are accessible via the NCBI database (http://www.ncbi.nlm.nih.gov/sra) as project number SRP070579.

Samples collected in 2014 were processed as follows. Samples were sent to the US Forest Service Northern Research station (Houghton, Michigan) for DNA extraction and purification. Approximately 10 cubic centimeters of collected peat were subsampled, placed in a 50ml falcon tube with twenty 3.2mm chrome-steel beads and pulverized for 2 minutes in a modified mini-beadbeater-96 (Biospect Products, Bartlesville, OK, USA). DNA was then extracted from 0.5 g of the pulverized peat using a MoBio PowerSoil® DNA Isolation Kit following the manufacturer's instructions, amended with an additional 30 minute incubation at 65° C following the addition of the C1 lysis buffer and 10 minutes of vortexing. DNA was cleaned with a MoBio PowerClean® Pro DNA Clean-Up Kit and quantified with a Qubit Flourometer (Invitrogen). DNA extracts were then sent to the U.S.

Department of Energy Joint Genome Institute (JGI, Walnut Creek, California) where they were subjected to PCR amplification with the primers 515F and 806R fitted with adaptors and unique 11bp barcode sequences. Amplified DNA was then sequenced on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using 2x250 chemistry. One sub-sample from the Sugar 30-40 cm range failed to sequence and is excluded from analyses. Data are accessible via the JGI portal under the DOE contract number: DE-AC02-05CH11231 (http://genome.jgi.doe.gov/).

All samples were aligned with PANDAseq (Masella *et al.* 2012), processed with QIIME (Caporaso *et al.* 2010) and USEARCH (Edgar 2010) (OTU assignment at a 97% cutoff value), and taxonomically identified via Greengenes database (DeSantiz *et al.* 2006) using the RDP method, described below. Pineola 2013 data were received as forward and reverse read sample pairs while JGI data was rearranged and quality filtered from raw read data using the BBMap package (BBMap_35.43.tar.gz accessed December 14, 2015). A total of 187,645 reads were entered for Pineola 2013, having a total of 16,567 unique reads. After analysis, a total of 30,843 singletons and chimeric sequences were removed. A total of 1,786 853 sequences were entered for JGI, having a total of 103,187 unique reads. After analysis 211,793 singletons and chimeric sequences were removed, resulting in 15,200 total OTUs.

Sequences in both the 2013 and 2014 data sets identified as Ellin 329 by the Greengenes database were collected. Additional unclassified sequences within *Alphaproteobacteria* were analyzed using RDP classifier and RDP Seq Match (Cole *et al.* 2005), and any sequences with *Rhizomicrobium spp.* top hits were included in the analysis (88-94% ID to strain CS4; 89-96% ID to Bacterium Ellin329 accession number: AF498711.1). To eliminate repetitive OTUs between the two datasets, DOTUR was used to

reassign OTUs using a 97% cut-off value. The Ellin 329 sequences, the sequence for the novel isolate CS4, (sanger sequencing) and top hit sequences from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), were aligned using the SILVA aligner of the ARB program (http://www.arb-silva.de/aligner/). Aligned sequences were edited utilizing the BioEdit software (Ibis Biosciences, Carlsbad, CA 92008). Phylogenetic trees were constructed with the neighbor-joining (Saitou & Nei 1987) and maximum likelihood methods using the PHYLIP package (http://evolution.gs.washington.edu/phylip.html). Sequences for Ellin 329 representative OTUs were deposited in GenBank with accession numbers KU705479–KU705504, and the sequence for strain CS4 with accession number KU738893.

Culture Growth and Isolation:

Cells were isolated in PM1 medium, prepared in anaerobic balch tubes as described in Bräuer *et al.* (2006), and flushed with ultra-high purity N_2 gas. Post sterilization, the following additions were made (final concentrations): ca. 0.5 mM Ti(III) Nitrilotriacetate (TiNTA) (preparation described in Bräuer *et al.* 2006), 20 mM MES (pH 5.7), Vitamin Solution (0.2, 1.0, 0.5, or 0.01 mgL^{-1} of each vitamin as described in Bräuer *et al.* 2006 and Balch *et al.* 1979), 40 mgL^{-1} yeast extract, 1 gL^{-1} glycerol, and 10 mgL^{-1} rifampicin. Tubes were inoculated with 0.5 ml of 0.45 µm filtrate from the 2013 collection of peat from Pineola Bog. Inoculated media was incubated in the dark at 28°C without shaking and growth was monitored by checking for turbidity in the liquid media by use of a spectrophotometer. Once isolated, cells were also grown in PM1 medium containing the addition of 500 mgL^{-1} peptone and/or with 500 mgL^{-1} sucrose or dextrose substituted for glycerol.

Colonies were isolated on 1.5% agar plates supplemented with glucose and peptone, streaked aerobically, but incubated anaerobically in a mason jar with an N_2 headspace. After approximately one week of growth, single colonies were transferred to new plates. Colonies were re-streaked three times to ensure purity and axenic colonies were transferred back to liquid media in the anaerobic chamber. Purity was visually inspected using fluorescence microscopy on an Olympus BX51 using acridine orange with a FITC filter. DNA was extracted, PCR amplified and sent to Beckman-Coulter Genomics (Danvers, MA) for Sanger sequencing.

SEM Imaging:

Strain CS4 was grown anaerobically on agar plates supplemented with the aforementioned media additions excluding TiNTA. Sterile 0.2 µm filters were cut into quarters and placed on the agar prior to inoculation with cells grown in liquid culture. After two weeks of growth in an anaerobic jar, the filter was removed and soaked in 2.5% gluteraldehyde for 2.5 hours. The filter was then dehydrated by soaking in a series of 50%, 75%, 85% 90%, and 95% ethanol-water solutions, followed by soaking two times in 100% ethanol for a minimum of one hour per soak, the final soak overnight. The filter was then critically point dried using liquid CO_2 in a Tousimis 931 CP drier. The dried filter was mounted on a metal stub with adhesive and gold coated. Images were collected at 25 kV with a 4 µm spot size.

Results

Bacterial and Archaeal Communities:

Bacterial sequences had the highest relative abundance, although relative abundance of archaeal sequences increased with depth. Archaeal sequences ranged from approximately 1% in the 10-20 cm depths to 3% in the 30-40 cm depths and 8.7% in the Pineola 60-70 cm depth. The highest archaeal relative abundance (18.7%) was detected in the Pineola 2013 data set in the 25-30 cm depth (Fig. 1-A). Among bacterial phyla, *Proteobacteria* had the highest abundance, (33-42%), with *Acidobacteria, Actinobacteria, Nitrospirae, Verrucomicrobia, Chloroflexi, Planctomycetes, Spirochaetes* and *Bacteroidetes* also present in all sample locations and dates (Fig. 1-B). Within *Proteobacteria,* the alphaproteobacterial and deltaproteobacterial sequences had the greatest abundance. *Alphaproteobacteria* were the most prevalent in the 10-20 cm depth in Pineola and Sugar Mountain Bog sites (40 and 39%, respectively) with alphaproteobacterial and betaproteobacterial relative abundance decreasing with depth (Fig. 1-C). *Deltaproteobacteria* became the most prevalent class in deep samples from Tater Hill and Pineola. Within *Alphaproteobacteria*, sequences related to *Rhizobiales* dominated in all sites, ranging from 68-77%, with sequences related to *Rhodospirillales* and the rarely cultivated candidate order, Ellin 329, the second and third most abundant (Fig. 1-D).

