

Revealing the Ecological Role of *Gemmatimonadetes*
Through Cultivation and Molecular Analysis of
Agricultural Soils

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DEDICATION

In loving memory of Najat Darwich, you taught me to be brave.

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I would like to thank Katharine Sides for actually cultivating KBS708 so that I could do the research I am doing today. I owe all of my successes to the opportunity my advisor, Mark Radosevich, provided me when he gave me my first solo research project as an undergraduate working in his laboratory. Mark I believe you saw something special in me and for this I am glad. Jennifer DeBruyn for guiding me on my journey first as a mentor and then as a member of my graduate committee. To my final committee member, Sean Schaeffer, thank you for taking me on as a student even though you did not know me well and helping me see the bigger picture.

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ABSTRACT

Bacteria belonging to phylum *Gemmatimonadetes* are frequently detected in a variety of environments using culture-independent methods. Despite their ubiquity and prevalence, almost nothing is known about their physiology or ecology because so few strains have been isolated. The first objective of this study was to determine the distribution of *Gemmatimonadetes* within soil aggregates and the response of the relative abundance of *Gemmatimonadetes* to dry/wet cycling and soil management. The second objective was to analyze the effects of soil management, aggregate size, and atmospheric conditions on cultivability of *Gemmatimonadetes*. Universal and *Gemmatimonadetes*-specific 16S rRNA gene primers were used to determine relative abundance of *Gemmatimonadetes* in whole soil, macro-, and micro-aggregates. The relative abundance of *Gemmatimonadetes* was not enriched in the aggregate fractions. Soil subjected to wet/dry cycling had a lower relative abundance of *Gemmatimonadetes* than control soil at constant moisture. Tillage alone did not affect the distribution of *Gemmatimonadetes* in the soil. Over 100 putative *Gemmatimonadetes* isolates were obtained in cultivation experiments. Amongst these, strains Gmat50, Gmat59 and Gmat410, were confirmed to be *Gemmatimonadetes* via 16S rRNA gene sequencing. Sequence analysis revealed that the 16S rRNA gene of Gmat50 and Gmat410 were 93% and 95% identity, respectively, isolate Ellin5290. The closest cultivated relative of Gmat59 was isolate Ellin7146 with an identity of 88% over the 16S rRNA gene fragment

analyzed. *Gemmatimonadetes* was successfully cultivated under both aeration conditions but was more frequently detected on plates incubated aerobically. Plates inoculated with tilled soil yielded more frequent detection of *Gemmatimonadetes* than plates inoculated with no-till soil. In conclusion the results indicated that the distribution of *Gemmatimonadetes* in soil was more dependent on moisture availability than aggregation and suggested members of this phylum are well adapted to low moisture conditions but unable to resist moisture fluctuations induced by wet/dry cycling. Furthermore, cultivability was improved by extended incubation time but not by reduced oxygen concentration and elevated CO₂. These results are consistent with a predominantly aerobic niche and homogenous distribution of *Gemmatimonadetes* at the aggregate scale.

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INTRODUCTION AND GENERAL OVERVIEW

Microbial life in soil is abundant and diverse with over a billion bacteria in one gram of soil and as many as 10,000 different prokaryotic species (Janssen, 2006). For example, microscopic examination of bulk soil from an Australian pasture revealed the presence of $1.33 \times 10^9 \pm 0.24$ cells per g of soil, and it also revealed that $64.3\% \pm 3.8\%$ of the observed cells were viable (Janssen et al., 2002). Typically, the efficiency of cultivating bacteria from soil using the viable plate count method is two orders of magnitude lower than total microscopic direct counts (Staley and Konopka, 1985). Advances in culture independent techniques have identified over 40 well-resolved bacterial phyla further supporting the notion that the vast majority of soil bacteria resist cultivation using standard methods (Hugenholtz et al., 1998). Among the total bacterial species that inhabit soil, less than 1% has been cultivated in the laboratory (Schoenborn et al., 2004). Many phyla, therefore, are known only based on their molecular signature and have no cultured representatives. As of 2003 there were an estimated 52 phyla of which 26 are candidate phyla with no cultured representatives (Rappé and Giovannoni, 2003). Estimates of the major division of bacterial lineages have grown to 85 with the majority having little or no cultured representatives (Stewart, 2012).

Many studies have evaluated a variety of approaches to improve the cultivability of soil and environmental bacteria. In one study, each 10-fold decrease in inoculum concentration, resulted in a 2-fold increase in final plate

count estimate (Davis et al., 2005). The disproportion seen in the final plate count estimate may be due a higher rate of competition present in the more concentrated inoculum, which have a negative impact on the community. Researchers were able to increase the mean viable count from 1.4 to 14.1% of the mean microscopically determined total cell count by using dilute nutrient broth (DNB) medium, gellum gum in place of agar, and sonication of the soil before serial dilution (Janssen et al., 2002). The use of the DNB medium may have increased mean viable counts of bacteria cultured by limiting the available nutrients to levels more consistent to what is available in the environment. Most environmental bacteria are believed to be oligotrophic in nature and would not survive well in a nutrient rich environment. When comparing gellum gum to noble agar solidifying agent, gellum gum was found to exclusively support growth of 44% of highly novel isolates (Tamaki et al., 2009). This suggests that even the purified form of agar may inhibit growth of highly novel isolates. Sonication was used to increase the inoculum concentration by releasing bacteria from water stable aggregates into solution (Janssen et al., 2002). Not only is increasing the number of cultivable bacteria important, increasing the cultivable bacterial diversity is important as well. Increasing the cultivable bacterial diversity increases the probability of cultivating a novel or rare organism. By simply extending incubation time the family level diversity of *Acidobacteria* was increased by 11%, subclass diversity of *Actinobacteridae* increased by 10% and the phylum *Proteobacteria*'s diversity was expanded by 8% (Joseph et al., 2003). In the same study, novel isolates from the widely distributed, yet rarely cultivated,

phylum *Verrucomicrobia* and *Gemmatimonadetes* were also obtained (Joseph et al., 2003).

Some approaches that have improved the cultivation of rarely isolated groups of bacteria include the use of low nutrient and low ionic strength media, alternative media solidifying agents, and extending the incubation time. The use of refined cultivation techniques has aided in cultivating the only 6 isolates from the phylum *Gemmatimonadetes*, of which only 2 have been characterized (Davis et al., 2005; DeBruyn et al., 2013, 2011; Joseph et al., 2003; Zhang et al., 2003). The phylum *Gemmatimonadetes* has been widely detected in soil, sediment, and wastewater environments from around the world. *Gemmatimonadetes* is one of the nine most commonly detected phyla in 16S rRNA gene libraries from soil (Janssen, 2006) accounting for 0.2 to 6.5% of all soil bacteria (mean = 2.2%) (DeBruyn et al., 2011). Molecular surveys of 16S rRNA gene libraries suggest this phylum is more abundant within the interior of soil micro-aggregates and is desiccation resistant (Aanderud and Lennon, 2011; Drees et al., 2006; Mummey et al., 2006). These findings provide clues to the ecological role of this phylum. More isolates from the phylum *Gemmatimonadetes* need to be cultivated and studied to allow us to increase our understanding of the phylum as a whole and further the elucidation of its ecological role.

Goals and Objectives

The overall goal of the research was to understand the effects of dry/wet cycling, soil aggregate size class, and atmospheric conditions on the growth and

isolation of bacteria from the phylum *Gemmatimonadetes*. Dry/wet cycling was chosen because strain KBS708 appeared to be desiccation resistant and the phylum *Gemmatimonadetes* has shown an inverse relation to moisture in soil. The whole soil, macro- and micro-aggregates fractions were tested to determine the distribution of the phylum within the soil and to illuminate what environmental conditions the bacteria prefer. Since KBS708 showed some sensitivity to oxygen cultivation under aerobic and microaerophilic (2.5% O₂ and 5% CO₂) conditions were used to determine if other members of the phylum were also sensitive to oxygen.

The specific objectives were:

1. To determine the distribution of *Gemmatimonadetes* within soil aggregates and to analyze the effects of dry/wet cycling on cultivability of members of this phylum.
2. To cultivate and characterize representatives from the phylum *Gemmatimonadetes*.

Hypotheses

Bacteria from the phylum *Gemmatimonadetes* are adapted to microaerophilic and low moisture conditions.

1. The relative abundance of *Gemmatimonadetes* is greater in micro- and macro-aggregates than in whole soil.
2. Plates incubated under microaerophilic conditions will enhance the cultivability of *Gemmatimonadetes*.

3. Dry/wet cycling of soil will enrich for *Gemmatimonadetes* by creating conditions favorable to desiccation resistant bacteria.

CHAPTER I

CHARACTERIZATION AND TAXONOMIC IDENTIFICATION OF STRAIN KBS708¹

¹ DeBruyn JM, MN Fawaz, AD Peacock, JR Dunlap, LT Nixon, KE Cooper, M Radosevich. *Gemmatirosa kalamazoonensis* gen nov. sp. nov., a member of the rarely-cultivated bacterial phylum Gemmatimonadetes. Journal of General and Applied Microbiology. (Accepted.)

ABSTRACT

Gemmatimonadetes are frequently detected in a variety of environments using culture-independent methods. Despite their ubiquity and prevalence, almost nothing is known about their physiology or ecology due to lack of cultured representatives: To date, only one bacterium in this phylum has been characterized (*Gemmatimonas aurantiaca* T-27, isolated from wastewater). The objective of this study was to expand the number of cultivated strains and characterize *Gemmatimonadetes* strain (KBS708) in order to determine its functional or ecological niche. KBS708 was randomly isolated from agricultural soil after 36 days of incubation on minimal media (VL55) supplemented with peptone. Culture purity was confirmed by 16S rRNA gene RFLP analysis. 16S rRNA gene sequencing placed KBS708 within the phylum *Gemmatimonadetes* with less than 94% similarity to other uncultivated *Gemmatimonadetes* and only 89% similarity to *G. aurantiaca* T-27, indicating it is a very novel representative within this phylum. KBS708 is a gram-negative chemoorganoheterotroph, which strongly flocculates when grown in static liquid cultures. Liquid culture growth under these conditions occurred at a pH range 4-7, with an optimum of pH 5.5- 6 and at a temperature range of 20-37°C, under light and dark conditions. Slow growth on the surface of plates under aerobic conditions and rapid growth under microaerophilic conditions (2.5% O₂ and 5% CO₂) was observed. KBS708 displays unique pleomorphic morphology with cells that range from 0.5-0.7 µm in diameter and 1-16 µm in length. Other interesting morphological features

revealed by SEM and TEM include evidence of an S layer, abundant membrane vesicles, and exopolysaccharide. Preliminary sequencing of the genome revealed a high GC content (72.6%) and large genome size (7.5 Mbp).

Introduction

The phylum *Gemmatimonadetes* is commonly found all over the world in various soil, sediment, and wastewater environments. In soil 16S rRNA gene libraries, *Gemmatimonadetes* is one of the nine most commonly detected phyla (Janssen, 2006). The relative abundance of *Gemmatimonadetes* in large 16S rRNA gene libraries from soil ranged between 0.2 to 6.5% (mean = 2.2%) (DeBruyn et al., 2011). Although *Gemmatimonadetes* is ubiquitous, in soils, there are only six cultured isolates (Davis et al., 2005; DeBruyn et al., 2011; Joseph et al., 2003; Zhang et al., 2003). *Gemmatimonas aurantiaca*, strain T-27 was the first to be cultivated. The bacterium is a gram-negative, aerobic, polyphosphate accumulating microorganism isolated from a wastewater bioreactor that was cycled between aerobic and anaerobic conditions to improve phosphorus removal (Zhang et al., 2003). Subsequently, strains Ellin5220 (AY234571), Ellin5290 (AY234641) and Ellin5301 (AY234652) were isolated from a rotationally grazed pasture soils at the Dairy Research Institute, Ellinbank, Victoria Australia (Joseph et al., 2003). The 16S rRNA gene analysis of these three isolates revealed they were 7 to 8% divergent from *G. aurantiaca* and 5 to 7% divergent from each other (Joseph et al., 2003). Another *Gemmatimonadetes* bacterium Ellin7146 (AY673312) was also isolated from a

control pasture soil at the Dairy Research Institute, Ellinbank, Victoria, Australia (Davis et al., 2005). Strain Ellin7146 was cultivated on dilute nutrient broth solidified using gellan and was found to be 9% divergent from strain T-27 (Davis et al., 2005).

In a previous study, our laboratory isolated an additional member of this phylum, designated strain KBS708, from an organically managed agricultural soil on a wheat-corn-soybean rotation at Kellogg Biological Research Station LTER in Hickory Corners, Michigan, USA. Strain KBS708 was distantly related to *G. aurantiaca* and the other soil isolates (Figure 1). In a seasonal study of the soils from the Kellogg Biological Station LTER, the phylum *Gemmatimonadetes* represented 0.05 to 5.31% of all bacteria (DeBruyn et al., 2011). The objective of this study was to characterize and classify a novel isolate from the phylum *Gemmatimonadetes* to further understand the ecological role of this phylum.

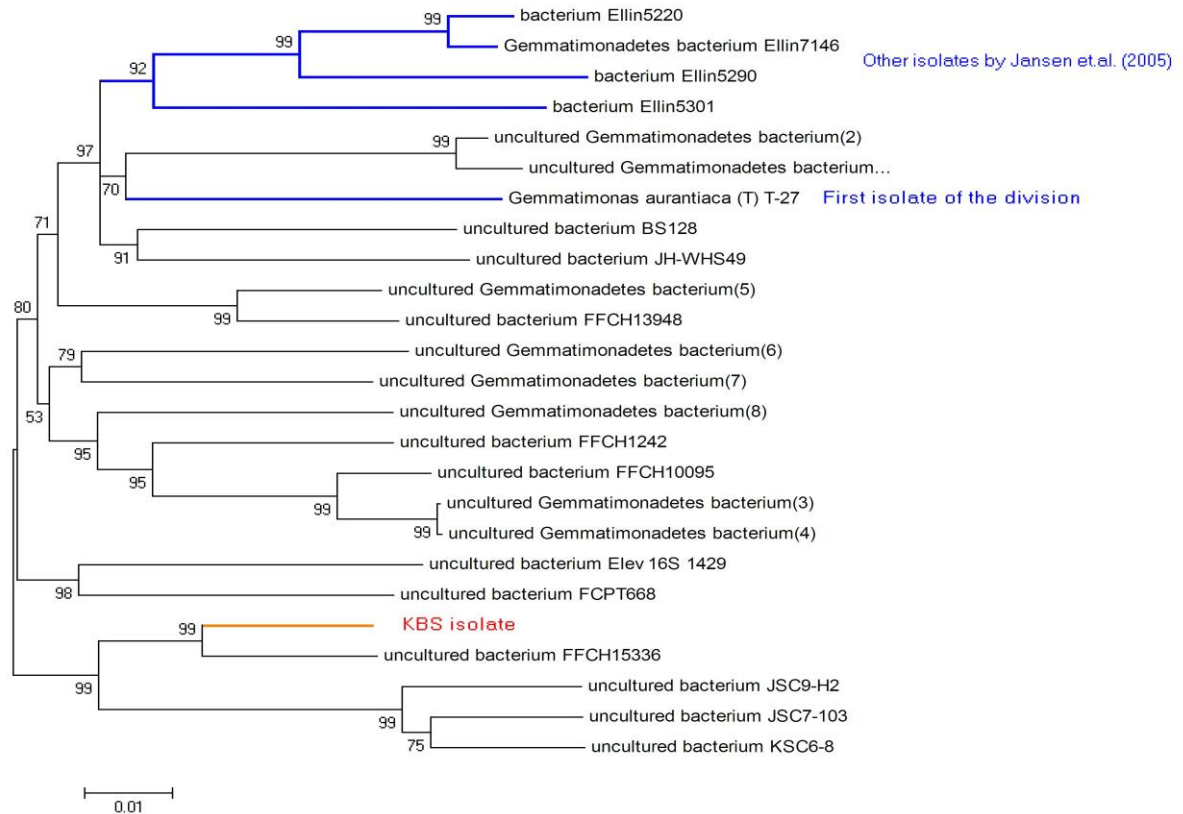


Figure 1 Neighbor joining tree with 1000 bootstraps, and *E. coli* as the root demonstrating the phylogenetic relationship of KBS708 (orange) and other isolates (blue) within the phylum Gemmatimonadetes by Dhritiman Ghosh.

Methods

Cultivation

Organically managed agricultural soil was sampled (0-10 cm) from the LTER at KBS in Kalamazoo, Michigan, USA. The samples were diluted in phosphate buffered saline (PBS) and cultivated on agar plates prepared in a basal salts media VL55 supplemented with peptone as a carbon source (Joseph et al., 2003). Colonies were randomly selected from VL55 agar spread plates at various time intervals up to 36 days. Fungal contamination was inhibited by adding 2 ppm cyclohexamide. Liquid cultures were grown in VL55 (pH 5.5) at 20°C at 120 RPM (Sides, 2010). The bacteria isolated from this study formed a large culture collection numbering approximately 750 isolated strains. One strain from the collection, KBS708, was placed within the phylum *Gemmatimonadetes* based on partial sequencing of the 16S rRNA gene (Sides, 2010). This isolate was selected for further characterization as part of this study.

Phylogeny based on 16S rRNA gene sequencing

DNA was extracted using MoBio Ultra Clean Microbial DNA Isolation Kit according to manufacturer's protocol. PCR was used to amplify the 16S rRNA gene with the universal bacterial primers 8f and 1392r. PCR products were cloned into pGEM T Easy Vector using the pGEM[®]-T Easy Vector System II Kit by Promega according to the manufacture's protocol. Plasmid was extracted using the Wizard[®] Plus SV Minipreps DNA Purification System by Promega and

were sequenced at the University of Tennessee Knoxville Molecular Biology Resource Facility.

Restriction Fragment Length Polymorphism Analysis (RFLP)

RFLP assay was designed for the strain KBS708 to determine the purity of the DNA without having to submit a sample for sequencing. The full-length 16S rRNA gene sequence was analyzed using NEBcutter V2.0 for the restriction enzyme analysis. The enzyme *AluI* (restriction site 5'...AG[↓]CT...3') was chosen based on its availability and the size of the predicted fragments. The predicted RFLP profile consisted of cuts at 226, 268, 627, 1009, 1041, 1307 and 1334 bp or fragments of 226, 42, 359, 382, 32, 266, 27 and 46 bp in length. The *AluI* digest was used to verify that the culture in use or the DNA extracted was pure in place of re-sequencing. The 16S rRNA was amplified by PCR from the extracted DNA using the universal eubacterial 16S rRNA primers 8F and 1392R. The PCR product was then digested by 1 µL of *AluI* enzyme in a solution of 10x NEBuffer 2 and 100x bovine serum albumin (BSA) at 37°C for six hours. The digested product was run on a 1.5% agarose gel in 1x TAE buffer. If the expected bands and no additional bands were present on the gel, it was deemed a pure culture.

Physiological characteristics

The pH and temperature optima for growth of KBS708 were determined in liquid cultures of VL55 with peptone amendment. Growth was scored as either positive or negative by the presence or absence of flocculated cell mass at the

base of the culture tube or flask. The media pH was adjusted to 4, 5, 6, 7, 8 and 9, using 1 M HCl or 1 M NaOH and incubated at room temperature with shaking at 120 rpm. Further analysis of growth as a function of temperature was performed at pH 5.5, 6.0 and 6.5. These cultures were statically incubated at 4, 22, 25, 28, 31, 34, 37 and 40°C.

KBS708 was grown in VL55 with peptone amendment on growth medium solidified with either 1.5% noble agar or 2.5% Gellrite was evaluated under both aerobic and microaerophilic conditions. Microaerophilic conditions were achieved by incubating the plates in gas pack jars equipped with BD BBL Campy PakPlus Microaerophilic System Envelope with Palladium Catalyst or GasPak EZ Campy Container Systems Sachets (6-16% O₂, 2-10% CO₂). Microaerophilic growth experiments were changed to a gas chamber under microaerophilic atmospheric conditions (2.5% oxygen and 5% carbon dioxide) to increase the incubation capacity of the experiments. Sensitivity to oxygen was tested by mixing cultures into semisolid VL55 amended with peptone in 0.5% gellan or 0.5% noble agar. Anaerobic growth was tested by incubating plates with GasPak EZ Anaerobic Systems Sachets with Indicator ($\leq 1\%$ O₂, $\geq 13\%$ CO₂).

Production of oxidase was tested by applying 2 drops of a 1% solution of 2,5-dimethyl-p-phenylenediamine to a flocculent from a liquid culture and incubated for 10 seconds. Sensitivity to hydrogen peroxide was tested with the addition of 50 units of catalase per μL volume of VL55 media with peptone amendment and 1.5% noble agar. Production of catalase was tested by applying hydrogen peroxide to mature colonies and observing bubble production.

Sensitivity of cell wall targeting enzymes was tested by growing KBS708 in the presence of 2 μ L of 100 ppm ampicillin or 50 μ L of Fast Plasmid Mini Lysis Solution by Eppendorf to VL55 liquid media.