Candidate Order Ellin 329:

A novel, acid-tolerant, fermentative, obligate anaerobe, designated strain CS4, was isolated from Pineola Bog. The culture contained gram negative, curved rods (~0.25 µm diameter and ~3.0 µm long; Fig. 2). Growth was initiated between pH 5-6.8; however, actively growing cultures continued to grow down to pH 4. Growth was also observed between 15-

35°C and with less than 100 mM NaCl. Phylogenetic analysis indicated that strain CS4 clusters with OTUs identified as Ellin 329 in the Greengenes database, and alphaproteobacterial order *incertae sedis* in RDP. Isolates *Rhizomicrobium palustre*, *Rhizomicrobium electricum,* Ellin 329, Ellin 332, and Ellin 5086 among others (Fig. 3), also clustered within this novel order. Clear clustering can be seen defining Ellin 329 from other well characterized orders, including *Rhizobiales*, *Caulobacterales* and *Sphingomondales*. From the Illumina sequencing data, 26 unique OTUs were retrieved, 22 of which clearly clustered within Ellin 329. Four of the OTUs, 5403, 7727, 238, and 439, may represent another novel order or a candidate family within Ellin 329 (Fig. 3, dashed line). Abundance of Ellin 329 sequences ranged from (4%-5%) across all sites, and decreased in abundance with depth even as a percentage of alphaproteobacterial reads (Table 1).

Discussion

Previous studies have shown that *Acidobacteria* dominate over the second most abundant phylum, *Proteobacteria*, in northern oligotrophic peatlands (Serkebaeva *et al.* 2013; Pankratov *et al.* 2011; Costello and Schmidt 2006). However, our study shows that *Proteobacteria* were the dominant bacterial phyla in all sites, potentially the result of higher pH (4.6-6.2) and nutrient content of the sites studied here, compared to many northern peatlands. At least one study has reported that abundance of *Acidobacteria* and *Proteobacteria* were inversely proportionate to each other (Urbanová & Bárta 2014) with acidobacterial abundance greater in bog environments with lower pH and lower nutrient concentrations (Lin *et al.* 2014). This trend is echoed in the relatively high microbial diversity shown here where each site has an average of 11 phyla (range 10-15) in contrast to

northern bogs which showed an average of eight phyla (range 6-9; as reviewed by Dedysh 2011) calculated for phyla with a relative abundance greater than 1% in both cases. This corroborates other wetland studies that have shown a positive correlation between pH and both bacterial diversity (Dorador *et al.* 2013) and archaeal diversity (Kotsyurbenko 2010). Indeed, even in a range of non-saturated soils, Fierer and Jackson showed that bacterial diversity and richness increased along a spectrum of acidic to more neutral conditions (Fierer & Jackson 2005). Overall, the sites analyzed here display high bacterial diversity, as seen by the 15 non-rare phyla detected in total across all three sites (Fig. 1-B).

Most notably, representatives of *Cyanobacteria* (Pineola 2014 30-40 cm, Sugar 2014 and Tater 2014 10-20 cm) and *Chlorobi* (Pineola 2013, Tater 2014, Sugar 2014; increasing in abundance with depth), both phototrophic organisms (Gupta 2004), were not only detected in the top layer, but in deeper layers as well. Although *Cyanobacteria* are not commonly detected in peatland soils, they have been detected in sphagnum moss (Berg *et al.* 2013). Interestingly, a number of rarely detected groups, candidate divisions WS3, NC10 and OP3 were detected in our sites, and they have been detected in previous analyses of high elevation wetlands (Dorador *et al.* 2013). Candidate division WS3, originally detected in a methanogenic aquifer (Dojka *et al.* 1998), represented (1.21%) in Tater 2014 at both depths, though it has previously only been detected in deeper peatland depths (Kirkpatrick *et al.* 2006). Candidate division NC10 was detected in Pineola 2014, 60-70 cm, and Tater 2014, 30-40 cm, and this group has been previously implicated in anaerobic methane oxidation coupled to denitrification (Ettwig *et al.* 2009). Candidate division OP3 was originally retrieved from the Obsidian Pool hot spring in Yellowstone National Park, later detected in flooded patty soils (Derakshani *et al.* 2001) and peatlands (Serkebaeva *et al.* 2013), and may

have similar metabolic capabilities to Deltaproteobacteria (Glöckner *et al.* 2010). OP3 was detected in our sites in Pineola 2014, 60-70 cm and Tater 2014, 30-40 cm.

The difference between archaeal sequence abundance in the 25-30 cm Pineola 2013 samples versus the 30-40 cm Pineola 2014 samples is striking. This difference may be attributed to the fact that 25 cm was previously identified as the zone of maximum potential for methane production for Pineola Bog (Hawkins *et al.* 2014), and may correlate with a greater abundance of methanoarchaeal sequences. In addition, this difference may be due to seasonal factors between sampling dates (Sun *et al.* 2012).

A novel isolate from the poorly-described, candidate alphaproteobacterial order Ellin 329

Phylogenetic analysis of the SSU rRNA gene of strain CS4 places it within a novel alphaproteobacterial order first recognized in a study of Australian pasture soils (Sait *et al.* 2002) from which several isolates were also obtained (Ellin329, Ellin332, Ellin335, Ellin359 and Ellin362, included in Fig. 2) on low nutrient, 5% xylan agar. Two additional cultured strains, *Rhizomicrobium electricum* and *Rhizomicrobium palustre*, (Kodama & Watanabe 2011; Ueki *et al.* 2010) also fall within this order, along with the 26 unique OTUs retrieved here in our study by Illumina sequencing (Fig. 3). According to the literature, the minimum sequence identity for order classification is 83.55% (Yarza *et al.*, 2014) supporting the proposition that all clones collected in this study (89-96% ID to Bacterium Ellin329) are members of the order, Ellin 329. Members of Ellin 329 are diverse in nature originating from many different sites such as rice paddies (Itoh *et al.* 2013; Kodama & Watanabe 2011), tropical soils (Ducey *et al.* 2013), volcanic soils (Zeglin *et al.* 2015), tundra soils (Männistö *et al.* 2009), freshwater (Bruns *et al.* 2003) grassland soils (GenBank accession number LN8828806) and other soils (Dunbar *et al.* 2012; Lesaulnier *et al.* 2008; Sait *et al.* 2002).

Interestingly, 44% of sequences identified in our sites as Ellin 329 (OTUs 54, 981, 5447, 6341) showed high identity to clones derived from soil samples collected on Kasatochi Island in Alaska (Zeglin *et al.* 2016). The isolates described by Sait *et al*. and the isolate *R. palustre* (Ueki *et al.* 2010), have been shown to utilize xylan as a growth substrate (Sait *et al.* 2002) among other carbon substrates. Additionally, *R. electricum* was isolated from a cellulose-fed microbial fuel cell (Kodama & Watanabe 2011), suggesting that it is involved in polysaccharide degradation, at least indirectly. Strain CS4, cultured here, is adapted to relatively low pH (6) and oligotrophic environments (Na^+ < 50mM), and utilizes both simple and more complex saccharides. Overall, the collective cultured strains indicate that members of Ellin 329 may be involved in primary plant litter decomposition, as well as sugar fermentation. Corroborating this idea, Ellin 329 was found to thrive in the shallow (10-20 cm) depths of all three peatlands, where fresh plant litter and exudates would be most prevalent. Our results show that representatives of Ellin 329 were present in moderately high abundance in these three peatlands, which vary in pH, hydrology, and nutrient concentrations. This finding, combined with data in the literature demonstrating Ellin 329 sequence presence in diverse sites globally, suggests that Ellin 329 may play an integral role in C cycling at global scales. As such, further studies should be conducted to examine the presence and functions of Ellin 329 in wetlands, and perhaps other soil types worldwide.