Substrate utilization

The ability of KBS 708 to metabolize various carbon and energy sources were evaluated using Phenotype Microarray Plates (BioLog) PM1 and PM2A. The inoculum consisted of 1.76 mL of suspended culture at 81% transmittance added to 22.24 mL of VL55 medium without peptone amendment, in MES buffer and nitrilotriacetic acid in the SL10 solution. From this cell suspension, 100 μ L was transferred to each of the wells in the PM1 and PM2A plates. The well plates were covered using Breathe-Easy membranes to prevent evaporation of medium and incubated statically at room temperature. The first evaluation in Phenotype Microarray Plates was done without any dye reagent and growth was determined by visual observation. The second and third studies were done with the addition of BioLog Redox Mix G (100x) for slow growing bacteria and growth and or metabolic activity was determined by color change in the wells.

In addition to the BioLog substrate utilization assays, other growth substrates, nitrogen sources, and modes of growth were also evaluated (Table 1). Unless otherwise noted, all of the growth experiments were conducting in the VL55 basal media. However, to evaluate autotrophic growth the MES buffer was replaced with either sodium or potassium phosphate since the MES contains carbon. Also, to assess growth in the absence of nitrogen sources other than N₂

gas, the VL55 media was prepared without addition of nitrotrilotriacetic acid or any other fixed nitrogen source. All cultures were incubated at room temperature on a platform shaker set at 120 rpm. Growth on the following carbon and energy sources were evaluated individually and also as a group with and without MES buffer: glycerol (1 mL/L), arabinose (751 mg/L), fructose (901 mg/L) and xylose (751 mg/L). Polymers (chitin, cellulose and N-acetylglucosamine) were tested as a carbon source with and without MES buffer. Methanol and sodium acetate was tested as a carbon sources. Autotrophy was tested by removing all carbon sources from the media including, MES buffer and nitrilotriacetic acid from the SL10 from the VL55 medium and by assessing growth Bristol's medium designed to cultivate photoautotrophs (Weis, 1975). Other media that were tested included NM-1 medium (Zhang et al., 2003), LB medium, ATCC Ammonia oxidizing bacteria (AOB) *Nitrosomonas* medium 221, and R2 medium (Reasoner and Geldreich, 1985).

Cell Morphology (Electron microscopic analysis)

KBS708 biomass was examined at the Advance Microscopy and Imaging Center at the University of Tennessee. Transmission electron microscopy (TEM) fixation was done by placing the sample into 3% glutaraldehyde in 0.1 M cacodylate and allowed to fix for one hour at room temperature. The sample was then rinsed three times for 10 minutes in buffer. Post-fix the sample with 2% OsO₄ in 0.1 M cacodylate buffer. The sample was then sequentially dehydrated for 15 minutes each in 25, 50, 70, 95, and 100% ethanol and then in a 100%

acetone solution for 15 minutes. The sample was further dehydrated in a 2:1 ethanol/Spurr's reagent mix (Electron Microscopy Sciences) for an hour, 1:2 ethanol/Spurr's reagent mixture capped overnight in the hood, and then in 100% Spurr's reagent for 4 hours. The complete Spurr's reagent mix and samples were put into the mold or beam capsules and placed in an oven for 24 hours at 68°C. Post fixation, samples were thin section with a Leica UC7 ultramicrotome. Sections were stained with uranyl acetate and lead citrate before examination in the TEM. TEM thin sectioning images were taken with Hitachi H-800 Electron Microscope at 100kV.

For the negatively stained samples, 5 µL of sample was spotted onto a freshly glow discharged carbon film, allowed to sit for one minute then stained with uranyl acetate for one minute. TEM negative stain images were taken with a Hitachi H-800 Electron Microscope.

Samples were fixed for SEM in 3% glutaraldehyde in 0.1M cacodylate and allowed to equilibrate for 1 hour at room temperature. The samples were rinsed in buffer at least 3 times for 10 minutes each. For secondary fixation, the samples were treated with 2% osmium tetroxide in 0.1M cacodylate and allowed to fix for an hour at room temperature. The sample was then washed in buffer 3 times for 10 minutes each. During the final wash step the sample was allowed to settle onto a 3x4 mm silicon chip. The chip and sample were then dehydrated in 25, 50, 70, 95, and 100% dry ethanol for 10 minutes each. After fixation the samples were coated with gold in a SPI sputter coater for examination. Scanning

Electron Microscope (SEM) standard images were taken with the Zeiss SEM. Images were taken between 248 X and 180,970 X magnification.

Physiology

Phase contrast and epifluorescent microscopic images were taken with a Nikon Eclipse E600 epifluorescence microscope. KBS708 was allowed to colonize the surface of sterile glass slides immersed in liquid cultures grown in VL55 medium with peptone amendment. After sufficient growth the media was carefully removed as to not disturb the culture and the slides were heat cured.

The slides were stained with a 1% solution of 4',6-diamidino-2-phenylindole (DAPI) and examined with a DAPI filter to determine the presence of potential polyphosphate accumulation (Serafim et al., 2002). The DAPI staining procedure has been successfully applied to study phosphorus accumulation in bacteria in wastewater bioreactors (Zhang et al., 2003). In this study, bright yellow fluorescence was indicative of polyphosphate accumulation, dull yellow fluorescence indicated the presence of lipids, and DNA fluoresces blue-white (Serafim et al., 2002). Additional slides were stained with Nile blue and examined through a CY5 filter to test for the presence of polyhydroxybutyrate (PHB), a polyhydroxyalkanoate produced by aerobic bacteria with oxygen limitations in the presence of excess carbon and an energy source (2007). If PHB were present, a bright orange fluorescent would be observed. The slide was stained for 10 minutes in 1% solution of Nile blue A, washed briefly in tap water, and fixed in 8% aqueous acetic acid solution (2007).

Desiccation resistance was tested by removing a flocculent of KBS708 from a liquid culture and placing it into sterile glass flasks and incubating in a vacuum desiccator under vacuum suction and in the presence of Drierite, anhydrous CaSO_4 . Flasks were removed from the desiccator and the cells were revived by adding VL55 liquid medium with peptone amendment after 1 and 2 days and 1 and 2 weeks of desiccation.

Respiratory Quinones

Liquid cultures of KBS708 were incubated at 37°C for 4 days with orbital shaking at 120 rpm. The flocculated biomass was then removed and washed in sterile water to remove residual media. The sample was then freeze dried using a speed vacuum and shipped over night on dry ice for processing. Respiratory quinones were analyzed using HPLC at the Complex Carbohydrate Research Center at the University of Georgia.

Genome sequencing

KBS708 genomic DNA was obtain by extraction with MoBio Ultra Clean Microbial DNA Extraction Kit. The genome was sequenced using a Roche 454 pyrosequencing instrument using titanium chemistry and Illumina HiSeq 2000 at the Joint Institute for Biological Sciences at Oak Ridge National Laboratory in Oak Ridge, Tennessee. Analysis of the sequence data was not part of the thesis research but the results were used to estimate the %G+C content and size of the KBS708 genome.

Results

Phylogeny based on 16S rRNA sequencing

Strain KBS708 was found to be a member of the phylum *Gemmatimonadetes* based on analysis of the full-length 16S rRNA gene using Basic Local Alignment Search Tool to complete homology searches within the National Center for Biotechnology Information (NCBI) database. The closest cultured relative was *Gemmatimonas aurantiaca* T-27 with a sequence identity of 89% over 1,381 bp.

Physiological characteristics

Plate cultures of KBS708 grown at room temperature under aerobic conditions formed few visible colonies after 30 to 60 days of incubation; however, the abundance of visible colonies that formed within 3 days under microaerophilic conditions with an atmosphere of 5% CO₂ and 2.5% O₂ was increased by greater than 10 fold when compared to plates incubated under aerobic conditions. Under microaerophilic conditions colonies were 1 to 6 mm in diameter, opaque light pink to pink in color, circular, convex elevation, and an entire margin (Figure 3). Colony characteristics varied with incubation time. For example, the color varied from opaque white to pink, the size ranged from 1 to 12 mm in diameter, the texture was smooth to buttery, and the shape was convex. The changes of colony characteristics over time may be due to changes in gene expression and adaptation to changes in micro-environmental conditions during age of the culture however they were not related to the growth conditions. The

growth rate of KBS708 was difficult to determine by optical density due to its flocculating growth habit in liquid media. Under aerobic conditions at room temperature growth in liquid was observed within 10 days and occurred between 20°C and 37°C; no growth was observed at 4°C or at 40°C. At 25°C growth was observed within 10 days and at 37°C growth was observed within 4 days. KBS708 grew between pH 4.0 and 7.0 but produced the largest floccules at pH 5.5 and 6.0. Liquid cultures grown with orbital shaking at 120 rpm formed a single mass that was slight pink to pink in color, opaque jellyfish-like consistency, irregularly shaped massive to cord like structures from 1 to 2.5 cm in length (Figure 4). The flocculation observed in liquid cultures was believed to be due to extracellular polysaccharide (EPS) production.

When grown under microaerophilic conditions, visible colonies appeared within three days. When KBS708 was suspended in semi-solidified growth medium prepared with 0.5% gellan, a distinct layer of denser growth was observed at a depth within the medium indicative of some degree of sensitivity to oxygen (Figure 2). These findings are supported by enhanced growth under microaerophilic conditions strongly suggesting that KBS708 is microaerophilic in nature. The microaerophilic preference of KBS708 could be an indicator of oxygen sensitivity as observed in *G. aurantiaca* T-27 the only other well characterized strain the phylum *Gemmatimonadetes*.

Strain KBS708 did not produce catalase as indicated by an absence of bubbles upon application of hydrogen peroxide. It appeared that oxidase was

produced because the culture changed to blue in color when 2,5-dimethy-p-phenylenediamine was applied.

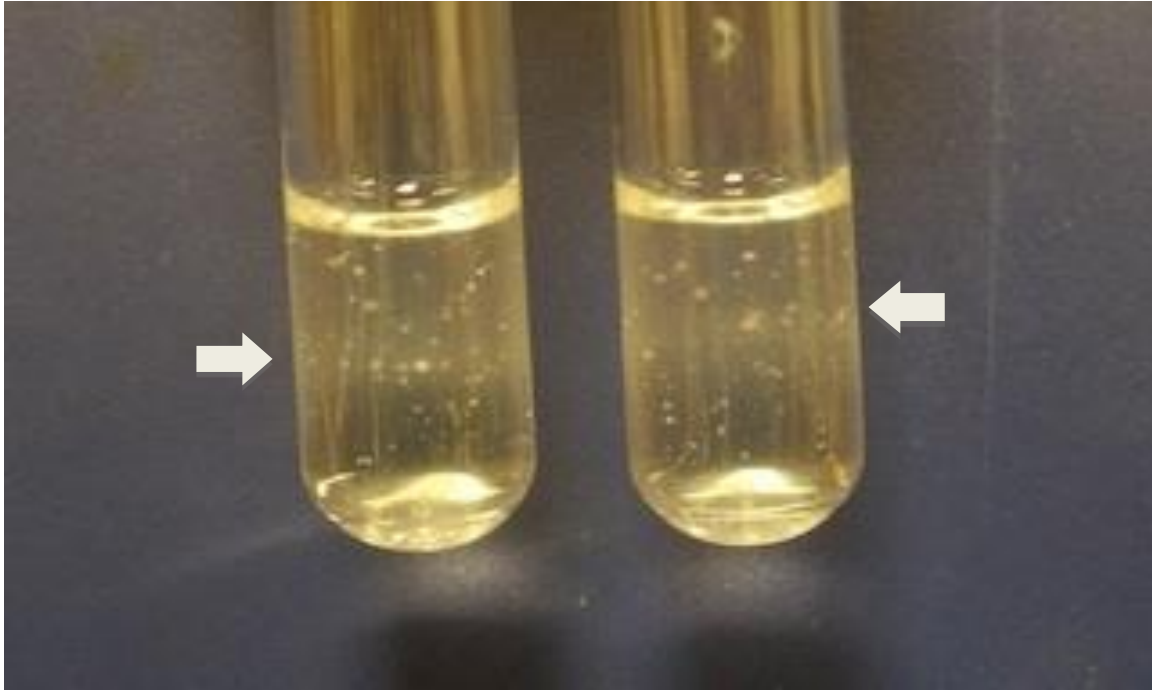


Figure 2 Digital image of KBS708 colonies embedded in VL55 media with 0.5% gellan. The faint white band at the center of the gelling medium shows the preferred depth of growth.

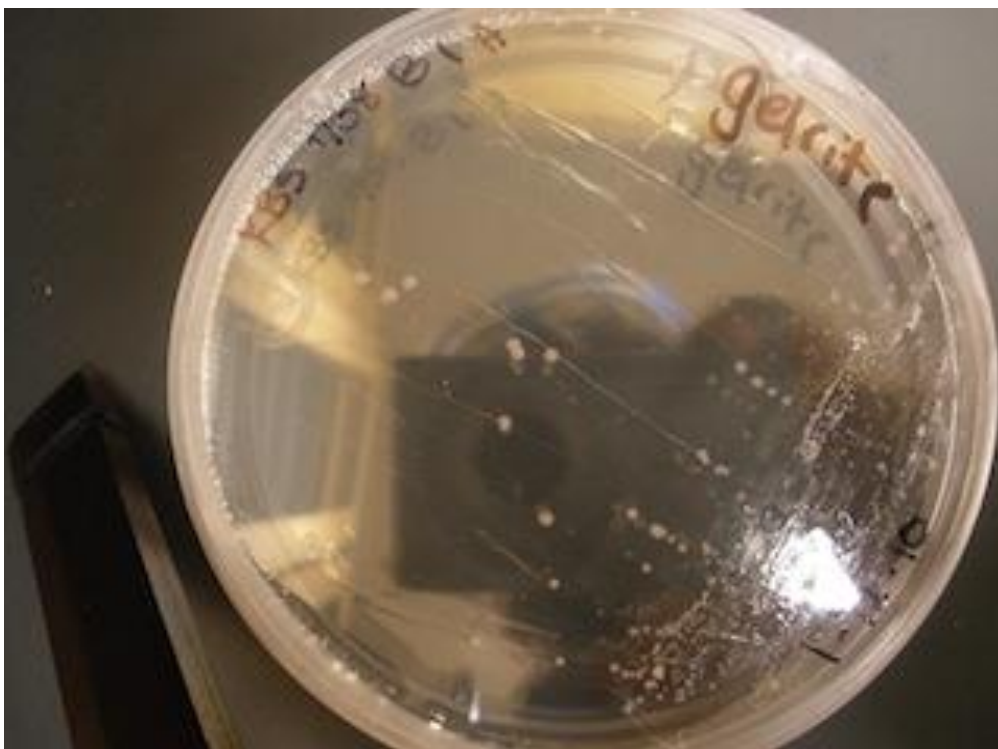


Figure 3 Digital image of plate colonies of KBS708 on VL55 media with 2.5% gellum.



Figure 4 Growth of strain KBS708 under aerobic conditions at room temperature with shaking at 120 rpm in liquid VL55 media.

Substrate utilization

The capacity of KBS708 to metabolize a range of carbon substrates was tested using BioLog Phenotype arrays and a variety of other media. Growth was observed in R2 medium and VL55 based medium with peptone, glycerol, arabinose, fructose, and xylose added as carbon and energy sources (Table 1). Strain KBS708 did not grow in LB rich medium, Bristol's medium, NM-1 medium, or AOB medium (Table 1). BioLog PM1 and PM2A MicroPlate Phenotype MicroArrays were used to determine a broader range of carbon sources that could be metabolized by KBS708 (Table 2). The BioLog metabolic activity assay results were not reproducible. It was difficult to determine growth because some wells had a plaque type build up while others had a faint purple color change. However, the redox sensitive dye contained in the microtitre plate wells clearly changed color in some wells and not in others indicating KBS708 was able to metabolize an array of organic substrates including a broad range of sugars, amino acids, and polymers (Table 2).

Table 1 KBS708 growth conditions and media list with various components of the media tested individually. ND stands for not determined.

Physiology/ Metabolism Tested	Media Type or Growth Conditions	Observed Growth	Days until Visible Growth	MES Buffer	Na ₂ HPO ₄ NaH ₂ PO ₄ Buffer	K ₂ PO ₄ Buffer	MgSO ₄	CaCl ₂	(NH ₄) ₂ HPO ₄	Selenite Tungstate	SL10	Adjusted SL10 (no nitritotriacetic acid)	Vitamin I and II
Carbon source – peptone and MES	VL55 (liquid)	Yes	3	X			X	X	X	X	X		X
	VL55 (liquid)	No	ND	X			X	X	X	X		X	X
	VL55 1.5% agar plates	Yes	22	X			X	X	X	X	X		X
	VL55 2.5% Gelrite plates	Yes	22	X			X	X	X	X	X		X
pH range	VL55 pH 4 - 7 (liquid)	Yes	ND	X			X	X	X	X	X		X
	VL55 pH > 7 (liquid)	No	ND	X			X	X	X	X	X		X
Temperature range	VL55 20°C - 37°C (liquid)	Yes	ND	X			X	X	X	X	X		X
	VL55 4°C (plates)	No	ND	X			X	X	X	X	X		X
Sensitivity to oxygen	Embedded in VL55 0.5% agar tubes	Yes	5	X			X	X	X	X	X		X
	Embedded in VL55 0.5% Gelrite	Yes	5	X			X	X	X	X	X		X
	VL55 1.5% agar plates in CampyPakPlus Microaerophilic System 5- 15% O ₂ , 5-12% CO ₂	Yes	4	X			X	X	X	X	X		X
	VL55 1.5% agar plates in GasPak EZ Campy Container Systems Sachets 6-16% O ₂ , 2-10% CO ₂	Yes	ND	X			X	X	X	X	X		X
	VL55 1.5% agar plates in GasPak EZ Anaerobic Systems Sachets with Indicator ≤1% O ₂ , ≥13% CO ₂	No	ND	X			X	X	X	X	X		X
	VL55 1.5% agar plates in 2.5% O ₂ , 5% CO ₂ , balance N ₂	Yes	3	X			X	X	X	X	X		X
Sensitivity to hydrogen peroxide	VL55 50 units/L catalase 1.5% agar plates	No	ND	X			X	X	X	X	X		X
	VL55 50 units/L catalase 2.5% Gelrite plates	No	ND	X			X	X	X	X	X		X
Sensitivity to cell wall-targeting enzymes	VL55 with 100 ppm ampicillin (liquid)	Yes	7	X			X	X	X	X	X		X
	VL55 with lysozyme (liquid)	Yes	7	X			X	X	X	X	X		X

Table 1 continued.

Physiology/ Metabolism Tested	Media Type or Growth Conditions	Observed Growth	Days until Visible Growth	MES Buffer	Na ₂ HPO ₄ NaH ₂ PO ₄ Buffer	K ₂ PO ₄ Buffer	MgSO ₄	CaCl ₂	(NH ₄) ₂ HPO ₄	Selenite Tungstate	SL10	Adjusted SL10 (no nitrilotriacetic acid)	Vitamin I and II
Carbon source - peptone	VL55 (liquid)	Yes	4			X	X	X	X	X	X		X
	VL55 (liquid)	Yes	4			X	X	X		X	X		X
	VL55 (liquid)	No	ND			X	X	X		X		X	X
	VL55 (liquid)	Yes	3	X			X	X	X	X		X	X
	VL55 (liquid)	ND	ND		X		X	X	X	X	X		X
	VL55 with peptone 0.025 g/L (liquid)	ND	ND			X	X	X	X	X		X	X
	VL55 (liquid)	slight	14			X	X	X		X		X	
Carbon source- glycerol	VL55 glycerol, phosphate buffer, SL10 (liquid)	stunted	ND			X	X	X	X	X	X		X
	VL55 glycerol, phosphate buffer, no (NH ₄) ₂ HPO ₄ , adjusted SLIO (liquid)	No	ND			X	X	X		X		X	
	VL55 glycerol, phosphate buffer, no (NH ₄) ₂ HPO ₄ , adjusted SLIO, no vitamins (liquid)	Yes	14			X	X	X		X		X	X
Carbon source- sugars	VL55 arabinose, fructose, xylose (liquid)	Yes	ND	X			X	X	X	X	X		X
	VL55 with arabinose 751 mg/L (liquid)	ND	ND		X		X	X	X	X		X	X
	VL55 with fructose 901 mg/L (liquid)	ND	ND		X		X	X	X	X		X	X
	VL55 with xylose 751 mg/L (liquid)	ND	ND		X		X	X	X	X		X	X
	VL55 with arabinose, fructose, xylose (liquid)	Yes	10			X	X	X	X	X	X		X

Table 1 continued.

Physiology/ Metabolism Tested	Media Type or Growth Conditions	Observed Growth	Days until Visible Growth	MES Buffer	Na ₂ HPO ₄ NaH ₂ PO ₄ Buffer	K ₂ PO ₄ Buffer	MgSO ₄	CaCl ₂	(NH ₄) ₂ HPO ₄	Selenite Tungstate	SL10	Adjusted SLIO (no nitrilotriacetic acid)	Vitamin I and II
Carbon source- polymers	VL55 with chitin, cellulose, n-acetylglucosamine (liquid)	Yes	ND	X			X	X	X	X	X		X
	VL55 with chitin, cellulose, n-acetylglucosamine (liquid)	Yes	10			X	X	X	X	X	X		X
Carbon source- other	VL55 with methanol (liquid)	No	ND		X		X	X	X	X		X	X
Carbon source- sodium acetate	VL55 with sodium acetate (liquid)	No	ND			X	X	X	X	X	X		X
Autotrophy (no carbon source)	VL55 without peptone (liquid)	No	ND			X	X	X	X	X	X		X
	VL55 without peptone (liquid)	No	ND		X		X	X	X	X		X	X

Morphology

Strain KBS708 exhibited long, irregular rod-shaped individual cells that were 460-440 nm in diameter and 1-16 μm in length (Figure 5-10). Images of KBS708 showed evidence of EPS production consistent with the clumping growth seen in liquid cultures (Figure 5-6). The cells of KBS708 appeared to be covered with bulbous vesicle-like protrusions (Figure 5, 9-10) similar to those seen on strain T-27 (Zhang et al., 2003). TEM thin sections of the bulbous structures appeared to be membrane vesicles with lipid bilayers budding off the cell (Figure 7). These structures may be evidence of EPS production, used to eliminate waste, or an artifact of the electron microscope preparation. SEM images of KBS708 showed multiple pinching along the cell that may suggest multiple fission or may be an artifact of the pleomorphic shape (Figure 6-8); however, the thin sectioning image does not show evidence of multiple fission (Figure 8). TEM and SEM images showed large protrusions from the center of the cell that may be evidence of budding cellular division (Figure 9). A regular pattern covering the cell suggests the presence of an S-layer cellular envelope (Figure 10). Thin sectioning electron microscopy revealed electron dense areas within the cell, which is characteristic of phosphorous accumulation (Figure 7-8). TEM images also indicate the presence of an s-layer (Figure 7-10).

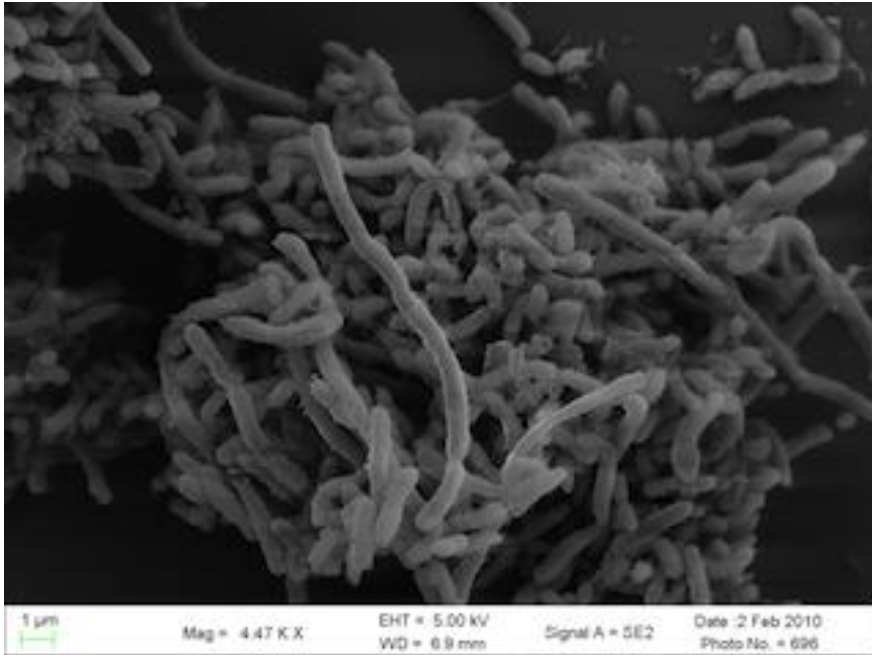


Figure 5 SEM image of KBS708. Clump of cells of various lengths and irregular shape (1-16 nm).

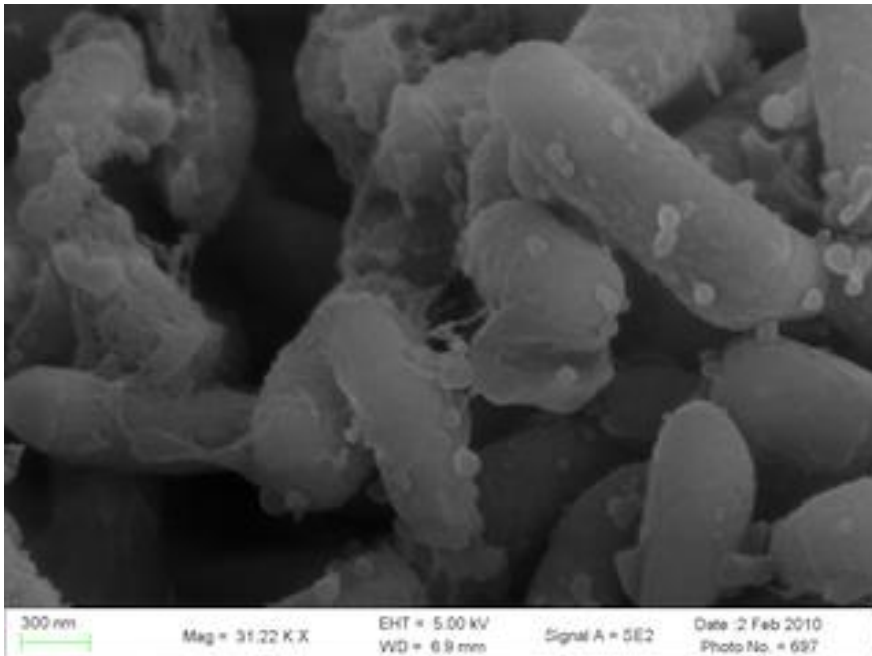


Figure 6 SEM image of KBS708. Cells are rod shaped and are covered in membrane bound vesicles. The web-like structure connecting the cells may be evidence of EPS production or an artifact.

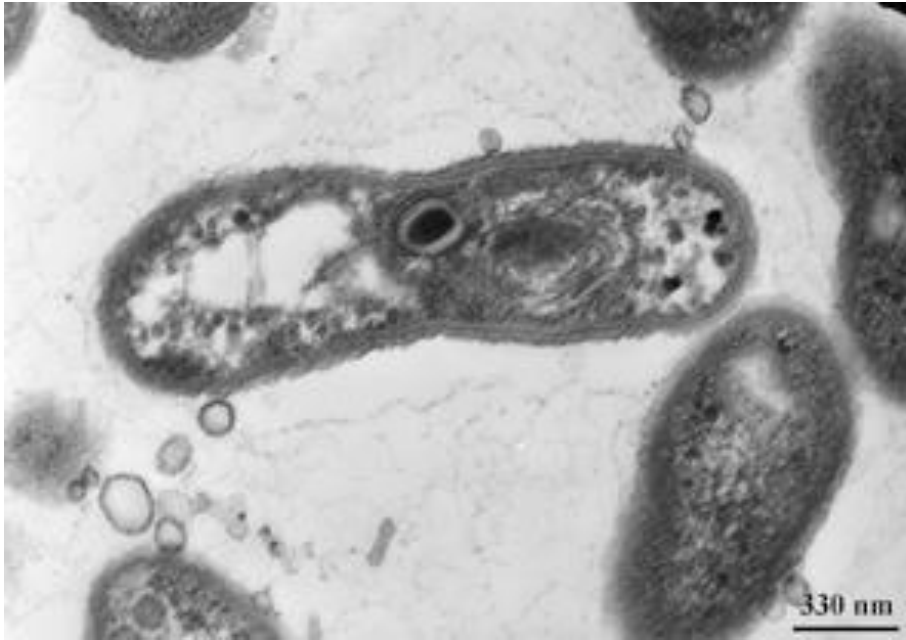


Figure 7 Thin sectioning TEM image of KBS708. Cells have a Gram-negative cell wall. Dark masses with in the cell are suggestive of polyphosphate accumulation. Vesicles covering the cells appear to be bound by membranes.

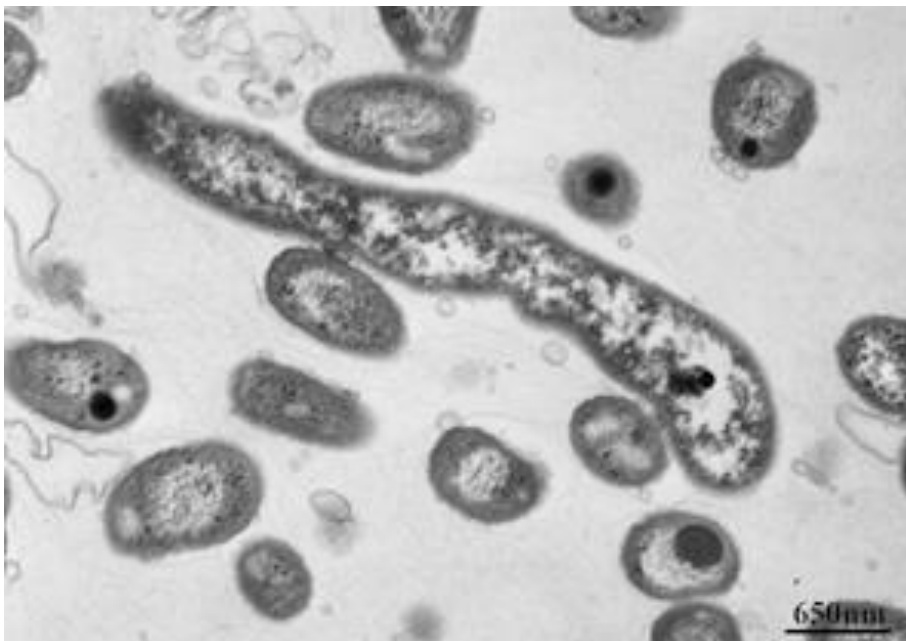


Figure 8 Thin sectioning TEM of KBS708. The long irregularly shaped cell appears to be a single cell with no evidence of division at pinching sites. Dense inclusions are suggestive of polyphosphate accumulation.



Figure 9 Negative stain TEM of KBS708. The cell and vesicles appear to be covered in an S-layer membrane as evident by its regular pattern.

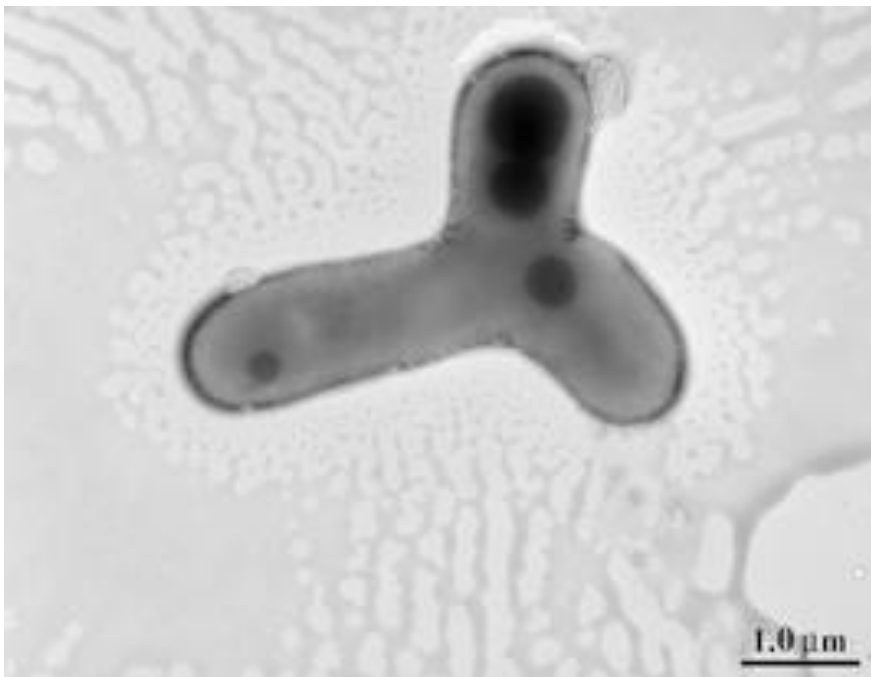


Figure 10 Negative stain TEM of KBS708. The center protrusion suggests the cell is dividing by budding.

Physiology

Microscope slides were immersed in static liquid cultures of KBS708 to allow examination for biofilm development across the slide (Figure 11). The DAPI stains of KBS708 produced very concentrated dots of bright yellow fluorescence and areas of dull yellow fluorescence, but sparse blue-white fluorescence was observed. This could suggest that the cells and the polyphosphate accumulations are not occurring at the same place and may be residual from the media. The bright yellow fluorescence supports the presence of polyphosphate bodies as suggested in the TEM images. In the present study, bright orange fluorescence was observed suggesting the presence of PHB. The presence of PHB typically appears when cells are grown under nitrogen or oxygen limitation with excess carbon (Reddy, 2007).

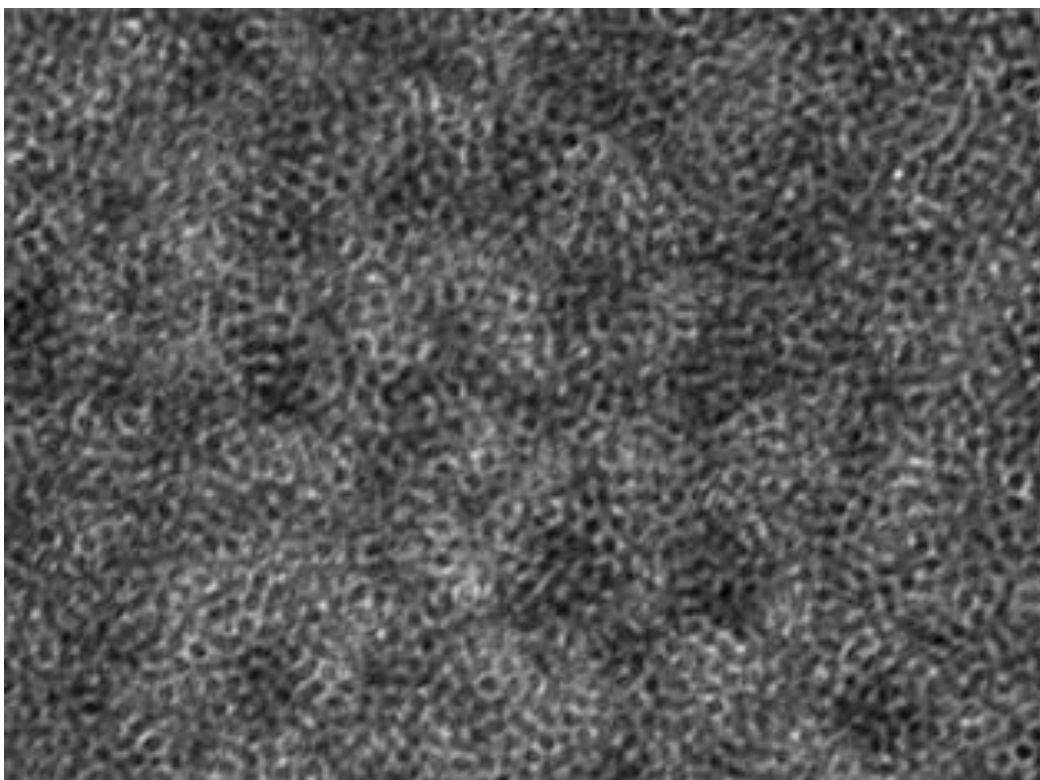


Figure 11 Contrast microscope image of KBS708 liquid culture grown onto a slide.

Respiratory Quinones

MK-9 was the major respiratory quinone.

Genome

The GC content of the genomic DNA was 72.6% based on preliminary sequencing of the KBS 708 genome. The preliminary genome size was 7.5 Mbp.

Comparison to Gemmatimonas aurantiaca T-27

Gemmatimonas aurantiaca T-27 was isolated in an enhanced biological phosphorus removal anaerobic-aerobic sequential wastewater reactor and incubated on NM-1 plates for 14 days (Zhang et al., 2003). KBS708 was isolated

from organically managed agricultural soil with a wheat-corn-soybean rotation at the Long Term Ecological Research Site at Kellogg Biological Station on VL55 agar plates after incubation for 36 days. The isolates share similar colony morphology with the exception of color; T-27 being faintly orange to pink and KBS708 being light to dark pink in color (Table 2). Both isolates are gram-negative and rod shaped, however KBS708 has a longer more pleomorphic shape than strain T-27. In liquid culture KBS708 flocculates into a single mass. Strain T-27 is motile where as KBS708 showed no evidence of motility. KBS708 can grow at a much wider temperature range than T-27 (20-37°C and 25-35°C, respectively), but T-27 can grow at a wider pH range than KBS708 (pH 6.5-9.5 and pH 5.0-7.0, respectively). Both isolates can grow aerobically, but KBS708 cannot grow anaerobically and prefers a microaerophilic atmosphere. Only T-27 can produce catalase and carotenoids but both can produce oxidase. Both isolates appear to have luxury phosphate uptake in the form of polyphosphate storage molecules. Strain KBS708 appears to be able to metabolize a much broader range of substrates. Both have menaquinone (MK)-9 respiratory quinones. KBS708's major fatty acid is iso-17:1 ω 7c and T-27's major fatty acid is iso-C15:0, and they do not appear to share any of the same fatty acids. Strain KBS708 has a higher GC content (72.6%) and larger genome size (7.5 Mbp) than T-27.

Table 2 Comparison of *Gemmatimonas aurantiaca* T-27 to KBS708. ND stands for not determined.

		<i>Gemmatimonas aurantiaca</i> T-27 (Zhang et al 2003)	Strain KBS708
Isolation	Isolated from	Enhanced biological phosphorus removal anaerobic-aerobic sequential wastewater reactor	Organically managed agricultural soil with a wheat-core-soybean rotation at the Long Term Ecological Research Site at Kellogg Biological Station
	Medium	NM-1 agar plates	VL55 agar plates
	Incubation	14 days	36 days
Morphology	Colony description (plate)	Circular, smooth, faintly orange to pink, 1-2 mm in diameter	Round, smooth and convex, opaque, light to dark pink color, <2 mm in diameter
	Colony description (liquid)	ND	Flocculates, cells held together by extra-cellular polysaccharide, light to dark pink color
	Shape	Rod-shaped	Long and irregular rod-shaped
	Size	0.7 um in width and 2.5-3.5 um in length	460-440 nm in diameter and 1-16 um in length
	Cellular envelope	Gram negative	Gram negative, S-layer
	Motility	Motile	Non-motile
	Reproduction	Binary fission and budding suggest asymmetrical cell division	Protrusions suggest division at center of cell
Physiology	Temperature	25-35°C	20-37°C
	pH range	6.5-9.5	5.0-7.0
	Respiration	Aerobic-anaerobic	Aerobic-microaerophilic
	Defense against oxygen radicals	Produces catalase, oxidase, and carotenoids	Produces oxidase
	Luxury phosphate uptake	Polyphosphate bodies	Polyphosphate bodies

Table 2 continued.

		<i>Gemmatimonas aurantiaca</i> T-27 (Zhang et al 2003)	Strain KBS708
Substrates	Carbon source	Yeast extract, polypepton, succinate, acetate, elatin, benzoate, glucose, sucrose, galactose, melibiose, maltose, formate and B-hydroxybutyrate	Peptone, arabinose, fructose, xylose, chitin, cellulose, N-acetyl-D-glucosamine, succinic acid, L-aspartic acid, D-mannose, L-lactic acid, formic acid, tween 20, 40 and 80, L-rhamnose, D-fructose, acetic acid, α -D-glucose, α -keto-glutaric acid, α -keto-butyric acid, sucrose, maltotriose, fumaric acid, bromo succinic acid, D-cellobiose, methyl pyruvate, D-malic acid, L-malic acid, pyruvic acid, γ -cyclodextrin, gelatin, 2-deoxy-D-ribose, l-erythritol, gentiobiose, γ -amino butyric acid, D-glucosamine, β -hydroxy butyric acid, γ -hydroxy butyric acid, α -keto valeric acid, 5-keto-D-gluconic acid, malonic acid, oxalomalic acid, sorbic acid, succinamic acid, L-tartaric acid, dihydroxy acetone
Chemotaxonomy	Respiratory quinone	Menaquinone (MK)-9	Menaquinone (MK)-9
	Fatty acids	iso-C15:0 (45% of total FAME) C16:1 (27%) C14:0 (12%) C13:0 (9%) C16:0 (7%)	iso-C17:1 ω 7c (36.2% of total FAME) isoC-15:0 (33.1%) 16:1 ω 7c (11.8%)
Genome	G+C content	66.00%	72.60%
	Genome size	4.6 Mbp (complete)	7.5 Mbp (preliminary)
	# 16s rRNA genes	1	2

Discussion

Strain KBS708 appears to prefer microaerophilic growth conditions but can grow under aerobic conditions. The microaerophilic preference is best seen when grown on plates. Under aerobic conditions it can take months for sparse

growth as indicated by only a few tiny colonies to appear, but plates under microaerophilic conditions robust growth consisting of more numerous and much larger colonies is seen within 3 days. The denser layer of growth about a centimeter below the surface of semi-solid gellan gum tubes in figure 2 supports KBS708 being sensitive to oxygen.

The ability to dry samples of KBS708 culture in a dissector for up to 2 weeks and revive the culture by adding VL55 medium suggests that KBS708 is desiccation resistant.

Comparison to Gemmatimonas aurantica T-27

Strain KBS708 is a gram-negative, oxygen sensitive, chemoheterotroph which likely accumulates phosphate and possibly PHB. When compared to strain T-27 (Table 2), there are a few similarities, but they are characteristically different. For example, there was no noticeable change in the rate of growth or colony formation when Strain KBS708 was grown on gellan gum; however, *G. aurantiaca* experienced increased growth rate and up to a 50-fold increase in colony formation (Tamaki et al., 2009).