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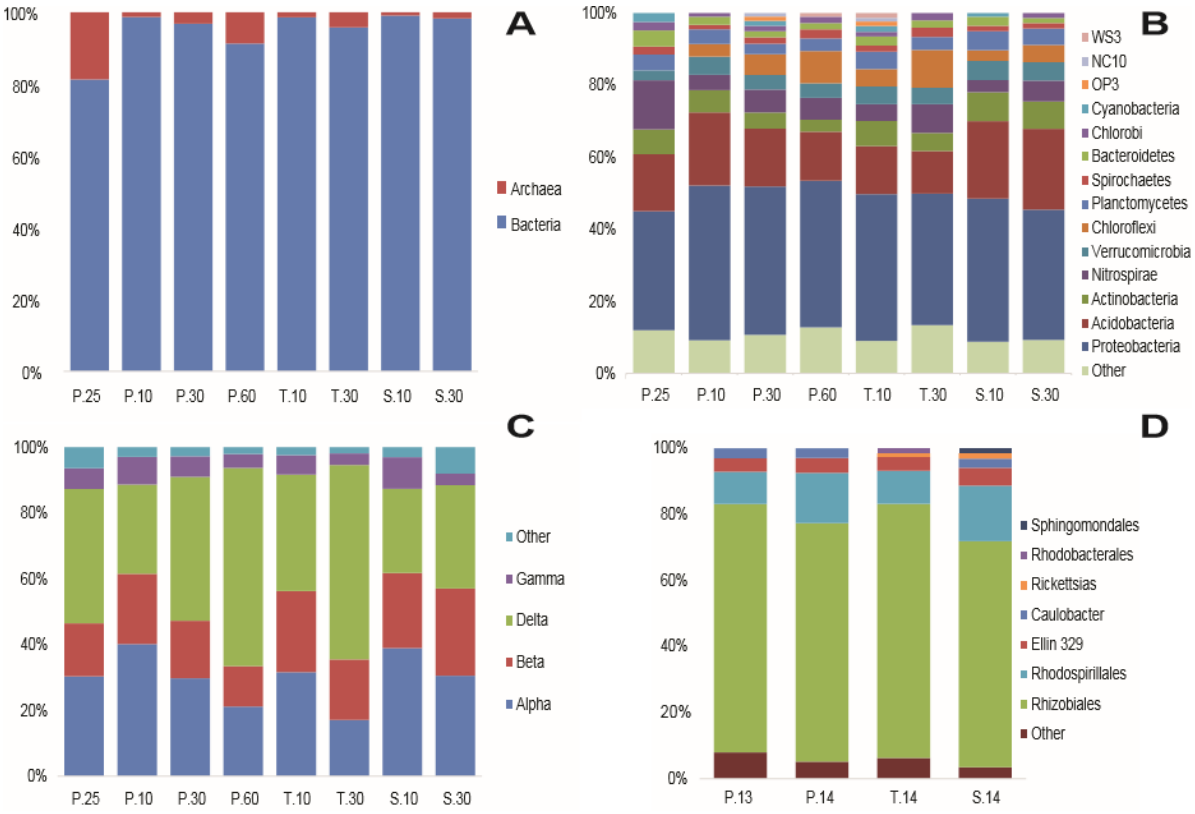

Figure. 3.1 (A-D). Relative abundance of sequences detected in Pineola 2013, (P.13 or P.25) Pineola 2014 (P.10, P.30, and P.60, or P.14), Tater 2014 (T.10 and T.30, or T.14) and Sugar 2014 (S.10 and S.30, or S.14). Relative abundance of domain-level (A), bacterial phylum-level (B) and proteobacterial class-level (C) sequences at every depth, in all sites examined. Alphaproteobacterial order relative abundances (D), averaged across all depths in all sites examined.

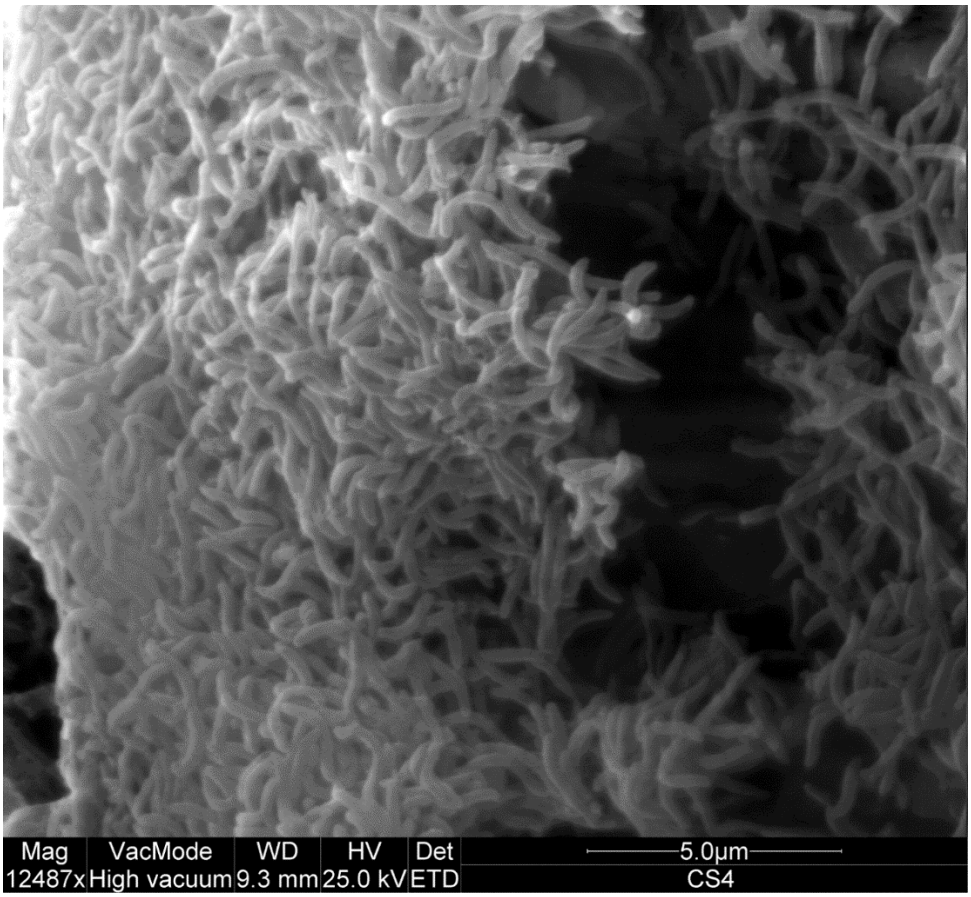

Figure. 3.2. SEM image of strain CS4. Image contrast and brightness were edited using Photoshop.