Both bacteria appeared to have bulbous vesicles on their cellular surface. Strain KBS708 appeared to be more irregularly shaped and be able to lengthen to a larger size than strain T-27. Their mode of respiration is completely different. Strain KBS708 respire aerobically but has a preference for low oxygen and high carbon dioxide conditions whereas strain T-27 can respire under anaerobic

and aerobic conditions (Zhang et al., 2003). Both isolates share the same respiratory quinone, menaquinone-9.

Isolate KBS708 appears to have a wider variety of substrate utilization but this may have been due to the examination of more substrates using the BioLog Phenotype Microarray testing, which allowed evaluation of 190 carbon substrates at once. The phenotype microarrays were not used in the characterization of T-27. Although the BioLog results varied it was clear that KBS708 could utilize a wide variety of carbon sources. Unlike strain KBS708, Strain T-27 was able to grow in nutrient rich media. The problems encountered in substrate utilization testing with KBS708 were largely due to the fact that this bacterium flocculated. Flocculation made cell density measurements completely impossible after a few days and also interfered with optimization of growth conditions.

The fatty acid profiles of T-27 and KBS708 shared isoC15:0. Interestingly KBS708's main fatty acid iso-17:1 ω 7c (36.2% of total FAME) is often used as a marker of *Desulfovibrio* (Edlund et al., 1985). Since there are no other studied isolates it is unclear what fatty acids are typical for this phylum.

DNA sequencing revealed that KBS708 had a much higher G+C content than T-27 and most bacteria in general. Strain KBS708's G+C content of 72.6% is close to the theoretical limit of 75%. The genome size is quite large as well at a 7.5 Mbp. Strain T-27 has a single copy while KBS708 has two identical copies of the 16S rRNA gene.

The vast majority of prokaryotic life in soil remains uncultivated. New isolates need to be cultivated to understand the depth of this diversity and what ecological role they play. The characteristics learned in study can be used to help isolate new bacteria from the phylum *Gemmatimonadetes*.

References

- Aanderud, Z.T., and Lennon, J.T. (2011). Validation of Heavy-Water Stable Isotope Probing for the Characterization of Rapidly Responding Soil Bacteria. *Applied and Environmental Microbiology* 77, 4589–4596.
- Anisimova, M., and Gascuel, O. (2006). Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst. Biol.* 55, 539–552.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552.
- Davinic, M., Fultz, L.M., Acosta-Martinez, V., Calderón, F.J., Cox, S.B., Dowd, S.E., Allen, V.G., Zak, J.C., and Moore-Kucera, J. (2012). Pyrosequencing and mid-infrared spectroscopy reveal distinct aggregate stratification of soil bacterial communities and organic matter composition. *Soil Biology and Biochemistry* 46, 63–72.
- Davis, K.E.R., Joseph, S.J., and Janssen, P.H. (2005). Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. *Applied and Environmental Microbiology* 71, 826–834.
- DeBruyn, J., Fawaz, M., Peacock, A., Nixon, L., Cooper, K., and Radosevich, M. (2013). *Gemmatirosa kalamazonensis* gen nov. sp. nov., a member of the rarely-cultivated bacterial phylum Gemmatimonadetes. *Journal of General and Applied Microbiology (Accepted)*.
- DeBruyn, J.M., Nixon, L.T., Fawaz, M.N., Johnson, A.M., and Radosevich, M. (2011). Global Biogeography and Quantitative Seasonal Dynamics of Gemmatimonadetes in Soil. *Applied and Environmental Microbiology* 77, 6295–6300.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36, W465–W469.
- Drees, K.P., Neilson, J.W., Betancourt, J.L., Quade, J., Henderson, D.A., Pryor, B.M., and Maier, R.M. (2006). Bacterial Community Structure in the Hyperarid Core of the Atacama Desert, Chile. *Applied and Environmental Microbiology* 72, 7902–7908.
- Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.

- Edlund, A., Nichols, P.D., Roffey, R., and White, D.C. (1985). Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *Journal of Lipid Research* 26, 982–988.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59, 307–321.
- Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.
- Janssen, P.H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719–1728.
- Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. (2002). Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology* 68, 2391–2396.
- Jiang, X., Wright, A.L., Wang, J., and Li, Z. (2011). Long-term tillage effects on the distribution patterns of microbial biomass and activities within soil aggregates. *CATENA* 87, 276–280.
- Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., and Janssen, P.H. (2003). Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Applied and Environmental Microbiology* 69, 7210–7215.
- Mummey, D., Holben, W., Six, J., and Stahl, P. (2006). Spatial stratification of soil bacterial populations in aggregates of diverse soils. *Microb. Ecol* 51, 404–411.
- Rappé, M.S., and Giovannoni, S.J. (2003). The uncultured microbial majority. *Annu. Rev. Microbiol.* 57, 369–394.
- Reasoner, D.J., and Geldreich, E.E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Applied and Environmental Microbiology* 49, 1–7.
- Schoenborn, L., Yates, P.S., Grinton, B.E., Hugenholtz, P., and Janssen, P.H. (2004). Liquid Serial Dilution Is Inferior to Solid Media for Isolation of Cultures Representative of the Phylum-Level Diversity of Soil Bacteria. *Applied and Environmental Microbiology* 70, 4363–4366.

- Serafim, L.S., Lemos, P.C., Levantesi, C., Tandoi, V., Santos, H., and Reis, M.A.M. (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Methods* 51, 1–18.
- Sexstone, A.J., Revsbech, N.P., Parkin, T.B., and Tiedje, J.M. (1985). Direct Measurement of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society Of America Journal* 49, 645–651.
- Sides, K. (2010). Agricultural Soil Bacteria; A Study of Collection, Cultivation, and Lysogeny. Master's Thesis, University of Tennessee.
- Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.* 39, 321–346.
- Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. (2004). New Strategies for Cultivation and Detection of Previously Uncultured Microbes. *Applied and Environmental Microbiology* 70, 4748–4755.
- Stewart, E.J. (2012). Growing Unculturable Bacteria. *Journal of Bacteriology* 194, 4151–4160.
- Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y., and Kamagata, Y. (2009). Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environmental Microbiology* 11, 1827–1834.
- Weis, D.S. (1975). A Medium for the Axenic Culture of Chlorella-Bearing Paramecium bursaria in the Light. *Transactions of the American Microscopical Society* 94, 109–117.
- Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., and Nakamura, K. (2003). Gemmatimonas aurantiaca gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. *International Journal Of Systematic And Evolutionary Microbiology* 53, 1155–1163.
- (2007). *Methods for general and molecular microbiology* (Washington, D.C: ASM Press).

CHAPTER II

DISTRIBUTION OF GEMMATIMONADETES IN AGRICULTURAL

SOIL

Abstract

Culture independent studies suggest that the phylum *Gemmatimonadetes* is abundant yet their ecology remains unknown. Previous studies suggest that *Gemmatimonadetes* and other microbial taxa are heterogeneously distributed at the aggregate scale and can respond quickly from a dehydrated state upon re-wetting. To further examine the spatial distribution of *Gemmatimonadetes* in soils and their resilience to drying and wetting, tilled and no-till managed soils were sampled from the University of Tennessee Research and Education Center (REC) in Jackson, Tennessee. In one experiment the soils were held at constant moisture and sampled periodically over 67 days. At each sampling point the soils were fractionated into whole soil, macro-aggregates (2000-250 μm) and micro-aggregates (<250-53 μm) by wet sieving and the relative abundance of *Gemmatimonadetes* was determined in each fraction using quantitative PCR (qPCR). In a second experiment, the soils were subjected to four dry/wet cycling treatments. The dry/wet cycled soils, dry control and moist control soils were sampled destructively at the end of each 2 week air dry period and after 72 hours of incubation post wetting. DNA was extracted from sampled soils and relative abundance of *Gemmatimonadetes* was analyzed by qPCR. The relative abundance of *Gemmatimonadetes* was greater in bulk soil ($9.1\% \pm 0.4$) than in macro- and micro-aggregates ($5.6\% \pm 0.4$ and $6.3\% \pm 0.4$), respectively. The relative abundance of *Gemmatimonadetes* was greatest in the dry control soils ($6.5\% \pm 0.9$) and lowest in soils that received dry/wet cycling treatment (2.9%

± 0.9); however, dry controls and the dry/wet cycled soils did not differ from the wet controlled soils ($4.4\% \pm 0.9$). In conclusion the results indicated that the distribution of *Gemmatimonadetes* in soil was more dependent on moisture availability than aggregation and suggested members of this phylum are well adapted to low moisture conditions but unable to resist moisture fluctuations induced by wet/dry cycling.

Introduction

Diversity and distribution of Gemmatimonadetes in soils

Most of what is known about the phylum *Gemmatimonadetes* comes from surveys of 16S rRNA genes amplified directly from environmental DNA. *Gemmatimonadetes* is one of nine numerically dominant phyla which together typically account for 90% of the total 16S rRNA gene sequences in libraries surveyed from soil (Janssen, 2006). The relative abundance of *Gemmatimonadetes* in these molecular surveys range from 0.2% to 6.5%, with a mean of 2.2% of total 16S rRNA gene sequences detected in agricultural and forest soils (DeBruyn et al., 2011). In these previous surveys, *Gemmatimonadetes* was frequently detected in terrestrial environments. For example, *Gemmatimonadetes* was detected at 26.4, 13.1, 11.1, 10.9, and 9.7% of total 16S gene sequences from grassland/ prairie/ pasture/ agriculture, forest, radioactive contaminated, and organics or hydrocarbons contaminated soil, respectively. *Gemmatimonadetes* has also been found in marine and freshwater sediments, activated sludge and wastewater treatment plants (Figure 12)

(DeBruyn et al., 2011). Overall this suggests that bacteria from the phylum *Gemmatimonadetes* are adapted to a wide range of environments.

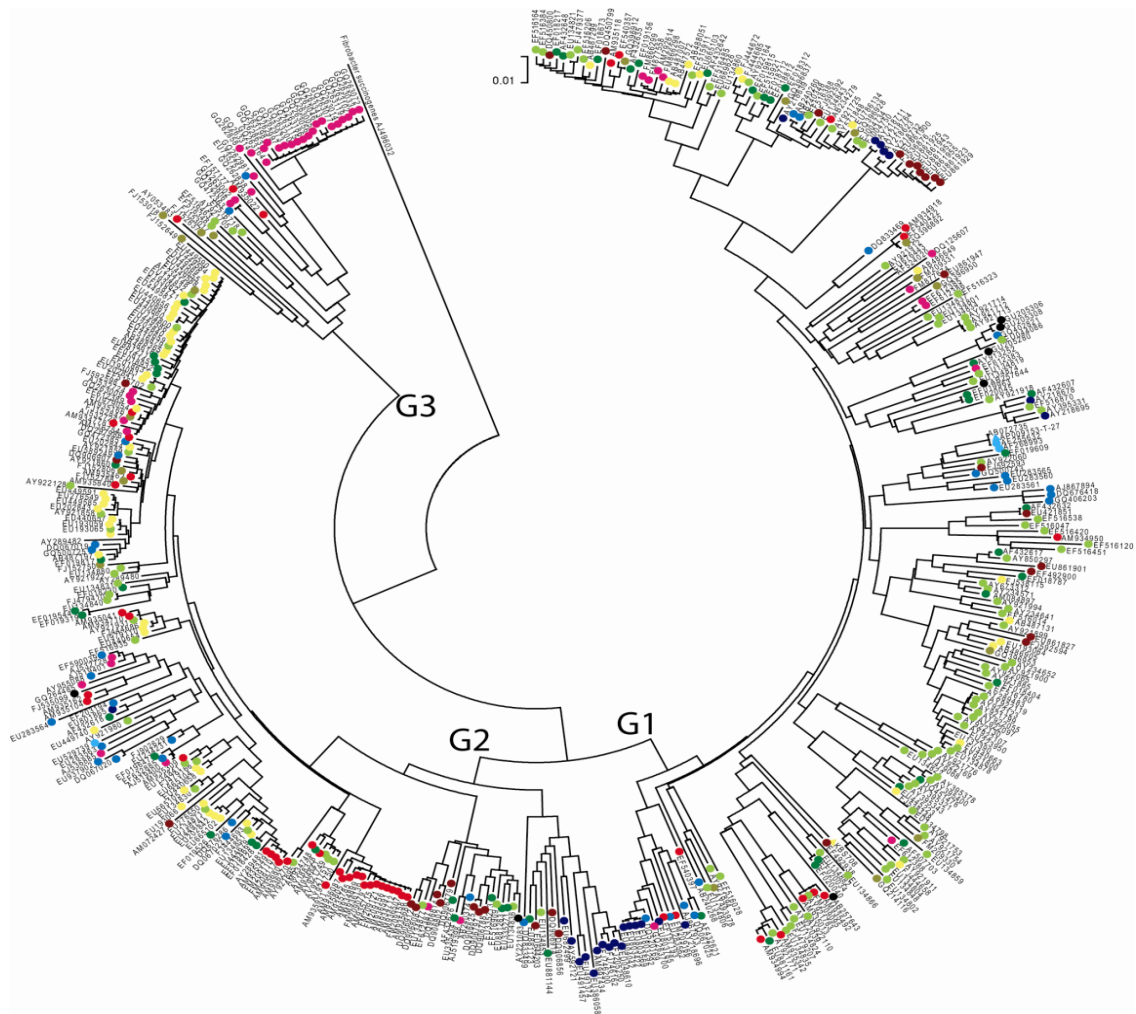


Figure 12 (DeBruyn et al., 2011) neighbor joining phylogenetic tree of 456 *Gemmatimonadetes* 16S rRNA sequences (>1200 bp) from RDP. Sequences are listed by their genbank accession number and color coded according to the environment the sample had taken from. Arid soils (tan); forest soil (dark green); prairie and grassland soil (light green); agricultural crop soil (yellow); alpine and tundra soil (brown); rock surfaces (black); soil contaminated with organics and hydrocarbons (pink); soil contaminated with radioactive waste (red); wastewater and activated sludge (light blue); freshwater sediments and saturated soils (medium blue); marine sediments (navy blue).

Phylogenic analysis of the 16S rRNA genes from environmental libraries revealed that the phylum *Gemmatimonadetes* is composed of three major clades (G1, G2, and G3). Clade G1 has the most random distribution pattern of the three clades, but the most sequences come from prairie and grassland soils. Clade G2 is mostly composed of 16S rRNA gene sequences found in agricultural and contaminated soil, and clade G3 is almost exclusively from genes detected in soils contaminated by organics and hydrocarbons (Figure 12) (DeBruyn et al., 2011). The retrieved sequences used to construct the phylogentic tree were also used to design the primer G1G3-673F for detecting environmental sequences of *Gemmatimonadetes*. When G1G3-673F was used with the universal eubacterial primer 907R, 95.6, 0, and 100% of the sequences in clades G1, G2, and G3, respectively could be detected, and matched less than a dozen sequences in non-*Gemmatimonadetes* phyla (n=7446) in *in silico* experiments (DeBruyn et al., 2011). Analysis of the relative abundance of *Gemmatimonadetes* in soils collected at the Kellogg Biological Station uncovered a mean relative abundance of G1 and G3 clades ranging from 0.9 to 5.31% of the total 16S rRNA gene copies in all soil types examined (Figure 13) (DeBruyn et al., 2011). The relative abundance of *Gemmatimonadetes* in all the soils was relatively constant except for a significant increase in the samples collected in September relative abundances were inversely correlated to soil moisture ($r = -0.336$) (DeBruyn et al., 2011).

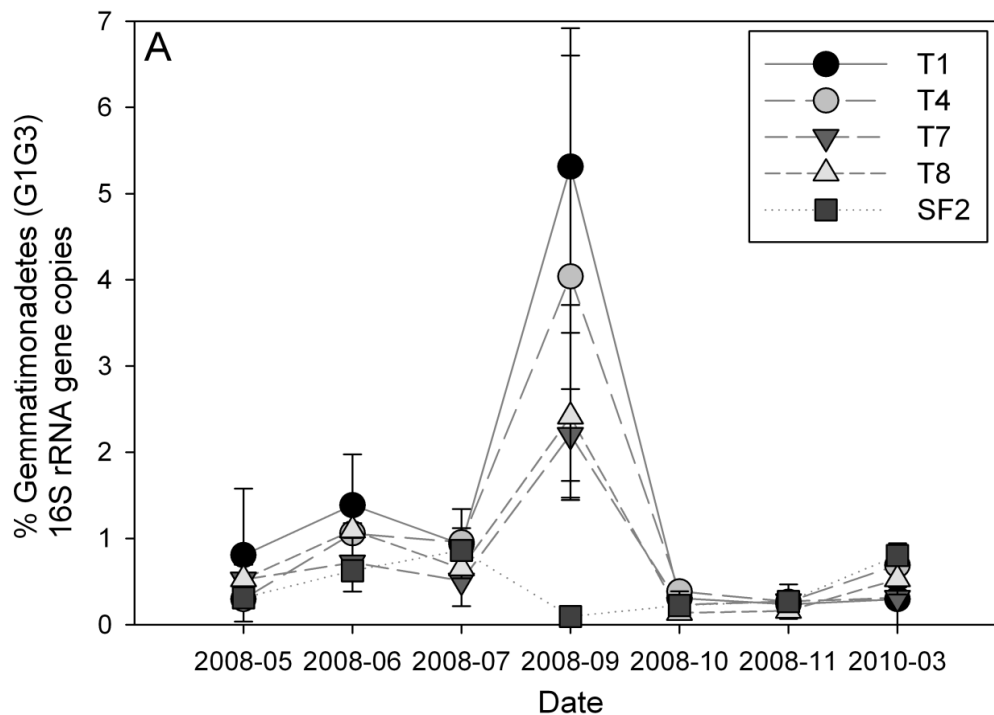


Figure 13 (DeBruyn et al., 2011) Relative abundance of *Gemmatimonadetes* 16S rRNA gene copies from clades G1 and G3 in five soils from the Kellogg Biological Station over time. Conventional agriculture (T1); organic agriculture (T4); early succession meadows (T7 and T8); mid succession forest (SF2).

Distribution within the soil aggregate

In a study by Mummey et al. (2006) which examined the distribution and activity of bacteria within whole macro-, whole micro-, and inner micro-aggregates it was found that *Gemmatimonadetes* was more abundant based on 16S rRNA clone libraries within inner micro-aggregates than in whole macro-aggregates and whole micro-aggregates. Analysis of 16S rRNA cDNA and 16S rRNA gene clone libraries shows that *Gemmatimonadetes* is active as well as abundant in the inner micro-aggregate (Mummey et al., 2006).

Gemmatimonadetes accounted for 10 to 32% of 16S rRNA genes cloned from

the inner micro-aggregate fraction relative to other bacterial clones. The relative abundance and activity of *Gemmatimonadetes* in the inner micro-aggregate may suggest the environmental conditions within soil to which they are well adapted. For example, the diffusion of oxygen was shown to be directly related to the radius of soil aggregates with oxygen concentration decreasing in the aggregate interior (Sexstone et al., 1985). The propensity of KBS708 to grow under microaerophilic conditions (see chapter I) may suggest that the increased relative abundance maybe due to lower oxygen concentration in the interior of soil aggregates. The objective of this study was to further examine this possibility by determining the distribution of clades G1 and G3 of the phylum *Gemmatimonadetes* were analyzed in whole soil, macro-aggregates and micro-aggregates for both till and no-till soil using *Gemmatimonadetes* specific primers. Soils from long-term tillage experiments conducted at the West Tennessee Research and Education Center (WTREC) were chosen because the degree of aggregation was expected to be substantially better in the no-till plots compared to the tilled plots (Jiang et al., 2011). In this study, the hypothesis that bacteria from the phylum *Gemmatimonadetes* are more abundant in micro-aggregates and macro-aggregates than in whole soil was evaluated.

Desiccation resistance

Some evidence has been presented from previous studies suggesting *Gemmatimonadetes* may be well-adapted to moisture-limited environments. For example, bacteria from the phyla *Gemmatimonadetes* and *Planctomycetes* were

prevalent members of bacterial communities in the hyperarid regions of the Atacama Desert, suggesting some members of these phyla maybe well adapted to low moisture availability (Drees et al., 2006). Aanderud and Lennon (2011) used H_2O^{18} to identify the which microbial taxa respond most readily to rewetting and revealed that the phylum *Gemmatimonadetes* responded positively and significantly to rewetting (Aanderud and Lennon, 2011). These findings suggest the phylum *Gemmatimonadetes* has adapted to surviving in arid environments and during drought conditions. In an experimental study, KBS708 cultures were desiccated. The desiccated cultures recovered weeks later following rewetting with VL55 liquid medium suggesting that KBS708 is desiccation resistant (see chapter I). Therefore a second objective for this study was to analyze the response of *Gemmatimonadetes* to changes in moisture availability was tested over time for till and no-till soils by determining the relative abundance by qPCR after wetting and drying cycles. We hypothesized that the relative abundance of *Gemmatimonadetes* would increase after dry/wet cycling of soil based on the expected resilience of *Gemmatimonadetes* to reduced moisture conditions and the reduction in abundance of less resilient taxa.

Methods

Site description

Field plots for this study were located at the WTREC in Jackson, Tennessee. The experimental plots established in conventional tillage and no-till management in 1978-1979 to study the effects of cultivation practices on highly

erodible soils. The soils in this area are classified as Grenada, Lexington, and Loring silt loams. The cropping system is cotton with rotating winter wheat cover, clover cover, vetch, and no cover.

Sampling: Aggregate scale distribution study

The research was initiated in Fall 2011 by collecting one soil sample from a tilled cotton plot with no cover and 0 lbs N amendment and no-till cotton plot with no cover and 30 lbs N amendment. The soils were sampled on November 13, 2011. There was no cover at the time of sampling except for the plant residue left over from the harvest and weeds. Using a clean trowel, samples were taken to a 6-in depth and placed in sterile whirl-pak™ bags. Samples were transported back to the laboratory and stored at 4°C until processing.

Sampling: Moisture availability study

Soil samples were taken from till and no-till cotton plots that were fertilized with 90 lbs N/acre with no winter cover crop at a depth of 0-6 in at the University of Tennessee WTREC on June 27, 2012. Samples were shipped overnight in a cooler with icepacks. Upon arrival, soils were processed with a 2 mm sieve and stored at 4°C.

Gravimetric Moisture Content

The gravimetric moisture content of the till and no-till soil samples was determined at the time of sampling and after adjustment to field capacity. All soil analysis was done in triplicate on 2mm sieved till and no-till soil. For the field

moist analysis, approximately 20 g of soil was loaded into pre-weighed tins, weighed and recorded. For the field capacity analysis, about 20 g of field moist soil was loaded into pre-weighed 3-in diameter brass sieves with an approximate mesh of 1000-500 μm in size set inside a catch tin. The soil and sieves were transferred into a basin where they were slowly wetted from the bottom up. After an hour the water was slowly pipetted from the basin to prevent loss of soil from the sieve. The soil and sieves were then allowed to drain until no drops formed under the sieve. At this point it was assumed that the samples were at field capacity. The sieves were returned to their catch tins, weighed and recorded. All soil samples were oven dried at 105°C overnight. After drying, the samples were cooled in a desiccator, weighed and recorded.

To confirm the method employed to determine the field capacity of the soils provided an accurate estimate of field capacity, 10 gram soil samples were brought to 75% of field capacity weighed and oven dried at 105°C overnight. The samples were cooled in a desiccator, weighed and the gravimetric moisture content was determined. The theoretical oven dry mass of each sample was calculated and compared to the actual oven dry mass of the samples.

Sample processing: Aggregate size

Field-moist samples were sieved with a 2000- μm sieve. The till and no-till soil was incubated at room temperature and maintained at constant moisture equivalent to the value measured at the time of sampling. Sterile water was added drop-wise in a spiral pattern to balance moisture loss due to evaporation.

Seedlings that sprouted during the incubation process were picked out to prevent weight change. Samples were taken on day 0, 17, 44, and 67. Only the whole soil was analyzed on day 17. The macro-aggregate (2000 to 250- μm) and micro-aggregate (250 to 53- μm) size fractions were obtained using a wet-sieving technique with 250- μm and 53- μm stacked sieves in sterile water to prevent introduction of foreign bacteria. The sieves were gently raised and lowered 3-cm to separate water-stable macro- and micro-aggregates in the 2000 to 250- μm and 250 to 53- μm ranges, respectively (Mummey et al., 2006).

Dry/Wet Cycling

The dry/wet cycling treatment consisted of four cycles of a two-week air-dry period, followed wetting to 75% of field capacity and incubation for 72 hours under air-dry conditions to allow the moisture content to slowly return to a desiccated state. Controls for this experiment were air-dry and soil adjusted to 75% of field capacity and held at constant moisture. Each treatment had a total nine replicates so that one sample could be destructively sampled at the end of each period and at time of zero.

DNA extraction

For both studies, DNA was extracted in triplicate from the whole (bulk), macro-aggregate, and micro-aggregate soil fraction using the MoBio Power Soil DNA Isolation Kit. Soil or aggregates (approx. 0.3 g) was placed into the provided tubes for extraction. The amount of fractionated soil used was only approximate because moisture content was not determined after the wet-sieving fractionation

procedure. The rest of the DNA extraction was done according to the manufacture's protocol and stored at -20°C. The DNA was quantified using Gen5 Nucleic Acid Quantification spectroscopy to determine the appropriate dilution. An initial dilution of the template DNA was done to insure the amplification of both the total and G1G3 *Gemmatimonadetes* 16S rRNA genes would be in range of the standard curve. A second 1:5 dilution was then done on all samples so that 5 µL of template could be used in all reactions to reduce pipetting error.

qPCR standard curve

The concentration of plasmid DNA containing the 16S rRNA fragment amplified with universal eubacterial primers 8F and 1392R from strain KBS708 was determined using Gen5 Nucleic Acid Quantification spectroscopy for the qPCR standard curve. The average concentration was found to be 83.21 ng/µL. The clone size was 4381 bp, made up of a 3000 bp vector and a 1381 bp insert, with a mass of 2847650 Daltons. The sample had 1.76×10^{10} copies/µL and was diluted 176 fold by diluting 2 µL of template into 350 µL of PCR water to make a 1×10^8 copies/µL. The resulting stock solution was further diluted 5-fold by mixing 352 µL into 1408 µL of PCR water resulting in a new stock solution containing 0.2×10^8 copies/µL. Adding this additional 5-fold dilution allowed a larger sample volume 5 µL, equivalent to 1×10^8 copies to be added when preparing further serial 10-fold dilutions, to minimize pipetting error. The resulting standards ranged from 1×10^8 copies to 10 copies per reaction.

Determining the relative abundance of Gemmatimonadetes (qPCR analysis)

The relative abundance of *Gemmatimonadetes* in soil was determined by the qPCR method described in the DeBruyn et al. (2011). The qPCR protocol for G1G3 consisted of an initial hot start interval of 95°C for 5 minutes to activate Sybr Green (Bio-Rad), followed by 41 cycles of 95°C for 5 seconds, 53°C for 20 seconds, and 72°C for 20 seconds. A melt curve was determined after completion of the final cycle. The primers used to determine the total 16S rRNA gene abundance were 1055F and 1392R and the PCR conditions were as follows: an initial hot start at 95°C for 5 minutes followed by 41 cycles of 95°C for 7 seconds, 50°C for 30 seconds, and 72°C for 20 seconds, ending in a final melt curve.

Each qPCR reaction was done in triplicate and consisted of 12.5 µL of Bio-Rad iQ™ SYBR® Green Supermix, 400 nM G1G3-637F primer, 400 nM 907R universal 16S rRNA gene primer, 5.5 µL of water, and 5 µL of 1:5 diluted template, resulting in a final volume of 25 µL per reaction. A negative control reaction was done without template. All reactions were performed using a Bio-Rad CFX96™ Real-Time system C1000™ Thermal Cycler. The relative abundance of *Gemmatimonadetes* was expressed as a percentage of the total 16S rRNA genes.

Correction of qPCR standard curve in moisture study

The standard curve used in the previous analysis of the effect of moisture on the relative abundance of *Gemmatimonadetes* in soil was found to be

inefficient. It was mistakenly diluted by 2 fold rather than by 10 fold. Although the standard curve was precisely linear with an R^2 value greater than 95, the efficiency was above 400%. To correct this error, a new standard curve was made with 10 fold serial dilutions. The new standard curve was found to have precise linearity and had an efficiency of 102%. Random samples were selected from each plate from the previous analysis were re-run and analyzed again using the new standard curve. The relative abundance of the new standard curve was then compared to those values of the previous analysis. The mean percent error was then used to correct the estimated relative abundance of *Gemmatimonadetes* in the remaining samples. The relative abundance resulting from the new standard curve and the previously used standard curve was found to have an average percent error of -97.19%.

Statistical analysis: Aggregate scale distribution study

A randomized design was used to compare the relative abundance of bacteria from the phylum *Gemmatimonadetes* in till and no-till soil at the whole, macro-aggregate, and micro-aggregate size fraction over 67 days. All factors were first run excluding day 17 since this sampling date only included the whole (bulk) sample analysis. Then the whole soil samples were run separately to including the day 17 samples. Analysis of variance was performed using mixed models (Proc Mixed, SAS 9.3), and least of squares means were computed and separated with LSD.

Statistical Analysis: Moisture variation study

Analysis of variance was done using mixed models (Proc Mixed, SAS 9.3), and least of squares means were computed and separated with LSD. Repeated measures was considered but it was found to be unnecessary because the likelihood change was only 2 as opposed to the required 5. Three-way analysis was used for the error term because there were not true replicates.

Results

Aggregate scale distribution of Gemmatimonadetes

When analyzing all factors, excluding day 17, the relative abundance of *Gemmatimonadetes* differed for soil management (till or no-till) ($p=0.01$), aggregate size class ($p<0.01$) and the interaction between soil management and the aggregate size class ($p=0.07$); however, it did not differ for sample time ($p=0.54$), the interaction between soil management and sample time ($p=0.15$), the interaction between aggregate size class and sample time ($p=0.06$) and the interaction between soil management, aggregate size class and sample time ($p=0.54$) (Table 6 Appendix). The relative abundance of *Gemmatimonadetes* was greater in tilled soils ($7.60\% \pm 0.31$) than for no-till management ($6.42\% \pm 0.31$) (Table 7 Appendix). At the aggregate size class, the relative abundance of *Gemmatimonadetes* was greater in whole soil ($9.15\% \pm 0.37$) than in macro- or micro-aggregates ($5.63\% \pm 0.37$ and $6.25\% \pm 0.40$, respectively) (Table 8 Appendix). For the interaction between management and sample time, in till soil

the relative abundance of *Gemmatimonadetes* was greater in whole soil (9.51% $\pm$ 0.53) than micro-aggregates (7.60% $\pm$ 0.57), but did not differ in no-till whole soil (8.78% $\pm$ 0.53). All were higher than till soil macro-aggregates (5.69% $\pm$ 0.53) and no-till soil macro- and micro-aggregates (5.58% $\pm$ 0.53 and 4.91% $\pm$ 0.57, respectively) as seen in Figure 14 (Table 9 Appendix).

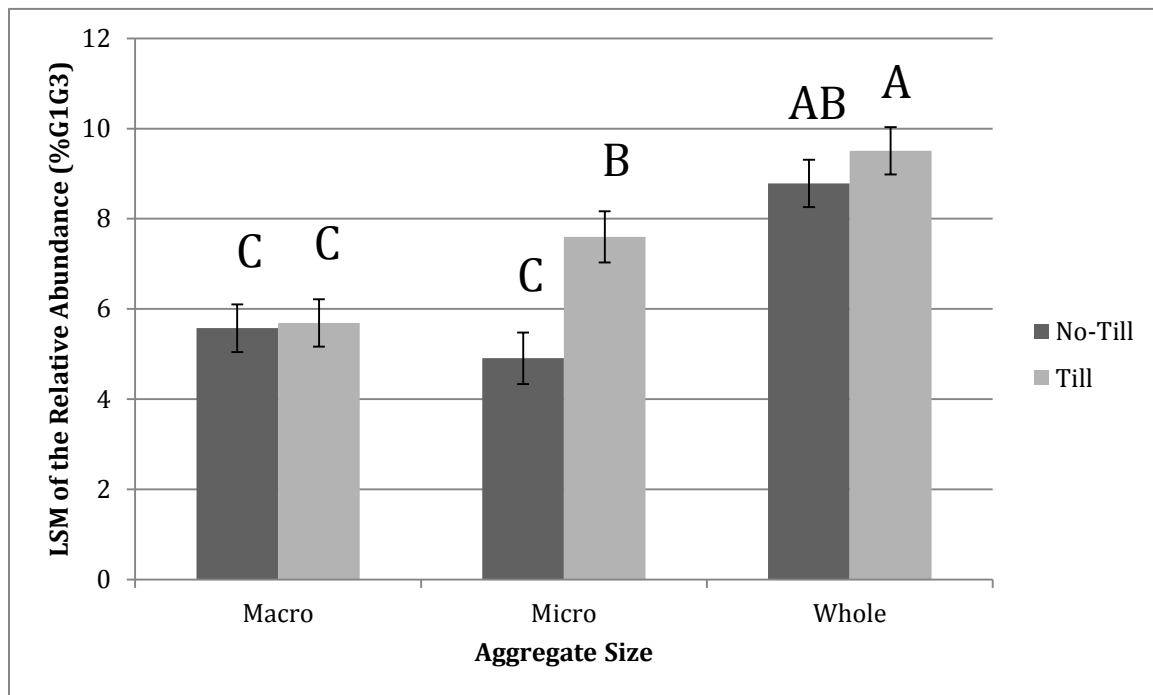


Figure 14 Least of squares means (LSM) of the relative abundance of clade G1 and G3 of *Gemmatimonadetes* for the interaction between management and aggregate size (n=9). The error bars indicate the standard error. Excluding whole soil sampled on day 17. See Table 9 in Appendix for statistics.

When analyzing only whole soil at all time points, the relative abundance of *Gemmatimonadetes* differed by soil management ($p < 0.01$), sample time ($p < 0.01$), and the interaction between soil management and sample time ($p = 0.01$) (Table 14 Appendix). *Gemmatimonadetes* was detected at a higher

relative abundance in till managed soil (9.56% $\pm$ 0.46) than in no till managed soil (6.80% $\pm$ 0.46) (Table 15 Appendix). The relative abundance differed for day 17 (11.51% $\pm$ 0.91) but not for any other sample time (6.79% $\pm$ 0.5246 to 7.43% $\pm$ 0.5407) (Table 16 Appendix). For the interaction between soil management and sample time, the relative abundance of *Gemmatimonadetes* in till soil sampled on day 17 (15.30% $\pm$ 1.29) differed from till and no-till managed soil on all other sampling days (5.89% $\pm$ 0.79 to 8.10% $\pm$ 0.74) as shown in Figure 15 (Table 17 Appendix).

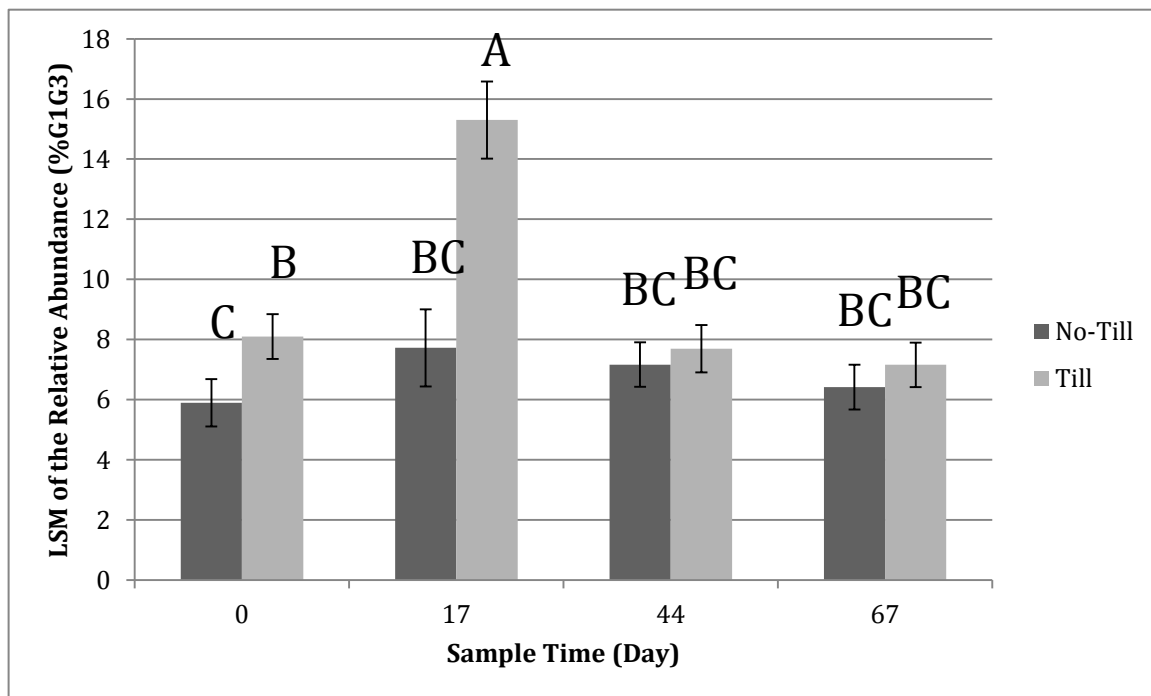


Figure 15 Analysis of whole soil at all time points. Least of squares means (LSM) of the relative abundance of *Gemmatimonadetes* for the interaction between management and sample time (n=3). Error bars indicate the standard error. Statistics in Table 17 in Appendix.

Moisture variation study

The relative abundance of *Gemmatimonadetes* in soil was greatest in the dry control soils (6.51%) and lowest in soils that received dry/wet cycling treatment (2.85%); however, dry controls and the dry/wet cycled soils did not differ from the wet controlled soils (4.38%) Figure 16 (Table 22 Appendix).

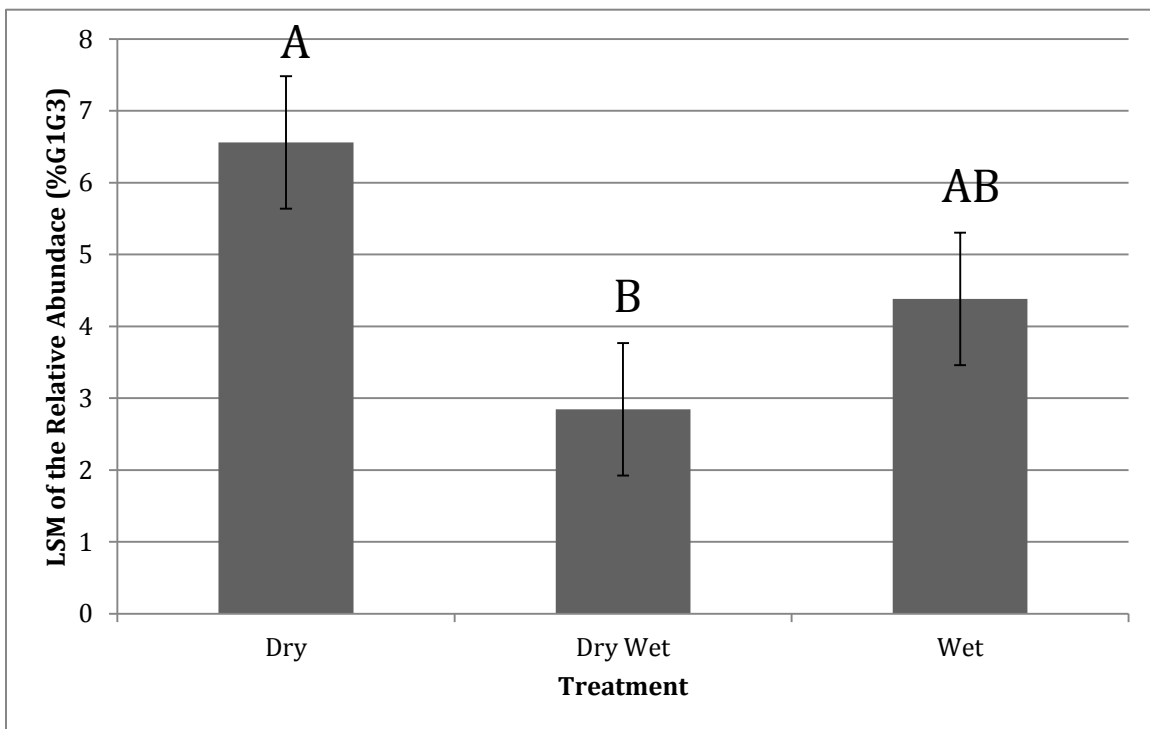


Figure 16 Least of squares means of the relative abundance of clades G1 and G3 based on treatment (n=54). The error bars are the standard error. See table 22 in Appendix for statistics.

The relative abundance of *Gemmatimonadetes* was greatest in no-till managed soil air-dry controls (8.34%), lowest in no-till and till soils that underwent dry/wet cycling and till soil wet controls (2.27-3.77%), but all were no

different in *Gemmatimonadetes* for no-till wet control soils and till dry control soils (4.78-4.99%) Figure 17 (Table 23 Appendix).

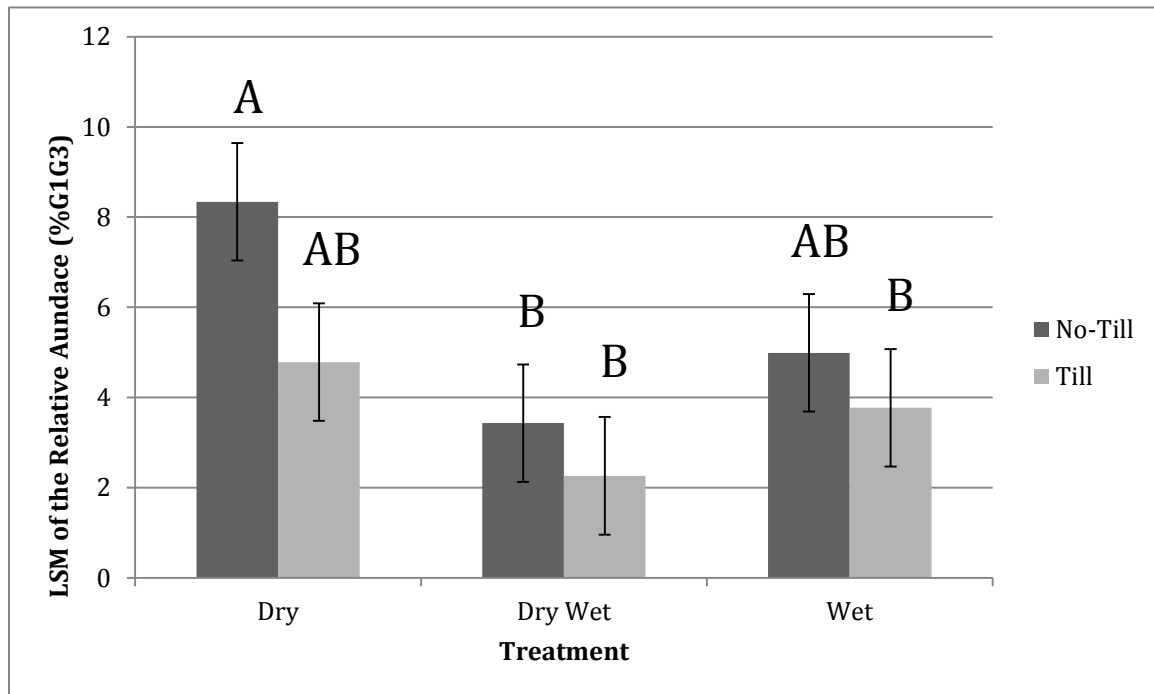


Figure 17 Least of squares means (LSM) of the relative abundance of clades G1 and G3 for the interaction of management and treatment (n=27). Error bars represent the standard error. See Table 23 in the appendix for the statistics.