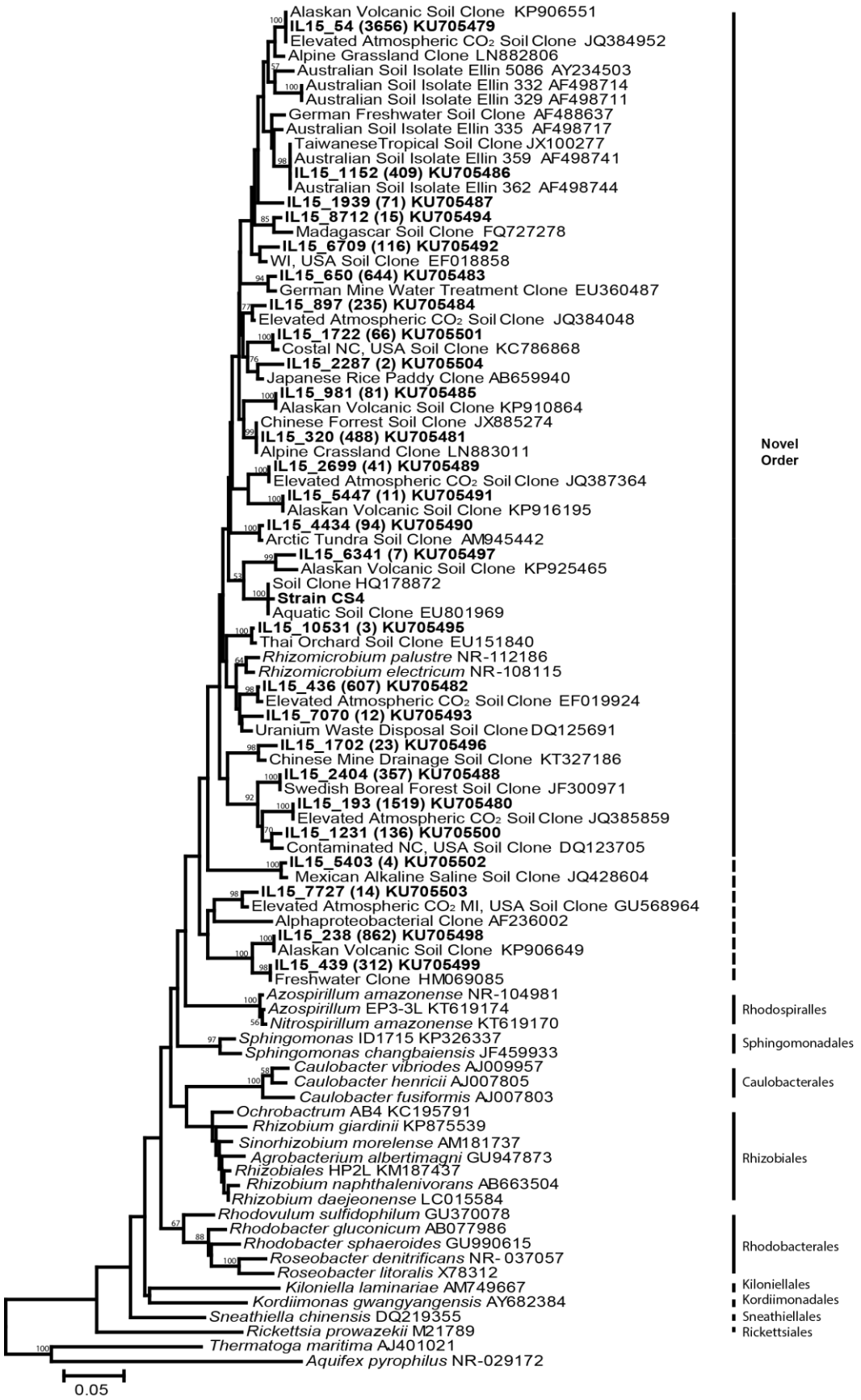
Alaskan Volcanic Soil Clone KP906551
IL15_54 (3656) KU705479
Elevated Atmospheric CO2 Soil Clone JQ384952
Alpine Grassland Clone LN882806
Australian Soil Isolate Ellin 5086 AY234503
Australian Soil Isolate Ellin 332 AF498714
Australian Soil Isolate Ellin 329 AF498711
German Freshwater Soil Clone AF488637
Australian Soil Isolate Ellin 335 AF498717
TaiwaneseTropical Soil Clone JX100277
Australian Soil Isolate Ellin 359 AF498741
IL15_1152 (409) KU705486
Australian Soil Isolate Ellin 362 AF498744
IL15_1939 (71) KU705487
IL15_8712 (15) KU705494
Madagascar Soil Clone FQ727278
IL15_6709 (116) KU705492
WI, USA Soil Clone EF018858
IL15_650 (644) KU705483
German Mine Water Treatment Clone EU360487
IL15_897 (235) KU705484
Elevated Atmospheric CO2 Soil Clone JQ384048
IL15_1722 (66) KU705501
Costal NC, USA Soil Clone KC786868
IL15_2287 (2) KU705504
Japanese Rice Paddy Clone AB659940
IL15_981 (81) KU705485
Alaskan Volcanic Soil Clone KP910864
Chinese Forrest Soil Clone JX885274
IL15_320 (488) KU705481
Alpine Crassland Clone LN883011
IL15_2699 (41) KU705489
Elevated Atmospheric CO2 Soil Clone JQ387364
IL15_5447 (11) KU705491
Alaskan Volcanic Soil Clone KP916195
IL15_4434 (94) KU705490
Arctic Tundra Soil Clone AM945442
IL15_6341 (7) KU705497
Alaskan Volcanic Soil Clone KP925465
Soil Clone HQ178872
Strain CS4
Aquatic Soil Clone EU801969
IL15_10531 (3) KU705495
Thai Orchard Soil Clone EU151840
Rhizomicrobium palustre NR-112186
Rhizomicrobium electricum NR-108115
IL15_436 (607) KU705482
Elevated Atmospheric CO2 Soil Clone EF019924
IL15_7070 (12) KU705493
Uranium Waste Disposal Soil Clone DQ125691
IL15_1702 (23) KU705496
Chinese Mine Drainage Soil Clone KT327186
IL15_2404 (357) KU705488
Swedish Boreal Forest Soil Clone JF300971
IL15_193 (1519) KU705480
Elevated Atmospheric CO2 Soil Clone JQ385859
IL15_1231 (136) KU705500
Contaminated NC, USA Soil Clone DQ123705
IL15_5403 (4) KU705502
Mexican Alkaline Saline Soil Clone JQ428604
IL15_7727 (14) KU705503
Elevated Atmospheric CO2 MI, USA Soil Clone GU568964
Alphaproteobacterial Clone AF236002
IL15_238 (862) KU705498
Alaskan Volcanic Soil Clone KP906649
IL15_439 (312) KU705499
Freshwater Clone HM069085
Azospirillum amazonense NR-104981
Azospirillum EP3-3L KT619174
Nitrospirillum amazonense KT619170
Sphingomonas ID1715 KP326337
Sphingomonas changbaiensis JF459933
Caulobacter vibriodes AJ009957
Caulobacter henricii AJ007805
Caulobacter fusiformis AJ007803
Ochrobactrum AB4 KC195791
Rhizobium giardinii KP875539
Sinorhizobium morelense AM181737
Agrobacterium albertimagni GU947873
Rhizobiales HP2L KM187437
Rhizobium naphthalenivorans AB663504
Rhizobium daejeonense LC015584
Rhodovulum sulfidophilum GU370078
Rhodobacter gluconicum AB077986
Rhodobacter sphaeroides GU990615
Roseobacter denitrificans NR- 037057
Roseobacter litoralis X78312
Kiloniella laminariae AM749667
Kordiimonas gwangyangensis AY682384
Sneathiella chinensis DQ219355
Rickettsia prowazekii M21789
Thermatoga maritima AJ401021
Aquifex pyrophilus NR-029172
0.05
Novel Order
Rhodospiralles
Sphingomonadales
Caulobacterales
Rhizobiales
Rhodobacterales
Kiloniellales
Kordiimonadales
Sneathiellales
Rickettsiales

Figure. 3.3. Neighbor-joining tree of select SSU rRNA gene sequences within several alphaproteobacterial orders . Representative OTUs (in bold) and top blast hits for sequences clustering in the Ellin 329 order that were retrieved from Pineola BogTater Hill Bog, Sugar Mountain Bog, and the sequence for strain CS4 (GenBank accession number KU738893). The number of sequences represented by each OTU (97%) is indicated in parenthesis. The solid line indicates clusters containing sequences clearly identified as Ellin 329 in the Greengenes database. The dashed lineindicates more divergent sequences that may represent novel order(s) or (a) novel family/families, within Ellin 329. *Thermatoga maritima* and *Aquifex pyrophilus* were used as an outgroup. Nodal support was determined using bootstrapping values of 100 replicates, in neighbor-joining, and values are displayed only for those nodes with support values >50% that were also supported by maximum likliehood analyses.

Table 3.1. Relative abundance of Ellin 329, as a percentage of total bacterial reads (% Total), and as a percentage of alphaproteobacterial reads (% Alphas), by depth for samples collected in Pineola Bog in 2013, as well as Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog in 2014. Samples were collected from 10-20, 30-40 and 60-70 cm depths, except for Pineola 2013, where samples were collected from a depth of 25-30 cm (30*). Read totals represent triplicate field replicates (n=3), except for Sugar 30, which had a failed sequencing reaction (n=2).

Site	**Pineola 2013**			**Pineola 2014**			**Tater 2014**			**Sugar 2014**		
Depth (cm)	10	30*	60	10	30	60	10	30	60	10	30	60
% Total	---	0.4	---	0.98	0.48	0.27	0.72	0.23	---	0.93	0.51	---
% Alphas	---	4.1	---	5.7	3.8	3.3	5.5	3.8	---	6	4.6	---
Total Reads	---	126707	---	244705	257117	154164	237402	236826	---	228133	162712	---
Alpha Reads	---	12695	---	42223	32193	13113	31384	14224	---	35288	1976	---
Ellin 329 Reads	---	516	---	2407	1237	427	1709	545	---	2118	828	---

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Chapter 4: Summary, Conclusions, and Broader Impacts

Introduction

Through the course of my research I sought to identify the key bacterial populations located in three southern Appalachian peatlands in an effort to better understand the processes and potential of mid-latitude wetlands. Specifically, my objectives were to identify and characterize a novel alphaproteobacterium, strain CS4 (Chapter 2). Additionally, my objectives were to evaluate microbial populations using Illumina sequencing of samples collected in 2013 and 2014 from Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog and compare the data to previously studied wetlands generally located in the North of North America, Europe, and Russia (Chapter 3). Data demonstrate that strain CS4 is a representative of a novel order, prevalent in all three sites.

Summary

Chapter 2 describes the characterization and identification of a novel bacterium, *Micropepsia pineolensis* strain CS4, as a novel member of *Alphaproteobacteria*. Findings from this investigation showed the need for the proposal of a novel order, *Micropepsiales* (formerly Ellin 329), a novel family, *Micropepsiaceae*, and a novel genus, *Micropepsia*, based on physiological results combined with phylogenetic analysis of 16S rRNA sequencing data. *M. pineolensis* utilizes a variety of mono- and di-saccharides including glucose, sucrose, maltose, lactose, trehalose and cellobiose, as growth substrates. The pH optimum was 5.6, temperature optimum 35°C and growth was seen up to 100 mM of NaCl. Strain CS4

also has a PLFA profile that includes the elevated presence of the saturated cyclopropane fatty acid $C_{19:0}$ ω8*c* demonstrating that it is uniquely adapted to low pH environments (Mykytczuk *et al.,* 2010). *M. pineolensis* displayed low identity (93-94%) to two cultured strains in the genus *Rhizomicrobium* (Kodama & Watanabe, 2011) and showed distinct physiological features distinguishing it from the *Rhizomicrobium* genus. Isolates identified in 2002 (Sait *et al.,* 2002), also displayed identity to strain CS4 (90-91%), which had been previously noted as a potential novel order. These findings, combined with physiological results and metabolic profiles, support the proposal of the novel order *Micropepsiales*. Additionally, based on the growth substrates utilized, and the data presented in Chapter 3, it is thought that members of *Micropepsiales* play an integral role in primary plant litter degradation in the top layer(s) of peatlands.

The data presented in Chapter 3 describe the relative abundance of bacterial populations within the three sites studied. Results show that *Micropepsiales* is the third most abundant alphaproteobacterial order and is a distinct order recognized by the Greengenes database. Additionally, *Deltaproteobacteria* were inversely proportionate with *Alphaproteobacteria* and increased with depth while *Alphaproteobacteria* decreased with depth. *Proteobacteria* had the highest relative abundance of bacterial classes (33-43%), in contrast to previously studied northern peatlands where *Acidobacteria* were shown to be the prominent bacterial population (Serkebaeva *et al.* 2013; Pankratov *et al.* 2011; Costello and Schmidt 2006). These differences may be due to ecological factors unique to the peatlands studied here compared to their northern counterparts including a more neutral pH value (4.6-6.2), longer growing season, higher nutrient concentrations and other characteristics that resemble a poor fen more than a bog. Additionally, the sites studied here displayed high

bacterial diversity, an average of 11 non-rare (>1%) phyla per site (range of 10-14), as compared to numerous other sites presented in the literature (as reviewed by Dedysh, 2011), which displayed an average of only 8 phyla (range of 6-9).

The archaeal relative abundance increased with depth; thus corroborating with previous reports. There was a substantial difference in the archaeal abundance displayed in 2013 as opposed to 2014. This may be due to depth and site of collection, as well as the season of the collection date. The samples collected in 2013 came from three locations within Pineola Bog titled "Ultra Sphagnum Mat," "Upper Creek," and "Methane Reservoir," and may have had a larger abundance of archaea, as the sampling depth of 25-30 cm was previously identified as the depth of optimum methane production (Hawkins *et al.,* 2014). This depth correlates with the level of the water table, where archaea can exist in anoxic environments but be close enough to the surface to utilize the nutrient pool produced by the breakdown of plant litter.

Conclusions and Broader Impacts

Overall, these two manuscripts presented here describe the bacterial and archaeal populations of three southern Appalachian peatlands. Much of the work previously conducted has specifically focused on microorganisms involved in methanogenesis, thus leaving vast gaps in data detailing the degradation of primary plant litter into useable compounds for methanogens. The literature has detailed that an estimated 29%-85% of the total anaerobic carbon mineralization is unable to be explained using the currently understood mechanisms (Keller & Bridgham, 2007). Members of the novel order proposed herein are of importance because of their prevalence in wetlands, at least in the sites studied,

and their potential role in primary plant litter degradation which is a poorly detailed process in peatlands. Microbes of the *Micropepsiales* (Ellin 329) order are thought to be involved in the initial steps in the anaerobic degradation of organic matter by hydrolyzing complex plant molecules into monomeric compounds to be utilized by these and other fermentative bacteria. It can be speculated that these hydrolytic and fermentative bacteria may also correlate with higher degradation rates, contributing to an environmental shift from a bog to a poor/intermediate fen by raising the rates of plant litter degradation as compared to NPP.

Bacterial diversity was shown to be higher in these southern sites compared to their northern counterparts, and *Proteobacteria* were the dominant phylum. A specific emphasis was placed on the novel order, *Micropepsiales,* for which I have isolated a novel representative.

The data collected in these studies will contribute to a growing collection of data used to promote the understanding of wetlands and the metabolic processes contained therein. Due to the fact that southern Appalachian wetlands are poorly studied, these data may contribute to a better understanding of the effects of temperature on microbial communities and potential CH_4 production as mid latitude wetlands are cited as significant contributors of methane (Walter *et al.,* 2001). The knowledge gained in these studies can be utilized by correlating previously collected data on key microbes involved in the methanogenic cycle to predict the potential effects of the studied sites on climate change. Furthermore, this data can begin to elucidate the lack of descriptive information of the hydrolytic bacteria and their metabolic processes utilized to degrade plant litter.

Future Work

In an effort to better understand the function and role of the members of *Micropepsiales*, further efforts need to be in place to isolate and characterize more members of *Micropepsiales*. Plating methods similar to Sait *et al.,* (2002) should be completed in the sites studied to isolate a wider variety of bacteria that utilize plant polysaccharide molecules such as xylan. Data from previously characterized sites could be mined to determine if members of *Micropepsiales* are present. With that, correlating ecological factors should be measures such as soil pH, temperature, nutrient content and CH_4 production. Additionally, a more complete analysis of the mechanisms and microbial assemblages involved in the anaerobic degradation of peat should be assessed. Such a lack of information has led to an incomplete understanding of wetland ecology, which is emerging as an important area of study as global temperatures increase.

Finally, further analysis on the microbial populations in the sites studied is necessary for a better understanding of the role mid-latitude wetlands play in global climate change. While this study focused on the proteobacterial population, there is still a significant amount of data remaining for further analysis.

Appendix A: Supplemental Characterization of *Micropepsia Pineolensis* Strain CS4

Summary

Classical microbiology methods and molecular techniques are widely utilized to identify and characterize novel microbial species. Described herein are specific details of the supplementary techniques and results of the determination of pH optimum, temperature optimum, NaCl tolerance, and G+C content of strain CS4 that are presented only briefly in Chapter 2.

Introduction

As global temperatures increase, a better understanding of the microbial processes in anaerobic soil environments, such as peatlands, is imperative because of the uncertain impact of temperature on organic matter degradation and gas flux rates (Gorham, 1991). It has been seen that, as temperatures rise, the decomposition processes in peatlands increase and the permafrost regions begin to thaw, overall releasing a significant amount of CH_4 into the atmosphere (Hodgekins *et al.,* 2014; McCalley *et al.,* 2014; Mackelprang *et al.,* 2011). Currently, CH_4 is 200 times less concentrated than CO_2 in the Earth's atmosphere (Aaronsen, *et al.,* 2013), but on a molecular level, CH4 can hold more heat than CO_2, thus making it approximately 30 times more effective at trapping heat as CO_2 (Cuff & Goudie, 2009).