For dry controlled soil sampled at day 48 (14.30%) there was a higher relative abundance of *Gemmatimonadetes* than the dry control soils sampled on days 0, 14, 17 and 34 (1.93-5.90%, respectively) Figure 18 (Table 27 Appendix).

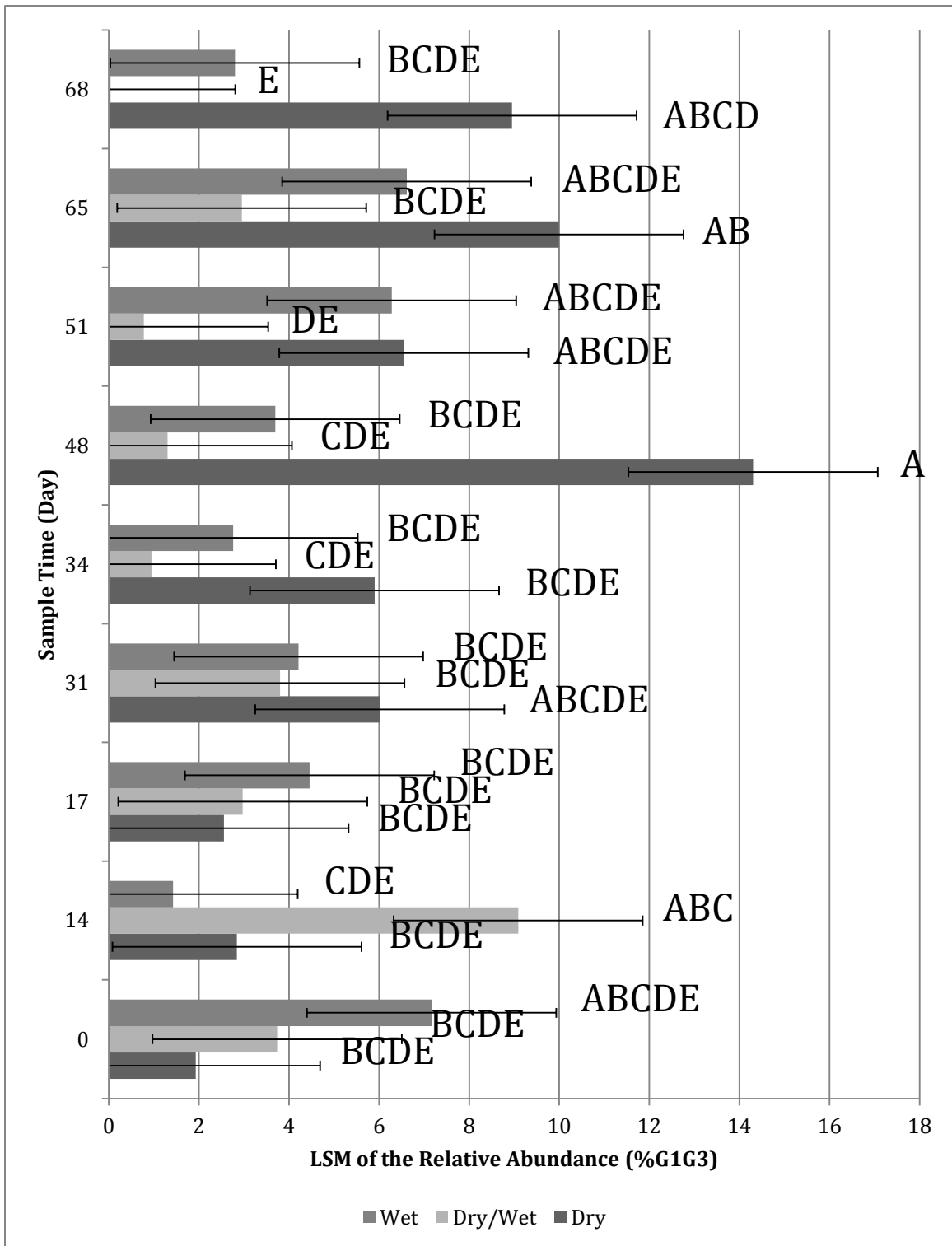


Figure 18 Least of squares means (LSM) of the relative abundance for clade G1 and G3 based on the interaction between sample time and treatment (n=6). The error bars represent the standard error. See Table 27 in the Appendix for statistics.

The dry/wet cycling has a downward trend over time but the control dry appears to have an upward trend over time (Figure 19). The relative abundance of *Gemmatimonadetes* appears to be increasing after 2 weeks of drying (days 14, 31, 48 and 65) and decreasing after wetting to 75% of field capacity and incubating for 72 hours under air dry conditions (days 17, 34, 51 and 68) (Figure 19). The difference between sample day 0 and 68 for the dry control increased (7.03%) but dry/wet and wet decreased (-3.70% and -4.37%, respectively). The relative abundance of *Gemmatimonadetes* was greatest in dry control soils.

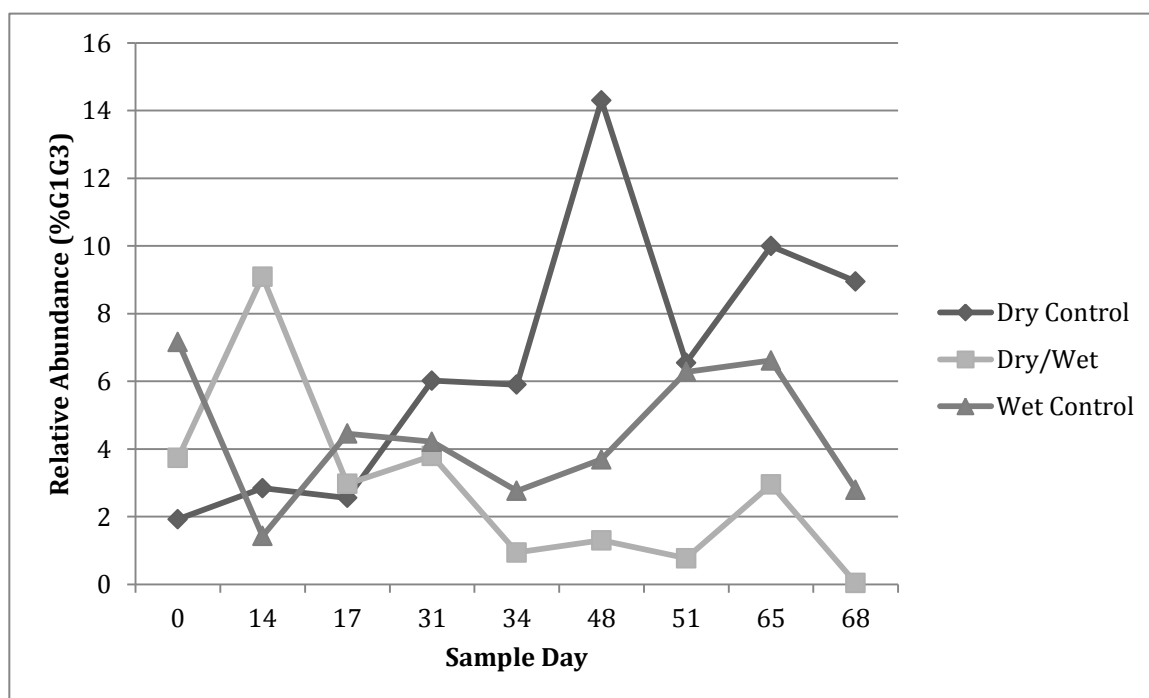


Figure 19 Relative abundance of clades G1 and G3 based on treatment over time. Table 18 in the Appendix.

Discussion

Aggregate scale distribution

Conventionally managed soil had a higher relative abundance of *Gemmatimonadetes* than no-till managed soil. It is unclear whether the different relative abundances of *Gemmatimonadetes* seen in till and no-till management was due to differences in the structural properties of the soils or the different amounts of nitrogen applied to the plots, 0 lb N (till) and 30 lb N (no-till). This effect may also be due to the lower aggregate stability in till soil, which would result in a higher micro-aggregate and silt and clay fraction. Analysis of the aggregate size class suggested that the whole soil had a greater relative abundance of *Gemmatimonadetes* than in both the macro- and micro-aggregates. The whole soil samples could be larger because they include the silt and clay fraction, which would have been washed away during wet-sieving of the macro- and micro-aggregates. The inner micro-aggregate fraction as well as the whole macro- and whole micro-aggregate fractions were tested in the Mummey et al. (2006) study and they found that *Gemmatimonadetes* represented 10, 18 and 32% of the sequenced clones from the inner micro-aggregate. In the macro- and whole micro- aggregate fractions *Gemmatimonadetes* had little to no representation in the sequenced clones when compared to the inner micro-aggregate fraction (Mummey et al., 2006). The lower representation in the macro- and micro- aggregates supports what was seen in this study. Conventionally managed soil differed at each aggregate size class with increasing abundances from macro-, micro-aggregates, to the whole soil. The

Davinic et al. (2012) study determined the mean relative abundance for soil bacteria in the whole soil, macro-, micro- and the silt and clay aggregate fractions. The mean relative abundance in the whole soil, macro-, micro-, and silt and clay aggregate fractions was between $4.38\% \pm 0.34\%$, $4.11\% \pm 0.24\%$, $4.76\% \pm 0.37\%$ and $5.91\% \pm 0.47\%$, respectively (Davinic et al., 2012). The mean relative abundance was highest in the silt and clay aggregate-size fraction ($5.91\% \pm 0.47\%$) where the both degradable and recalcitrant carbon forms were found (Davinic et al., 2012). The whole soil, macro-, whole micro-, inner micro-, and the silt and clay aggregate size fraction need to be tested together to further understand the distribution of *Gemmatimonadetes* with in soil.

When the DNA from whole soil was analyzed for all sample days, the differences in management type followed the previous analysis but a difference was seen for till soil at day 17. It is unclear why till soil sampled on day 17 had a greater relative abundance of *Gemmatimonadetes* than no-till soil.

Moisture

Based on the decrease of relative abundance over time, the dry/wet cycling did not enrich for *Gemmatimonadetes* as hypothesized. The moisture treatments were the only factors shown to be significantly different. There was no other difference between sampling time, management, or any combination of all the factors together. However, the higher relative abundance observed in the dry control samples is consistent with the results reported by DeBruyn et al. (DeBruyn et al., 2011) where the relative abundance of *Gemmatimonadetes* was

inversely correlated with moisture. *Gemmatimonadetes* accounted for half of the DGGE sequenced clones from two sites in the Atacama Desert (Drees et al., 2006). These findings continue to suggest that bacteria from the phylum *Gemmatimonadetes* are adapted to life in low moisture environments. The Aanderud and Lennon (2011) study analyzed the response of soil bacteria at 0 hours and 72 hours after rewetting with H_2^{18}O . *Gemmatimonadetes* was found to have a positive and significant response to moisture stress meaning that it produced new cells labeled with ^{18}O and increased in relative recovery upon rewetting (Aanderud and Lennon, 2011). This suggests that bacteria from the phylum *Gemmatimonadetes* are resistant to moisture stress. The results of the Aanderud and Lennon (2011) study disagreed with the findings of this study because the relative abundance of *Gemmatimonadetes* decreased after wet dry cycling. However the increase in the relative abundance of *Gemmatimonadetes* in the dry control study suggest that this phylum is resistant to moisture stress.

References

- Aanderud, Z.T., and Lennon, J.T. (2011). Validation of Heavy-Water Stable Isotope Probing for the Characterization of Rapidly Responding Soil Bacteria. *Applied and Environmental Microbiology* 77, 4589–4596.
- Anisimova, M., and Gascuel, O. (2006). Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst. Biol.* 55, 539–552.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552.
- Davinic, M., Fultz, L.M., Acosta-Martinez, V., Calderón, F.J., Cox, S.B., Dowd, S.E., Allen, V.G., Zak, J.C., and Moore-Kucera, J. (2012). Pyrosequencing and mid-infrared spectroscopy reveal distinct aggregate stratification of soil bacterial communities and organic matter composition. *Soil Biology and Biochemistry* 46, 63–72.
- Davis, K.E.R., Joseph, S.J., and Janssen, P.H. (2005). Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. *Applied and Environmental Microbiology* 71, 826–834.
- DeBruyn, J., Fawaz, M., Peacock, A., Nixon, L., Cooper, K., and Radosevich, M. (2013). *Gemmatirosa kalamazonensis* gen nov. sp. nov., a member of the rarely-cultivated bacterial phylum Gemmatimonadetes. *Journal of General and Applied Microbiology (Accepted)*.
- DeBruyn, J.M., Nixon, L.T., Fawaz, M.N., Johnson, A.M., and Radosevich, M. (2011). Global Biogeography and Quantitative Seasonal Dynamics of Gemmatimonadetes in Soil. *Applied and Environmental Microbiology* 77, 6295–6300.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36, W465–W469.
- Drees, K.P., Neilson, J.W., Betancourt, J.L., Quade, J., Henderson, D.A., Pryor, B.M., and Maier, R.M. (2006). Bacterial Community Structure in the Hyperarid Core of the Atacama Desert, Chile. *Applied and Environmental Microbiology* 72, 7902–7908.
- Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.

- Edlund, A., Nichols, P.D., Roffey, R., and White, D.C. (1985). Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *Journal of Lipid Research* 26, 982–988.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59, 307–321.
- Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.
- Janssen, P.H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719–1728.
- Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. (2002). Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology* 68, 2391–2396.
- Jiang, X., Wright, A.L., Wang, J., and Li, Z. (2011). Long-term tillage effects on the distribution patterns of microbial biomass and activities within soil aggregates. *CATENA* 87, 276–280.
- Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., and Janssen, P.H. (2003). Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Applied and Environmental Microbiology* 69, 7210–7215.
- Mummey, D., Holben, W., Six, J., and Stahl, P. (2006). Spatial stratification of soil bacterial populations in aggregates of diverse soils. *Microb. Ecol* 51, 404–411.
- Rappé, M.S., and Giovannoni, S.J. (2003). The uncultured microbial majority. *Annu. Rev. Microbiol.* 57, 369–394.
- Reasoner, D.J., and Geldreich, E.E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Applied and Environmental Microbiology* 49, 1–7.
- Schoenborn, L., Yates, P.S., Grinton, B.E., Hugenholtz, P., and Janssen, P.H. (2004). Liquid Serial Dilution Is Inferior to Solid Media for Isolation of Cultures Representative of the Phylum-Level Diversity of Soil Bacteria. *Applied and Environmental Microbiology* 70, 4363–4366.

- Serafim, L.S., Lemos, P.C., Levantesi, C., Tandoi, V., Santos, H., and Reis, M.A.M. (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Methods* 51, 1–18.
- Sexstone, A.J., Revsbech, N.P., Parkin, T.B., and Tiedje, J.M. (1985). Direct Measurement of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society Of America Journal* 49, 645–651.
- Sides, K. (2010). Agricultural Soil Bacteria; A Study of Collection, Cultivation, and Lysogeny. Master's Thesis, University of Tennessee.
- Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.* 39, 321–346.
- Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. (2004). New Strategies for Cultivation and Detection of Previously Uncultured Microbes. *Applied and Environmental Microbiology* 70, 4748–4755.
- Stewart, E.J. (2012). Growing Unculturable Bacteria. *Journal of Bacteriology* 194, 4151–4160.
- Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y., and Kamagata, Y. (2009). Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environmental Microbiology* 11, 1827–1834.
- Weis, D.S. (1975). A Medium for the Axenic Culture of Chlorella-Bearing Paramecium bursaria in the Light. *Transactions of the American Microscopical Society* 94, 109–117.
- Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., and Nakamura, K. (2003). Gemmatimonas aurantiaca gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. *International Journal Of Systematic And Evolutionary Microbiology* 53, 1155–1163.
- (2007). *Methods for general and molecular microbiology* (Washington, D.C: ASM Press).

CHAPTER III
CULTIVATION OF GEMMATIMONADETES

Abstract

Bacteria from the phylum *Gemmatimonadetes* have resisted cultivation despite their ubiquity in soil. The goal of this study was to cultivate more representatives from this phylum for future study to gain insight into their potential ecological role(s). Soil sampled from till and no-till cotton plots at the University of Tennessee Research and Education Center (REC) in Jackson, Tennessee. The soils were incubated at constant moisture for 67 days. Whole soil, macro-aggregate and micro-aggregate size fractions were used as inocula and incubated under microaerophilic (2.5% O₂ and 5% CO₂) or aerobic atmospheric conditions for 12 weeks. The plates were screened for the presence of *Gemmatimonadetes* by plate-wash PCR and all colonies on positive plates were further screened by direct PCR. Over 100 putative *Gemmatimonadetes* isolates were obtained and among these strains Gmat50, Gmat59 and Gmat410, were confirmed to be *Gemmatimonadetes* via 16S rRNA gene sequencing. Sequence analysis revealed that the 16S rRNA gene of Gmat50 and Gmat410 were 93% and 95% identical, respectively, to soil isolate Ellin5290. The closest cultivated relative of Gmat59 was Ellin7146 with an identity of 88% over the 1,289 bp 16S rRNA gene fragment analyzed. *Gemmatimonadetes* was successfully cultivated under both aeration conditions but was more frequently detected on plates incubated aerobically. Plates inoculated with tilled soil yielded more frequent detection of *Gemmatimonadetes* than plates inoculated with no-till soil. Cultivability was improved by extended incubation time but not by reduced

oxygen concentration and elevated CO₂. These results are consistent with a predominantly aerobic niche and homogenous distribution of *Gemmatimonadetes* at the aggregate scale.

Introduction

It is estimated that 0.1 to 1% of bacteria from mesotrophic and oligotrophic environments are typically enumerated using the viable plate count method– the standard for cultivating and isolating bacteria (Staley and Konopka, 1985). The use of solid media over liquid serial dilution enrichment prior to cultivation on plates has been more successful for isolating a broader range bacterial phyla and for the isolation of lineages with few cultivated representatives (Schoenborn et al., 2004).

The vast majority of bacteria in the environment resist cultivation. One possible cause is the inability to identify and provide all the essential growth factors and nutrients that are likely present in complex environments (Stewart, 2012). This includes nutrients, atmospheric conditions, pH, electron acceptors, and interactions with other organisms. Some bacteria are uncultivable because they may be out competed by other bacteria in the culture (Davis et al., 2005; Janssen et al., 2002; Joseph et al., 2003). Slow growers often go undetected due to insufficient incubation time.

Researchers have made advances in cultivating bacteria from rarely cultivated phyla and novel lineages by improving traditional cultivation methods. The use of nontraditional media, like Dilute Nutrient Broth (DNB) solidified with

gellan gum and media based on VL55, a more complete, basal salts media supplements with vitamins and growth factors, have resulted in higher viable counts and allowed the detection and isolation of members of rarely isolated taxa (Davis et al., 2005). In contrast to nutrient rich media, VL55 medium was made to mimic the lower concentration of inorganic ions in soil with sufficient ammonia and phosphate ions for bacteria to produce sufficient biomass (Davis et al., 2005). The combination of nontraditional media with an alternative solidifying agent has lead to increased cultivation of bacteria from the environment. One study was able to increase cultivation using a DNB solidified with agar and gellan gum, resulting in cultivation of 5.2% and 7.5% of mean determined microscopic cell count, respectively (Janssen et al., 2002). The gellan gum is a bacterial polysaccharide derived from *Sphingomonas* ssp. used in place of agar as a solidifying agent (Tamaki et al., 2009). In a comparison of solidifying agents, researchers found that 44% of rarely cultivated taxa only grew on gellan solidified media (Tamaki et al., 2009). It is not understood why some bacteria grew more readily on gellan than agar but Gellan has advantages as a solidifying agent at higher temperature incubations because of its higher melting point compared to agar (Tamaki et al., 2009).

It is difficult to replicate an environment as complex as the soil. One way to overcome this limitation is the use of semipermeable diffusion chambers incubated in situ that allows all the nutrients and community interactions within the environment without allowing cells to pass through the membrane (Stewart, 2012). Microscopic direct cell counts of microcolonies growing within the

chambers had recovery rates of up to 40% for the initial inoculum as opposed to standard petri plates which had a recovery rate of 0.05% (Stewart, 2012). Once microcolonies formed within the semipermeable chamber they could be further isolated and propagated by inoculating new chambers (Stewart, 2012). In cases where the bacteria are dominant in the environment yet resist cultivation; dilution to extinction can be employed (Stewart, 2012).