In order to better understand the processes, it is necessary to identify the key microbial members of peatland environments. At this time, very little is known about the microbial populations of peatlands (Dedysh, 2011), thus highlighting the importance of

isolating and characterizing microbes from wetland environments. For example, *Acidobacteria* identified in peatlands worldwide are representatives of subdivisions 1, 3, 4, and 8. Of these subdivisions, only subdivision 1 is well represented by cultured and characterized bacteria; subdivisions 3 and 8 contain only a few cultured bacteria, and subdivision 8, none (Dedysh, 2011). This point is particularly concerning, because *Acidobacteria* have been identified as one of the more prevalent bacteria populations in northern peatlands (Serkebaeva *et al.,* 2013; Pankratov *et al.,* 2011; Costello & Schmidt, 2006).

The data presented here was utilized to characterize and support the proposal of strain CS4 as a representative of a novel genus and species within a proposed novel order and family (Harbison *et al.,* in revision). These data indicate that members of the candidate order 'Micropepsiales' play a significant role in primary plant litter degradation by degrading poly- and di-saccharide molecules. As one of three described and cultured members, strain CS4 serves an important role by elucidating the characteristics of bacteria in the novel order.

Experimental Procedures

pH optimum, NaCl tolerance and temperature optimum:

For tests of pH optimum, strain CS4 was grown in amended PM1 media that contained a range of buffered pH from 3.88–7.51. The specific buffers (final concentration 0.5 M) and initial measured pH values were as follows: Homopipes, pH 3.88; Homopipes, pH 5.05; Homopipes, pH 5.38; MES, pH 5.7 and HEPES, pH 5.8 at a final pH of 5.48; MES, pH 5.62; MES pH 5.84; MES, pH 5.84; MES, pH 6.78; HEPES, pH 6.38; HEPES, pH 6.81; Homopipes, pH 7.51. For temperature optimum analyses, strain CS4 was grown in PM1

media buffered with MES to pH 5.6 and incubated at either 4°C, 15°C, 24°C, 28°C, 35°C, 42°C, or 52°C. For tests of NaCl tolerance, strain CS4 was grown in amended PM1 media that contained a range from 0-250 mM NaCl. All three experiments (pH, temperature and NaCl tolerance) were conducted in triplicate in 10 ml borosilicate clear glass bottles. Growth was measured using a ThermoSpectronic Genesys 20 photospectrometer on samples, with data collected every 4 hours from 12-24 hours post inoculation (hpi) and then every 12 hours following until a plateau was achieved. Any variables that did not display growth were again tested at one month post-inoculation to ensure there was no growth.

Fe (III) reduction:

Strain CS4 was grown in PM1 media with glucose (1 g l^{-1}) in triplicate with ferric ammonium citrate at a final concentration of 1, 2, 4, or 8 mM, or ferrous chloride at a final concentration of 0.06 mM or 0.24mM. Fe (III) reduction was tested colorimetrically by adding 1 drop of culture medium to a 0.01X solution of ferrozine (FerroZine Iron Reagent Solution Pillows, Hach) buffered to pH 7.0.

Sulfate reduction:

Strain CS4 was grown in PM1 medium with glucose (1 g l^{-1}) in triplicate supplemented with a final concentration of 20mM Na_2SO_4 and ca. 1 mM $FeSO_4$. Sulfate reduction was tested by visually observing for the presence of a black precipitate formed by iron-sulfide-complexes.

G+C content:

G+C content was determined according to work previously detailed by González & Jiménez (2004) using an Applied BioSystems RT-PCR 7500 machine and the v2.0.6 platform. Microbial DNA was extracted using a MP Bio Fast Spin DNA Kit and quantified

using a nanodrop. All reactions contained 0.8-5 µg of DNA and 25 µl of 1X Fermentas Maxima SYBR Green/ROX qPCR Master Mix. The final volume of each reaction was 50 µl, prepared in triplicate, in 96 well plates. The thermal ramp was from 25°C to 99.9°C at a ramp rate of 0.5%.

Six reference strains with known genomic G+C content were utilized to establish a regression line, including *Clostridium acetobutyliticum* DSM 1731, *Flavobacterium capsulatum, Bacillus subtilis, Methanoregula boonei, Micrococcus luteus* strain 4698, and *Escherichia coli.* The melting temperature (T_m) collected by the machine was utilized to determine the G+C content by plotting known values from cultured strains and determining a regression line.

Transmission electron microscopy:

Cell morphology was determined by inverting 100 mesh formvar coated copper grids on 5 µl of 1X concentrated culture medium for 5 minutes. The grids were subsequently inverted onto 5 µl of 2.5% glutaraldehyde and allowed to sit for one hour. The grids were washed with DI water for 10 seconds and then negatively stained by inverting onto 5 µl of 1% phosphotungsic acid for 5 minutes. Excess acids was wicked away and the grids were left to dry, with the sample side up, for 48 hours. Cells were imaged using a JEM-1400 Transmission Electron Microscope.

Results and Discussion

The pH range of strain CS4 is seen in Figure 1, with the optimum at approximately 5.6 with a range of 5.0-6.8. The temperature optimum is seen in Figure 2, displaying an optimal temperature of 35°C and a range of 15-35°C. NaCl tolerance is seen in Figure 3, with

growth observed at concentrations of $\leq$ 100 mM NaCl and optimal growth observed with $\leq$ 25 mM NaCl NaCl. Strain CS4 did not display Fe (III) or sulfate reduction.

Based on these values, it is seen that strain CS4 is acid-tolerant and is distinct from members of the most closely related genus *Rhizomicrobium*, which both have pH ranges of 5.0-7.5 and can tolerate up to 1% NaCl compared to only 0.6% tolerated by strain CS4 (Chapter 2; Kodama & Watanabe, 2011; Ueki *et al.,* 2010). It is thought that strain CS4 failed to meet a high optical density because of acidification of the media, preventing further growth. Final pH values were measured at pH 4, due to the production of ethanol, formate, acetate, lactate, CO_2 and H_2. Tests were completed with higher concentrations of MES buffer (50 mM, pH 5.7), though no marked differences were noted. Higher concentrations of MES buffer prohibit growth due to the presence of sodium salts in the buffer. The G+C content of strain CS4 was determined to be 62 mol % (Figure 4).

It is worthwhile to note that strain CS4 was not the sole isolate obtained from the Pineola 2013 samples. Cultures of interest included, strain PC4 which displayed high identity (100%) to cultured strains of *Clostridium beijerinckii* (KJ194928), strain CS3-SC2 which displayed high identity (97 %) to cultured strains of *Uliginosibacterium gangwonense* (AB682440) and strain CS3 which displayed high identity to cultured strains of *Actinobacterium acnes* strain Nb34MB-2 (KP296220). An unidentified culture that did not persist, strain G5, had rod shaped cells that grew in a rosette formation similar to many *Plancotomycete* strains. All of the strains mentioned above were cultivated at 28°C in PM1 media including 20 mM MES (pH 5.7), Vitamin Solution (Chapter 2), 40 mg l^{-1} yeast extract, and 1 g l^{-1} glycerol. Strains CS3 and CS3-SC2 also had additions of 0.5 mM TiNTA (Chapter

2) and 10 mg l^{-1} rifampicin. Strain CS3-SC2 could be grown in both an anaerobic and partially aerobic environment.

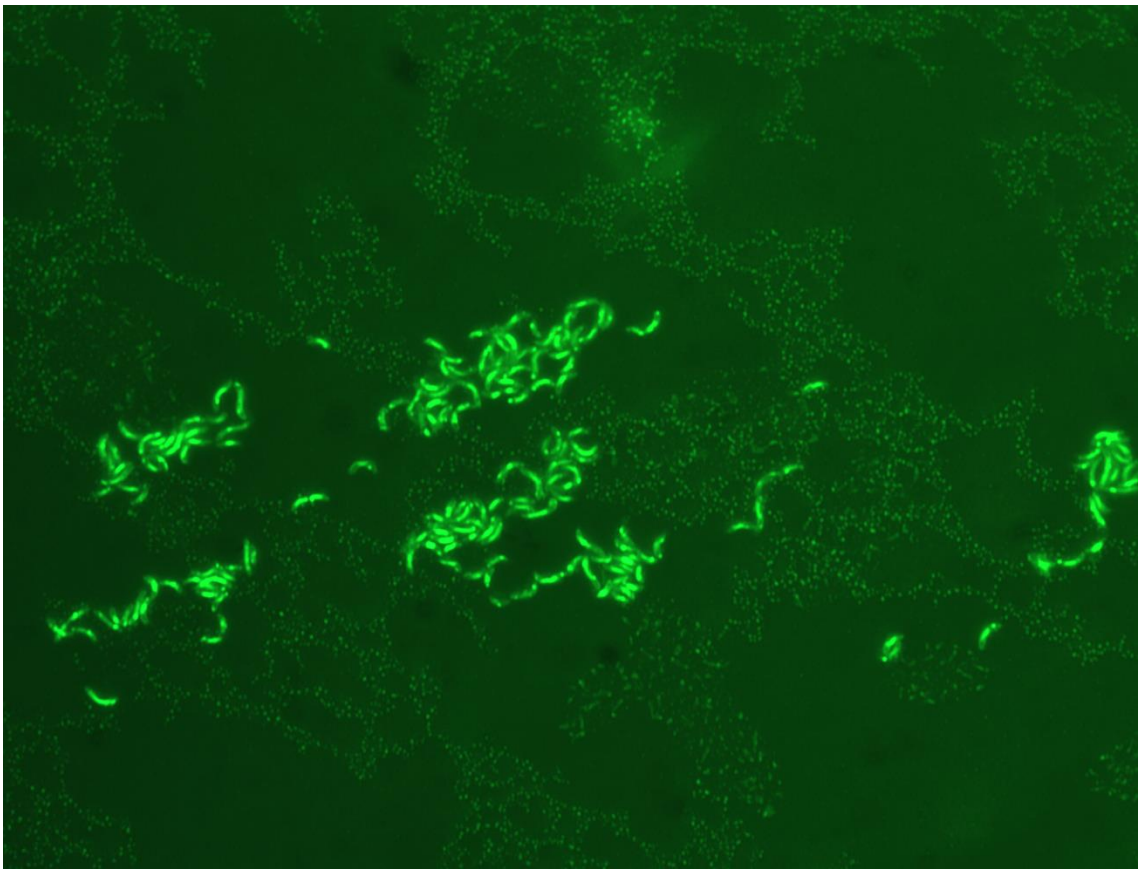

Figure A1. Fluorescence micrograph of strain CS4. Cell were grown on glycerol and imaged using acridine orange and a FITC filter on an Olympus BX51 microscope.

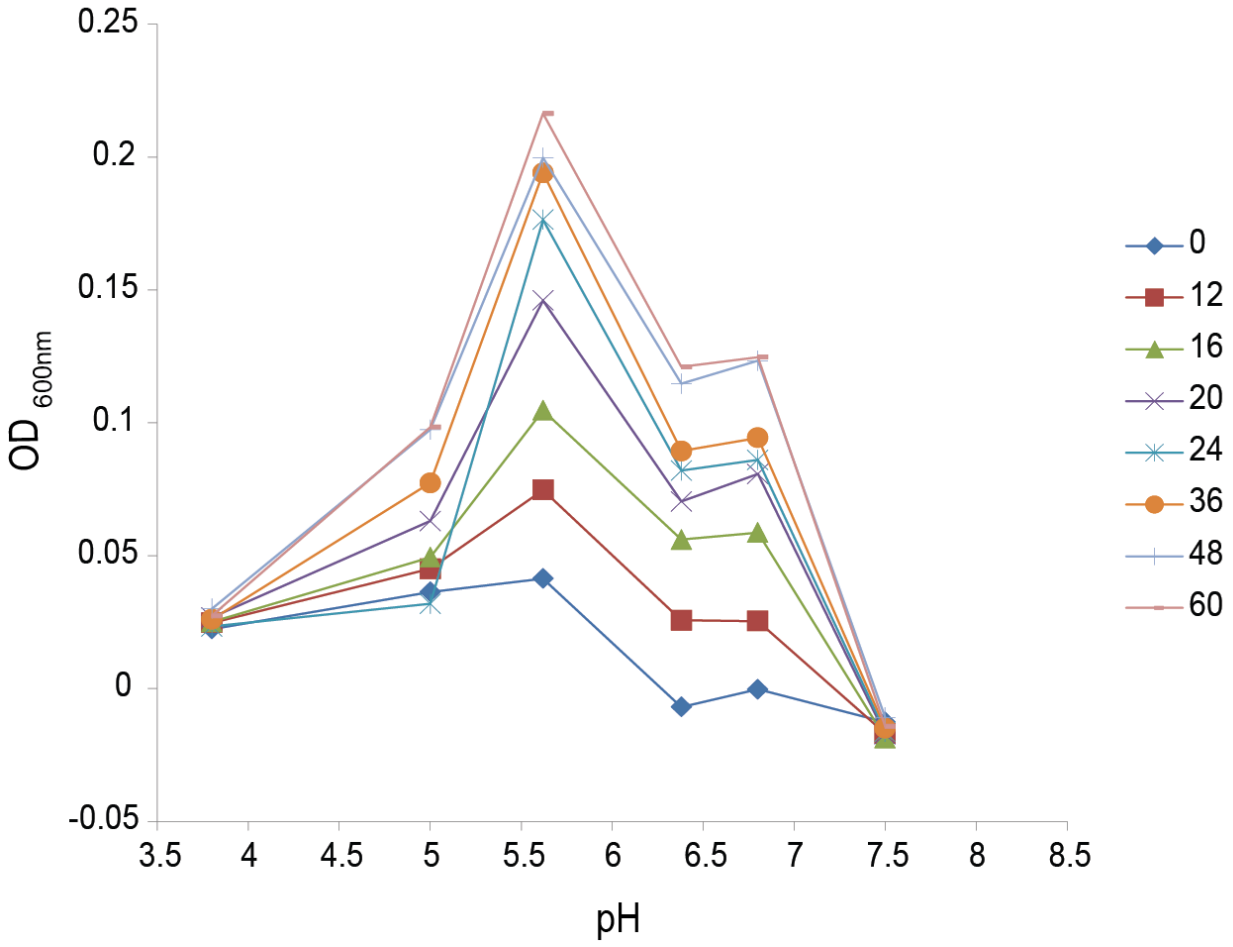

Figure A2. pH optimum of *Micropepsia pineolensis* strain CS4. Strain CS4 displayed growth at a pH range of 5.0-6.8, with optimal growth observed at 5.6.