Extended incubation time has also shown great promise in cultivating rarely isolated bacteria. Rarely isolated groups grew significantly slower on all media types than commonly isolated groups (Davis et al., 2005). Bacteria that are rarely isolated may be naturally slow growers or have a long lag time. In one study, 10-weeks of incubation was required to reach maximum colony development (Janssen et al., 2002). Another study observed that the number of colonies continued to increase past 12 weeks on VL55 based media (Davis et al., 2005). Thirty-six percent of isolates appearing after 8 weeks belonged to rarely isolated groups (Davis et al., 2005).

Co-culture dependent bacteria have evolved with the bacteria they depend on. To separate dependent bacteria the factor causing the dependency must first be discovered (Stewart, 2012). The dependency factor like a siderophore can be incorporated into the growth medium allowing the bacteria to be isolated. Changing the iron source from oxidized iron (Fe^{3+}) to soluble iron (Fe^{2+}) allows bacteria to bypass the use of specific siderophores and has resulted in the isolation of many rare bacteria (Stewart, 2012).

The objective of this study was to examine factors that may influence the cultivability of the phylum *Gemmatimonadetes* to gain further insight to their potential ecological role(s) in terrestrial ecosystems. Based on previous molecular surveys along moisture gradients and aggregate scale distribution studies, as well as characteristics of strain KBS708, a *Gemmatimonadetes* strain described in Chapter 1, it was hypothesized that plates incubated under microaerophilic atmospheric conditions and inner-aggregate fractions used as inocula would enhance the cultivability of phylum *Gemmatimonadetes*.

Methods

Cultivation conditions

Samples taken on day 67 (the conclusion of the experiment) from aggregate fractionation experiments described in chapter II were also used for cultivation studies. VL55 media with peptone (0.25 g/ L) amendment and 1.5% noble agar was prepared and amended with cyclohexamide to inhibit fungal growth. The plates were inoculated in triplicate with 100 μ l from the 10^{-4} , 10^{-5} , and 10^{-6} dilutions. The spread plates were incubated at 25°C for 12 weeks under microaerophilic (2.5% O₂ and 5% CO₂) or aerobic conditions resulting in a total of 56 initial primary spread plates.

Plate wash-PCR

After incubation, plate-wash PCR was performed on one plate from each group of triplicates (Stevenson et al., 2004). This was achieved by flooding plates

with 2 mL of MicroBead Solution from the MoBio Ultraclean[®] Microbial DNA Extraction Kit, followed by swabbing the agar surface with sterile cotton swabs to suspend bacteria from the colonies and 500 µL of the resulting cell suspensions were placed into MicroBead Tubes from the MoBio UltraClean[®] Microbial DNA Isolation Kit, according to the manufactures protocol. PCR of DNA extracts was performed using the *Gemmatimonadetes* specific primer G1G3-637f (5'-GAATGACGTAGAGATCC-3') and universal 16S rRNA gene primer 907r (5'-CCGTCAATTCMTTTRAGTTT-3') to identify the plates that had colonies from clade 1 and/or 3 of the phylum *Gemmatimonadetes* (see Chapter II). The PCR protocol consisted of an initial heating at 95°C for 30 seconds followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 53°C for 20 seconds and extension at 72°C for 20 seconds, and a final extension step at 72°C for 3 minutes. Positive amplification was determined by visualization PCR products using agarose gel electrophoresis. Gels with bands located between 300-200 base pairs like the positive control prepared from KBS708, indicated the samples originated from plates with positive *Gemmatimonadetes* colonies.

Isolation and identification

Replicate plates of those plates that tested positive for the presence of *Gemmatimonadetes* were selected as candidates to further isolate new strains of *Gemmatimonadetes*. Individual colonies were given a strain number (cataloged), picked and inoculated into single wells of a 96-well plate containing VL55 media supplemented with peptone and incubated under aerobic or microaerophilic

conditions accordingly. Direct PCR of the resulting cultures was preformed when sufficient growth was observed in all of the wells. This was achieved by adding 12.5 µL of Takara Premix Taq, 1 µL of 10 µM G1G3-637f primer, 1 µL of 10 µM 907r primer, 5.5 µL of water, and 5 µL of liquid culture. The reaction conditions consisted of heating at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 5 minutes.

Positive wells and the respective original colony were isolated on VL55 1.5% noble agar plates amended with peptone and cyclohexamide. Isolates were allowed to incubate up to 12 weeks and subsequently screened with additional direct PCR using the *Gemmatimonadetes* specific primers. When co-cultures were found during the incubation, they were separated onto different plates until each isolate appeared pure. Each colony grown was described in detail by the size, color, form, elevation, margin, surface, and opacity under the microscope and to the naked eye. This information was logged into a spreadsheet and used to differentiate the previously screened bacteria.

Gemmatimonadetes positive isolates underwent direct PCR using 1 µL each of the universal 16S rRNA primers (10 µM 8R and 10 µM 536R), 25 µL of Takara Premix Taq, 5 µL of culture, and 18 µL of water. The reaction consisted of 95°C for 10 minutes followed by 40 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 90 seconds, ending in 72°C for 10 minutes. The reaction was confirmed successful by the presence of a band about 500 base pairs long as determined using gel electrophoresis. The PCR product was then cleaned

using the Wizard[®] SV Gel and PCR Clean-Up System (Promega) according to manufactures protocols and sequenced at the University of Tennessee Molecular Biology Recourse Facility. Confirmation of the isolation of a member of the phylum *Gemmatimonadetes* was conducted by a BLAST homology search through the National Center for Biotechnology Information.

Isolates were grown in liquid culture under aerobic conditions, preserved in 15% glycerol, and stored at -80°C. DNA was extracted from the isolates using the MoBio UltraClean[®] Microbial DNA Isolation Kit according to the manufactures protocol. The full 16S rRNA gene was amplified using universal primers, 8F and 1392R 25 µL of Takara Premix Taq, 5 µL of culture, and 18 µL of water. The reaction consisted of 95°C for 10 minutes followed by 40 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 90 seconds, ending in 72°C for 10 minutes. The PCR product was then cloned using the Promega pGEM[®]-T Easy Vector System according to the manufactures protocol. The plasmid was isolated using the Promega Wizard[®] SV Minipreps DNA Purification System.

Phylogentic tree

Full 16S rRNA sequences from the isolates were compiled other known isolates and 181 uncultured sequences greater than 1200 bp using the Browser from the Ribosomal Database Project from Michigan State University. *E. Coli* was used as the out-group for the phylogenetic tree. All sequences were uploaded into the “One Click” phylogeny analysis program on the Phylogeny.fr website. The alignment was done by MUSCLE program (Dereeper et al., 2008;

Edgar, 2004). The Gblocks program was used to remove poorly aligned positions and divergent regions (Castresana, 2000; Dereeper et al., 2008; Edgar, 2004). Maximum-Likelihood phylogeny with bootstrapping was used to assemble the phylogenetic tree (Anisimova and Gascuel, 2006; Castresana, 2000; Dereeper et al., 2008; Edgar, 2004; Guindon et al., 2010).

New Isolates

Isolates were inoculated in triplicate in VL55 liquid medium with peptone amendment and incubated at room temperature and shaking at 120 rpm. Cell growth was measured by absorbance at 600 nm and plotted over time to determine growth rate. The growth curve was plotted as absorbance over time. To determine the growth rate constant, the logarithm of the absorbance was plotted over time and the slope was taken.

Results

Plate wash PCR with *Gemmatimonadetes* specific primers indicated that *Gemmatimonadetes* was present on 21 of 36 plates (Table 3).

Gemmatimonadetes was positively detected more often on plates inoculated with tilled soil than plates made from no-till soil. There was no difference in the total number of colonies on the plates based soil tillage management. Aerobic incubation had more positively identified plates than those incubated under microaerophilic conditions. There were a greater number of total colonies on plates incubated under aerobic conditions than on plates incubated under microaerophilic conditions.

Table 3 Plate wash PCR with *Gemmatimonadetes* specific primers. Positive (+) indicates that *Gemmatimonadetes* colonies were present on the plate. Negative (-) indicates that no reaction occurred.

Incubation	Management	Aggregate Size	Dilution Series		
			1×10^{-4}	1×10^{-5}	1×10^{-6}
Microaerophilic	Till	Whole	-	+	+
		Macro	+	-	+
		Micro	-	+	-
	No-Till	Whole	-	-	-
		Macro	+	+	-
		Micro	+	-	-
Aerobic	Till	Whole	+	+	+
		Macro	+	+	+
		Micro	+	+	-
	No-Till	Whole	+	-	-
		Macro	+	-	-
		Micro	+	+	+

The trends seen in plate wash PCR testing were also seen during the screening of individual colonies on the replicate plates (Table 4). On average, there were more positively identified colonies on aerobic plates than on microaerophilic plates (11.8% and 6.3%, respectively). Plates inoculated with tilled soil had more positive colonies than plates inoculated with no-till soil (9.7% and 7.0%, respectively).

Table 4 Direct PCR screening of all colonies with *Gemmatimonadetes* specific primers. Total colonies are the total colonies on the plate. Total G1G3 is the total number of colonies that amplified using the *Gemmatimonadetes* specific primers. %G1G3 detected is the percentage of Total G1G3 divided by total colonies. ND, not determined.

Incubation	Management	Aggregate Size	Dilution	Plate	Total Colonies	Total G1G3	%G1G3 Detected
Microaerophilic	Till	Whole	1x10 ⁻⁵	B	91	0	0
				C	88	3	3
			1x10 ⁻⁶	B	12	1	8
				C	18	2	11
		Macro	1x10 ⁻⁶	B	20	6	30
				C	18	1	6
		Micro	1x10 ⁻⁵	B	100	2	2
				C	114	0	0
	No-Till	Macro	1x10 ⁻⁵	B	78	1	1
				C	60	1	2
Aerobic	Till	Whole	1x10 ⁻⁵	E	92	4	4
				F	117	0	0
			1x10 ⁻⁶	E	17	1	6
				F	31	1	3
		Macro	1x10 ⁻⁵	E	114	ND	ND
				F	135	14	11
			1x10 ⁻⁶	E	35	7	20
				F	35	4	11
		Micro	1x10 ⁻⁵	E	117	52	44
				F	109	7	6
	No-Till	Micro	1x10 ⁻⁵	E	200	22	11
				F	117	ND	ND
			1x10 ⁻⁶	E	>200	ND	ND
				F	29	4	14

Positively identified bacteria were picked directly from the original spread plate for microaerophilic growth instead of inoculating from the positive well that the *Gemmatimonadetes* was detected in from 96-well plate. Many of the microaerophilic cultures were difficult to purify, occurring predominantly as co-cultures, of *Gemmatimonadetes* growing in the presence of a yellow bacterium or a spreading iridescent bacterium. A majority of the putative cultures were lost when attempting to isolate a pure culture. After some time fungal contamination became a primary concern. Thus, 2 mg/L of cyclohexamide was used to inhibit fungal growth. A number of plates were ultimately lost to fungal contamination. It seemed that some fungi were resistant to cyclohexamide, because the plates had fungal growth present within two days of inoculation. The cyclohexamide may have also been degraded by the light and was ineffective at inhibiting fungal growth; therefore, the plates were stored in the dark to prevent photodegradation.

Of the remaining plates, four putative *Gemmatimonadetes* isolates were brought into culture under microaerophilic conditions. The one characteristic that the isolates had in common was their pink color, which set them apart from the other bacteria on the plate. In other cases there were multiple pink colonies, a dissecting microscope was used to determine differences between colonies that appeared to be the same to the naked eye. The way the light passed through colonies that appeared the same to the naked eye looked like different colors under the microscope.

Isolate Gmat50 was isolated from tilled soil macro-aggregates at the 10^{-6} dilution incubated under microaerophilic atmospheric conditions. The strain Gmat50 was 93% identical based on the 1,224 bp 16S rRNA gene fragment to its nearest cultivated relative Ellin5290 isolated from a rotationally grazed pasture soils at the Dairy Research Institute, Ellinbank, Victoria Australia (Joseph et al., 2003). The natural log of growth rate constant was 0.22.

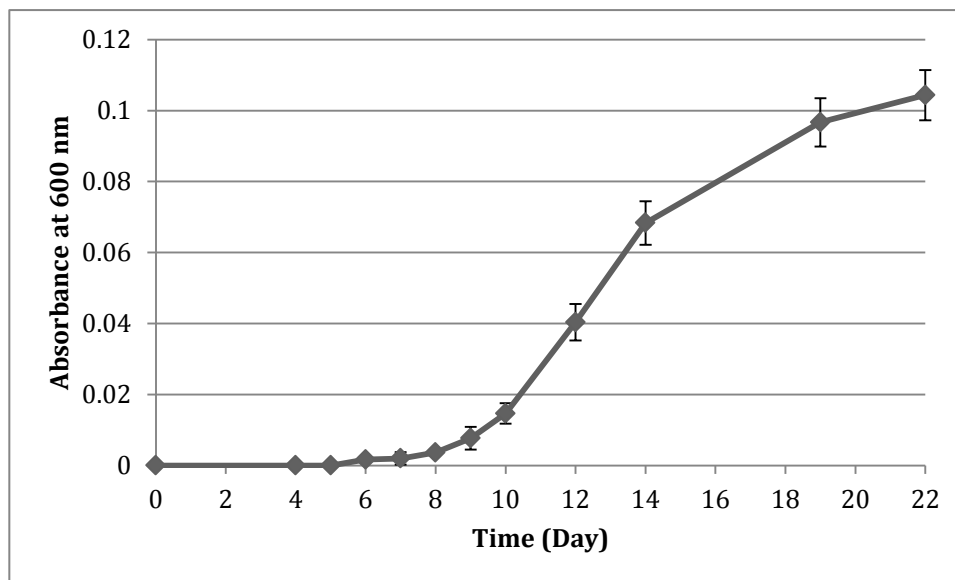


Figure 20 Growth curve of strain Gamt50 in VL55 liquid medium with peptone amendment under aerobic conditions on a shaking incubator at 120 rpm. Each point shows the mean and standard deviation of triplicate cultures.

Gmat59 was isolated from plates originally inoculated with macro aggregate size fraction of conventionally managed (tilled) soil and incubated at room temperature under microaerophilic conditions. Based on the 1,313 bp

sequence of the 16S rRNA gene, strain Gmat59 is 89% identical to its nearest cultivated relative Ellin7146 isolated from a control paddock soil at the Dairy Research Institute, Ellinbank, Victoria, Australia (Davis et al., 2005). Strain Gmat59 had the fastest growth rate of the isolates, reaching full growth in liquid culture in 12 days (Figure 17). The natural log of growth rate constant is 0.53.

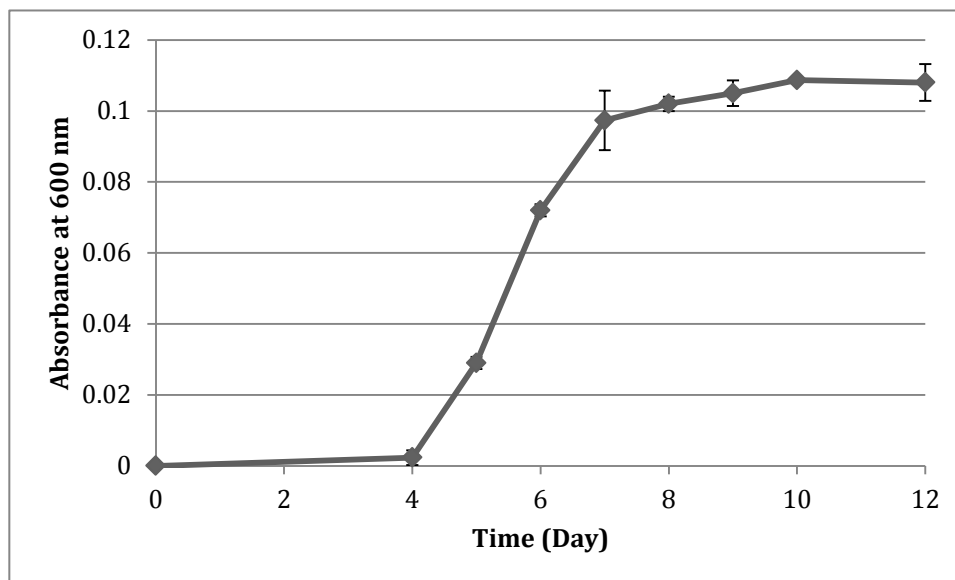


Figure 21 Growth curve of strain Gamt59 in VL55 liquid medium with peptone amendment under aerobic conditions on a shaking incubator at 120 rpm. Each point shows the mean and standard deviation of triplicate cultures.

Gmat410 was isolated from plates inoculated with the micro-aggregate size fraction of conventionally managed (tilled) soil and incubated at room temperature under microaerophilic conditions. Isolate Gmat410 appeared pink in liquid culture. Strain Gmat410 had a max identity of 95% to its nearest cultivated

relative Ellin5290 isolated from a rotationally grazed pasture soils at the Dairy Research Institute, Ellinbank, Victoria Australia (Joseph et al., 2003). Strain Gmat50 had a similar growth rate to Gmat410, but Gmat410 grew to a greater cell density (Figure 16 and Figure 18). The natural log of growth rate constant is 0.23.

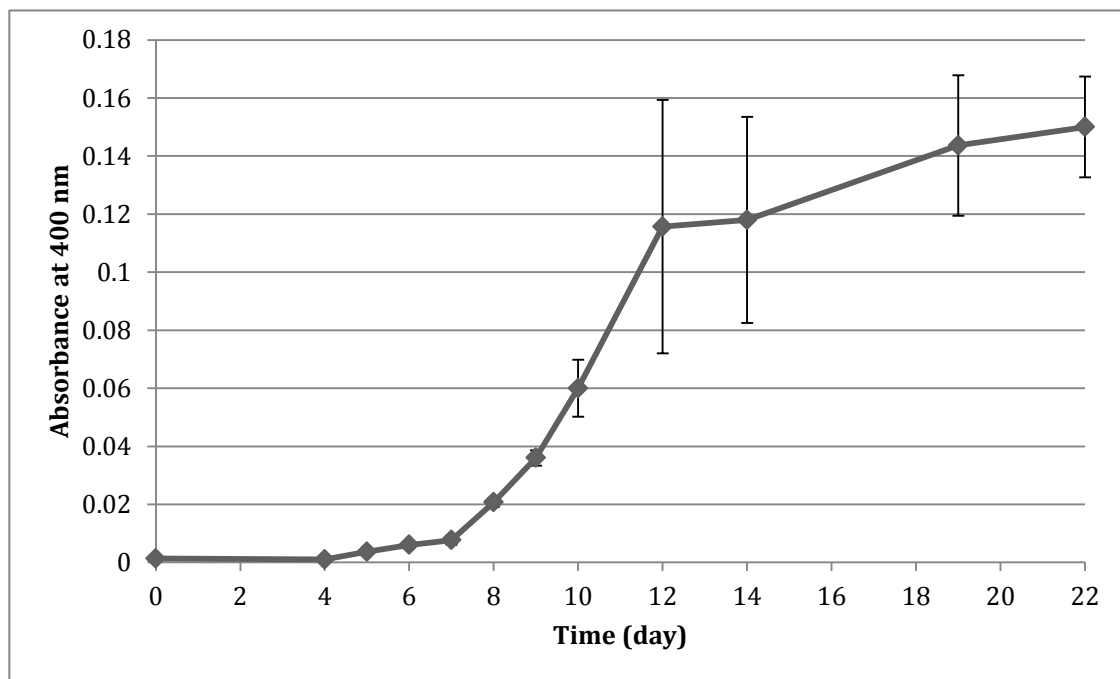


Figure 22 Growth curve of strain Gamt410 in VL55 liquid medium with peptone amendment under aerobic conditions on a shaking incubator at 120 rpm. Each point shows the mean and standard deviation of triplicate cultures.

Gmat210 isolated was from plates originally inoculated with conventionally managed (tilled) soil and incubated at room temperature under microaerophilic

conditions. This was the only isolate of the four that flocculated in liquid culture, but not to the same extent as KBS708.

Phylogenetic analysis of the isolates that were sequenced places all of them in the same clade as the previously cultured members of this phylum (Figure 19). The new isolates are less divergent from the Ellin5220, Ellin5290, and Ellin7146 isolates than the other isolates in this phylum.

Aerobic 96-well plates took longer to incubate for enough growth to test using direct PCR than microaerophilic. The positively detected wells were closely clustered on the plates suggested there may have been some false positives due to cross-contamination of the wells. Fungal contamination was evident in many of the aerobically incubated plates so the concentration of cyclohexamide was increased from 2 mg/L to 100 mg/L. Aerobic isolates were screened as they grew to rule out the fast growing bacteria first and then slower growing isolates as they grew.

Discussion

Despite acquisition of four new isolates from microaerophilically incubated plates, it appeared that microaerophilic conditions did not enhance the overall cultivation of bacteria from the phylum *Gemmatimonadetes*. On average, there were more positively identified colonies on aerobic plates than on microaerophilic plates (11.8% and 6.3% of total colonies, respectively). Plates inoculated with till soil had more positive colonies than plates inoculated with no-

till soil (9.7% and 7.0% of the total colonies, respectively). Tillage treatment was not found to be a statistically significant factor in the distribution of *Gemmatimonadetes* in soils based on molecular detection, but it appears to be an important factor for cultivability. This may be because *Gemmatimonadetes* is more accessible in tilled soil than in no-till soil which may give clues as to where this bacteria lives in soil.