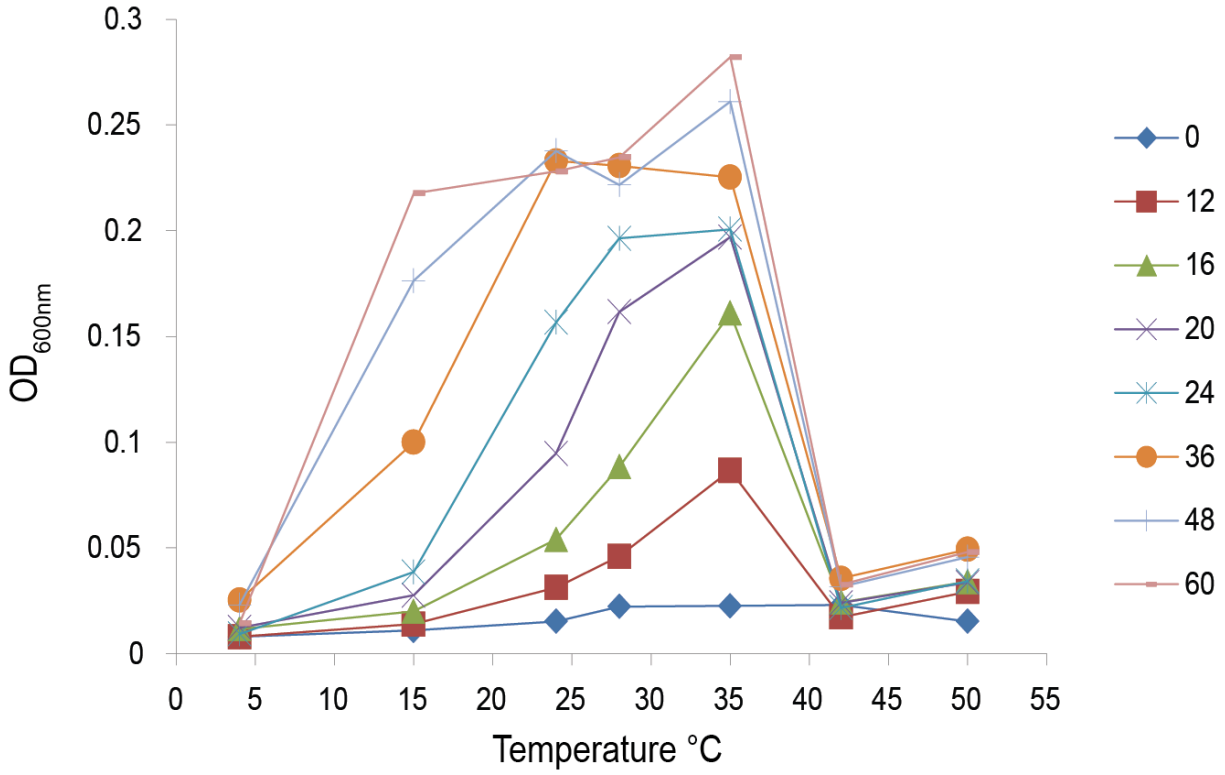

Figure A3. Temperature optimum of *Micropepsia pineolensis* strain CS4. Strain CS4 grew at a temperature range of 15-35°C, with an optimal growth temperature of 35°C.

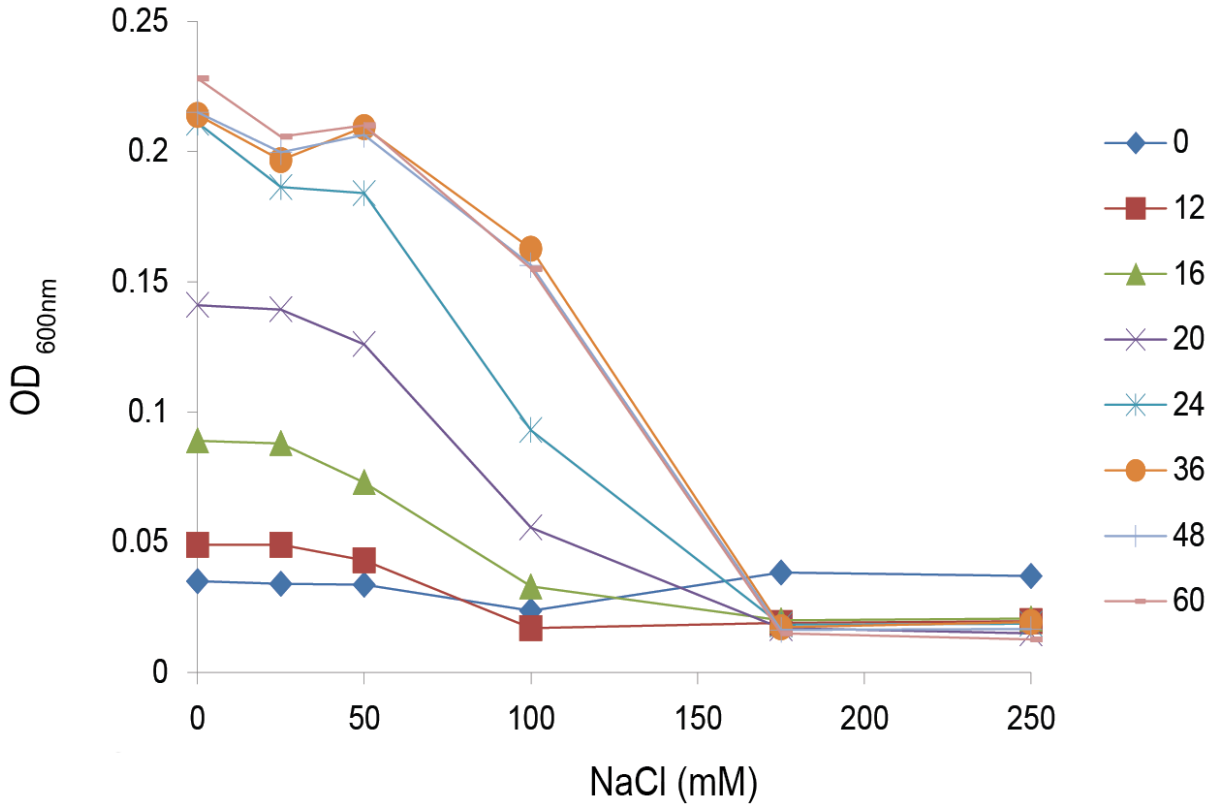

Figure A4. NaCl tolerance of *Micropepsia pineolensis* strain CS4. Growth was observed in media with ≤ 100 mM NaCl, with optimal growth at ≤25 mM NaCl.

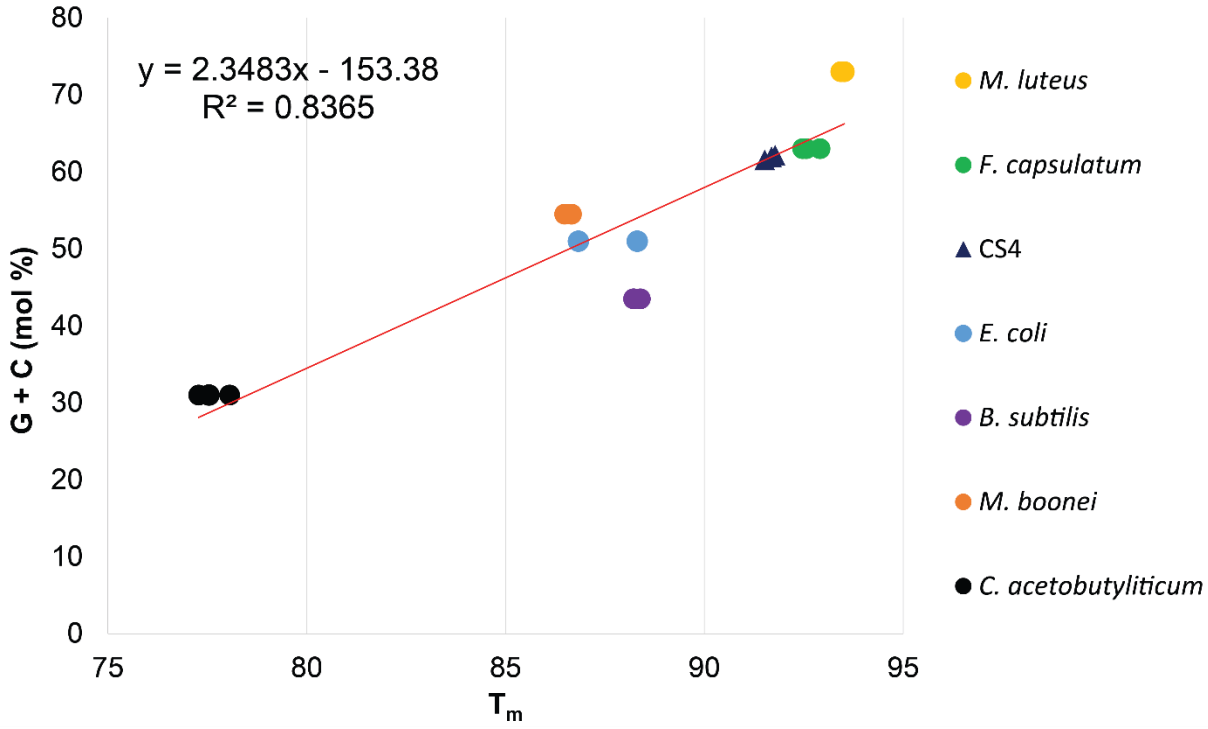

Figure A5. Regression line utilized to determine G+C content of *Micropepsia pineolensis* strain CS4. The T_m values of gDNA extracted from five bacterial strains, *Clostridium acetobutyliticum* DSM 1731, *Flavobacterium capsulatum*, *Bacillus subtilis*, *Micrococcus luteus* strain 4698, *Methanoregula boonei*, and *Escherichia coli*, were plotted against the G+C content (mol %) to determine the G+C content of strain CS4.

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Vita

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