Gemmatimonas aurantiaca T-27, the first cultivated member of the *Gemmatimonadetes*, was isolated from activated sludge in an anaerobic-aerobic batch reactor and was discovered to possess phosphorous removal capabilities via intra-cellular accumulation of polyphosphate (Zhang et al., 2003). The first soil strains (Ellin5220, Ellin5301 and Ellin7146) were isolated from rotationally grazed pasture at the Dairy Research Institute, Ellinbank, Victoria, Australia (Joseph et al., 2003). This study used sonication in addition to VL55 based media and extended incubation time to enhance the overall cultivability of soil bacteria yielding three new strains of *Gemmatimonadetes* (Joseph et al., 2003). Davis et al. (2005) examined the influence of growth media, inoculum size, and incubation time on the cultivation and isolation of soil bacteria. They found that members of rarely isolated groups appeared mostly after 2 months of incubation (Davis et al., 2005). The growth medium VL55 and dilute nutrient broth were found to be the best choices for isolating soil bacteria (Davis et al., 2005). Isolate Ellin5290 was isolated on dilute nutrient broth medium with gellan solidifying

agent from a control paddock at the Dairy Research Institute, Ellinbank, Victoria, Australia (Davis et al., 2005).

The broader ecological role of bacteria within the phylum *Gemmatimonadetes* remains an open question. The research set out to assess the aggregate scale distribution, aeration requirements, and resilience to moisture stress of *Gemmatimonadetes* using culture-dependent and – independent approaches. In this study, extended incubation time, microaerophilic conditions, and aggregate size distribution were the primary variables that were adjusted to improve the cultivability of *Gemmatimonadetes*. Based on the results of this research and previous work of others, we can conclude that the phylum is diverse, perhaps as diverse as the *Proteobacteria* and *Actinomycetes*, and includes slow-growing heterotrophic bacteria with versatile metabolism with respect to the range of organic substrates utilized as carbon and energy sources. Efforts to cultivate new members of the phylum yielded aerobes and microaerophilic members but neither facultative nor obligate anaerobes were identified. KBS 708 and *Gemmatimonas aurantiaca* T-27 have very large genome sizes with rather high GC-content but currently it is not known if these are common traits throughout the phylum. DeBruyn et al. (2011) determined that all environmental sequences from a wide array of environments and the limited number of sequences from the cultivated strains form three distinct clades within the phylum *Gemmatimonadetes*. During this study, four new members of the phylum were isolated and determined to be phylogenetically

distinct from those previously reported though all currently cultivated strains lie within clade 1 of the phylum. At present there are no known cultivated strains within clades 2 and 3. Like strain KBS708, these new strains all exhibited slow growth rates and a pink color, and in one case a flocculating growth habit in liquid culture. Further characterization of these isolates will undoubtedly lead to a better understanding of the genetic potential and the ecological role of the phylum and possibly provide clues to improve isolation techniques.

The aggregate scale distribution of *Gemmatimonadetes* remains unclear though some evidence suggesting an enrichment in the relative abundance within intra-aggregate spaces was obtained consistent with the previous findings of Mummey et al. (2003). This is also in keeping with the notion that some members are microaerophilic. Though some microaerophiles or at least microaerotolerant, strains were obtained, overall the cultivation studies did not indicate that microaerophily was a predominant lifestyle among members of the phylum. Moisture manipulation via wet/dry cycling resulted in a clear decrease in the relative abundance of *Gemmatimonadetes* in soil suggesting an inability or lack of resilience to rapid changes in moisture content. These findings were in contrast to those of Aanderud and Lennon (2011) that showed *Gemmatimonadetes* responded rather quickly to addition of moisture to dry soils. However, soils held in an air-dried state for extended periods of time exhibited consistently higher relative abundances of *Gemmatimonadetes* than in soils held at 75% of field capacity suggesting members of the phylum are well adapted to

resist moisture limitation. These findings agree well with those of Drees et al. (2006) that showed a consistent increase in the relative abundance of *Gemmatimonadetes* along a moisture gradient increasing from wet to dry in the Atacama Desert in Chile.

In conclusion, the results presented in this thesis represent the first detailed investigation of the ecology of terrestrial *Gemmatimonadetes*. The large collection of isolates obtained is a significant contribution to this end and will provide a strong foundation upon which future studies can be built.

References

- Aanderud, Z.T., and Lennon, J.T. (2011). Validation of Heavy-Water Stable Isotope Probing for the Characterization of Rapidly Responding Soil Bacteria. *Applied and Environmental Microbiology* 77, 4589–4596.
- Anisimova, M., and Gascuel, O. (2006). Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst. Biol.* 55, 539–552.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552.
- Davinic, M., Fultz, L.M., Acosta-Martinez, V., Calderón, F.J., Cox, S.B., Dowd, S.E., Allen, V.G., Zak, J.C., and Moore-Kucera, J. (2012). Pyrosequencing and mid-infrared spectroscopy reveal distinct aggregate stratification of soil bacterial communities and organic matter composition. *Soil Biology and Biochemistry* 46, 63–72.
- Davis, K.E.R., Joseph, S.J., and Janssen, P.H. (2005). Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. *Applied and Environmental Microbiology* 71, 826–834.
- DeBruyn, J., Fawaz, M., Peacock, A., Nixon, L., Cooper, K., and Radosevich, M. (2013). *Gemmatirosa kalamazoonensis* gen nov. sp. nov., a member of the rarely-cultivated bacterial phylum Gemmatimonadetes. *Journal of General and Applied Microbiology (Accepted)*.
- DeBruyn, J.M., Nixon, L.T., Fawaz, M.N., Johnson, A.M., and Radosevich, M. (2011). Global Biogeography and Quantitative Seasonal Dynamics of Gemmatimonadetes in Soil. *Applied and Environmental Microbiology* 77, 6295–6300.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36, W465–W469.
- Drees, K.P., Neilson, J.W., Betancourt, J.L., Quade, J., Henderson, D.A., Pryor, B.M., and Maier, R.M. (2006). Bacterial Community Structure in the Hyperarid Core of the Atacama Desert, Chile. *Applied and Environmental Microbiology* 72, 7902–7908.
- Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.

- Edlund, A., Nichols, P.D., Roffey, R., and White, D.C. (1985). Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *Journal of Lipid Research* 26, 982–988.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59, 307–321.
- Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.
- Janssen, P.H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719–1728.
- Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. (2002). Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology* 68, 2391–2396.
- Jiang, X., Wright, A.L., Wang, J., and Li, Z. (2011). Long-term tillage effects on the distribution patterns of microbial biomass and activities within soil aggregates. *CATENA* 87, 276–280.
- Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., and Janssen, P.H. (2003). Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Applied and Environmental Microbiology* 69, 7210–7215.
- Mummey, D., Holben, W., Six, J., and Stahl, P. (2006). Spatial stratification of soil bacterial populations in aggregates of diverse soils. *Microb. Ecol* 51, 404–411.
- Rappé, M.S., and Giovannoni, S.J. (2003). The uncultured microbial majority. *Annu. Rev. Microbiol.* 57, 369–394.
- Reasoner, D.J., and Geldreich, E.E. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Applied and Environmental Microbiology* 49, 1–7.
- Schoenborn, L., Yates, P.S., Grinton, B.E., Hugenholtz, P., and Janssen, P.H. (2004). Liquid Serial Dilution Is Inferior to Solid Media for Isolation of Cultures Representative of the Phylum-Level Diversity of Soil Bacteria. *Applied and Environmental Microbiology* 70, 4363–4366.

- Serafim, L.S., Lemos, P.C., Levantesi, C., Tandoi, V., Santos, H., and Reis, M.A.M. (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Methods* 51, 1–18.
- Sexstone, A.J., Revsbech, N.P., Parkin, T.B., and Tiedje, J.M. (1985). Direct Measurement of Oxygen Profiles and Denitrification Rates in Soil Aggregates. *Soil Science Society Of America Journal* 49, 645–651.
- Sides, K. (2010). Agricultural Soil Bacteria; A Study of Collection, Cultivation, and Lysogeny. Master's Thesis, University of Tennessee.
- Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.* 39, 321–346.
- Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. (2004). New Strategies for Cultivation and Detection of Previously Uncultured Microbes. *Applied and Environmental Microbiology* 70, 4748–4755.
- Stewart, E.J. (2012). Growing Unculturable Bacteria. *Journal of Bacteriology* 194, 4151–4160.
- Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y., and Kamagata, Y. (2009). Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environmental Microbiology* 11, 1827–1834.
- Weis, D.S. (1975). A Medium for the Axenic Culture of Chlorella-Bearing Paramecium bursaria in the Light. *Transactions of the American Microscopical Society* 94, 109–117.
- Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., and Nakamura, K. (2003). Gemmatimonas aurantiaca gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. *International Journal Of Systematic And Evolutionary Microbiology* 53, 1155–1163.
- (2007). *Methods for general and molecular microbiology* (Washington, D.C: ASM Press).

CONCLUSION

The relative abundance of *Gemmatimonadetes* was greater in whole soil than in micro- or macro-aggregates. These results are difficult to interpret. For the relative abundance to decrease in the inter-aggregate fractions suggests the *Gemmatimonadetes* were preferentially lost during the fractionation process or other taxa were enriched in the aggregate fractions. Thus, the aggregate-scale distribution of *Gemmatimonadetes* remains an open question and further investigation will be needed. *Gemmatimonadetes* were not enriched by dry/wet cycling treatments. It was believed that *Gemmatimonadetes* would be resilient to moisture stress; however, constant moisture conditions were preferred over moisture cycling conditions. The relative abundance of *Gemmatimonadetes* was highest in the dry control soils. The higher relative abundance observed in the dry control samples is consistent with the results reported by DeBruyn et al. (2011) where the relative abundance of *Gemmatimonadetes* was inversely correlated with moisture. These results suggest that *Gemmatimonadetes* are well adapted to persist in arid conditions.

The cultivation study was largely successful due to the cultivation of four new isolates under microaerophilic incubation. In addition, 113 putative isolates under aerobic incubation remain to be confirmed and characterized. Although the cultivation of isolates was not completed, observation of plate growth characteristics was informative. For example, many of the pink colonies examined resulted in positive amplification of 16S rRNA gene fragments using

Gemmatimonadetes-specific primers suggesting that production of this pink pigment maybe a widely conserved phenotype among members of *Gemmatimonadetes*. Plates incubated under microaerophilic conditions did not appear to enhance the cultivability, but aerobic incubation and inoculation from tilled soil seemed to result in more frequent cultivation of *Gemmatimonadetes*. The future characterization of these new isolates will lead to an improved understanding of the role of this phylum in the soil ecosystem, and it will broaden the approaches used to cultivate *Gemmatimonadetes*.

APPENDIX

Table 5 Aggregate size analysis relative abundance of *Gemmatimonadetes* based on qPCR analysis with *Gemmatimonadetes* specific primers and universal 16S rRNA primers.

Management	Aggregate	Sample	Time (days)	G1G3 SQ Mean	16S SQ Mean	% G1G3
No-Till	Whole	1	0	7.86E+04	8.46E+05	9.29
No-Till	Whole	2	0	4.80E+04	8.10E+05	5.92
No-Till	Whole	3	0	1.38E+05	1.58E+06	8.72
No-Till	Whole	1	17	1.20E+05	1.48E+06	8.11
No-Till	Whole	2	17	1.90E+05	2.26E+06	8.40
No-Till	Whole	3	17	2.72E+05	4.09E+06	6.65
No-Till	Whole	1	43	1.47E+05	1.50E+06	9.84
No-Till	Whole	2	43	2.07E+05	1.99E+06	10.37
No-Till	Whole	3	43	3.49E+05	3.87E+06	9.03
No-Till	Whole	1	66	8.56E+04	1.08E+06	7.89
No-Till	Whole	2	66	1.12E+05	1.35E+06	8.24
No-Till	Whole	3	66	1.64E+05	1.68E+06	9.76
Till	Whole	1	0	6.37E+05	6.28E+06	10.14
Till	Whole	2	0	1.45E+05	1.53E+06	9.46
Till	Whole	3	0	2.69E+05	2.71E+06	9.91
Till	Whole	1	17	3.56E+05	2.69E+06	13.25
Till	Whole	2	17	6.29E+05	4.01E+06	15.69
Till	Whole	3	17	4.34E+05	2.56E+06	16.96
Till	Whole	1	44	7.62E+05	5.31E+06	14.34
Till	Whole	2	44	3.55E+05	3.86E+06	9.19
Till	Whole	3	44	2.92E+05	3.60E+06	8.10
Till	Whole	1	67	7.13E+04	9.15E+05	7.79
Till	Whole	2	67	6.97E+04	8.12E+05	8.59
Till	Whole	3	67	1.11E+05	1.38E+06	8.04
No-Till	Macro	1	0	1.88E+05	2.73E+06	6.87
No-Till	Macro	2	0	2.10E+05	4.97E+06	4.22
No-Till	Macro	3	0	1.41E+05	3.49E+06	4.05
No-Till	Macro	1	43	2.00E+05	3.05E+06	6.55
No-Till	Macro	2	43	1.86E+05	3.64E+06	5.11
No-Till	Macro	3	43	1.70E+05	3.00E+06	5.66
No-Till	Macro	1	66	7.37E+04	1.39E+06	5.29
No-Till	Macro	2	66	7.31E+04	7.24E+05	10.09
No-Till	Macro	3	66	3.65E+04	1.58E+06	2.31
Till	Macro	1	0	4.67E+05	7.99E+06	5.85
Till	Macro	2	0	2.37E+05	4.10E+06	5.79

Table 5 continued. ND is not determined.

Management	Aggregate	Sample	Time (days)	G1G3 SQ Mean	16S SQ Mean	%G1G3
Till	Macro	3	0	3.58E+05	5.26E+06	6.81
Till	Macro	1	44	2.73E+05	4.12E+06	6.64
Till	Macro	2	44	2.17E+05	3.91E+06	5.56
Till	Macro	3	44	1.89E+05	3.79E+06	4.97
Till	Macro	1	67	1.06E+05	2.08E+06	5.09
Till	Macro	2	67	6.46E+04	1.20E+06	5.39
Till	Macro	3	67	9.25E+04	1.82E+06	5.1
No-Till	Micro	1	0	8.02E+04	2.32E+06	3.46
No-Till	Micro	2	0	9.65E+04	2.10E+06	4.59
No-Till	Micro	3	0	1.07E+05	2.63E+02	40521
No-Till	Micro	1	43	1.26E+05	2.41E+06	5.23
No-Till	Micro	2	43	2.17E+05	3.16E+06	6.87
No-Till	Micro	3	43	2.38E+05	4.08E+06	5.83
No-Till	Micro	1	66	3.12E+04	6.94E+05	4.49
No-Till	Micro	2	66	4.44E+04	1.00E+06	4.44
No-Till	Micro	3	66	6.93E+04	1.33E+06	5.22
Till	Micro	1	0	2.13E+05	2.05E+06	10.4
Till	Micro	2	0	1.90E+05	2.58E+06	7.35
Till	Micro	3	0	3.20E+05	4.47E+06	7.16
Till	Micro	1	44	2.92E+05	5.23E+06	5.59
Till	Micro	2	44	6.33E+04	0.00E+00	ND
Till	Micro	3	44	2.19E+05	3.08E+06	7.12
Till	Micro	1	67	5.42E+04	6.56E+05	8.26
Till	Micro	2	67	4.70E+04	5.00E+05	9.4
Till	Micro	3	67	5.95E+04	8.81E+05	6.75

Table 6 Aggregate size analysis Type 3 Test of Fixed Effects of all factors excluding analysis of day 17.

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Management	1	34	7.09	0.0117
Aggregate	2	34	25.05	<.0001
Management*Aggregate	2	34	2.96	0.0655
Time	2	34	0.62	0.5436
Management*Time	2	34	1.98	0.1541
Aggregate*Time	4	34	0.67	0.6183
Management*Aggregate*Time	4	34	0.78	0.5432

Table 7 Aggregate size analysis Effect=Management Method=LSD (P<0.05) Set=1 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
1	No-Till		—	6.4209	0.3125	B
2	Till		—	7.5980	0.3125	A

Table 8 Aggregate size analysis Effect=Aggregate Method=LSD (P<0.05) Set=2 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
3		Macro	—	5.6306	0.3725	B
4		Micro	—	6.2522	0.4023	B
5		Whole	—	9.1456	0.3725	A

Table 9 Aggregate size analysis Effect=Management*Aggregate Method=LSD (P<0.05) Set=3 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
6	No-Till	Macro	—	5.5722	0.5268	C
7	No-Till	Micro	—	4.9061	0.5690	C
8	No-Till	Whole	—	8.7844	0.5268	AB
9	Till	Macro	—	5.6889	0.5268	C
10	Till	Micro	—	7.5983	0.5690	B
11	Till	Whole	—	9.5067	0.5268	A

Table 10 Aggregate size analysis Effect=Time Method=LSD (P<0.05) Set=4 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
12			0	6.8897	0.3877	A
13			44	7.3531	0.3877	A
14			67	6.7856	0.3725	A

Table 11 Aggregate size analysis Effect=Management*Time Method=LSD (P<0.05) Set=5 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
15	No-Till		0	5.6828	0.5690	C
16	No-Till		44	7.1656	0.5268	ABC
17	No-Till		67	6.4144	0.5268	BC
18	Till		0	8.0967	0.5268	A
19	Till		44	7.5406	0.5690	AB
20	Till		67	7.1567	0.5268	ABC

Table 12 Aggregate size analysis Effect=Aggregate*Time Method=LSD (P<0.05) Set=6 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
21		Macro	0	5.5983	0.6452	B
22		Macro	44	5.7483	0.6452	B
23		Macro	67	5.5450	0.6452	B
24		Micro	0	6.1642	0.7213	B
25		Micro	44	6.1658	0.7213	B
26		Micro	67	6.4267	0.6452	B
27		Whole	0	8.9067	0.6452	A
28		Whole	44	10.1450	0.6452	A
29		Whole	67	8.3850	0.6452	A

Table 13 Aggregate size analysis Effect=Management*Aggregate*Time Method=LSD (P<0.05)
Set=7 excluding analysis of day 17.

Obs	Management	Aggregate	Time	Estimate	Standard Error	Letter Group
30	No-Till	Macro	0	5.0467	0.9124	D
31	No-Till	Macro	44	5.7733	0.9124	CD
32	No-Till	Macro	67	5.8967	0.9124	CD
33	No-Till	Micro	0	4.0250	1.1175	D
34	No-Till	Micro	44	5.9767	0.9124	CD
35	No-Till	Micro	67	4.7167	0.9124	D
36	No-Till	Whole	0	7.9767	0.9124	ABC
37	No-Till	Whole	44	9.7467	0.9124	A
38	No-Till	Whole	67	8.6300	0.9124	AB
39	Till	Macro	0	6.1500	0.9124	BCD
40	Till	Macro	44	5.7233	0.9124	CD
41	Till	Macro	67	5.1933	0.9124	D
42	Till	Micro	0	8.3033	0.9124	ABC
43	Till	Micro	44	6.3550	1.1175	BCD
44	Till	Micro	67	8.1367	0.9124	ABC
45	Till	Whole	0	9.8367	0.9124	A
46	Till	Whole	44	10.5433	0.9124	A
47	Till	Whole	67	8.1400	0.9124	ABC

Table 14 Aggregate size analysis Type 3 Test of Fixed Effects of whole soil only at all time points.

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Management	1	50	18.12	<.0001
Time	3	50	7.41	0.0003
Management*Time	3	50	4.22	0.0097

Table 15 Aggregate size analysis Effect=Management Method=LSD (P<0.05) Set=1 whole soil only at all time points.

Obs	Management	Time	Estimate	Standard Error	Letter Group
1	No-Till	—	6.7975	0.4590	B
2	Till	—	9.5605	0.4590	A

Table 16 Aggregate size analysis Effect=Time Method=LSD (P<0.05) Set=2 whole soil only at all time points.

Obs	Management	Time	Estimate	Standard Error	Letter Group
3		0	6.9933	0.5407	B
4		17	11.5100	0.9086	A
5		44	7.4272	0.5407	B
6		67	6.7856	0.5246	B

Table 17 Aggregate size analysis Effect=Management*Time Method=LSD (P<0.05) Set=3 whole soil only at all time points.

Obs	Management	Time	Estimate	Standard Error	Letter Group
7	No-Till	0	5.8900	0.7869	C
8	No-Till	17	7.7200	1.2850	BC
9	No-Till	44	7.1656	0.7419	BC
10	No-Till	67	6.4144	0.7419	BC
11	Till	0	8.0967	0.7419	B
12	Till	17	15.3000	1.2850	A
13	Till	44	7.6888	0.7869	BC
14	Till	67	7.1567	0.7419	BC

Table 18 Moisture study table of the relative abundance of *Gemmatimonadetes* based on qPCR analysis with *Gemmatimonadetes* specific primers and universal 16S rRNA primers.

Management	Treatment	Sample	Time (days)	%G1G3	Corrected %G1G3
Till	Dry Wet	1	0	0.084	2.98
Till	Dry Wet	2	0	0.076	2.71
Till	Dry Wet	3	0	0.094	3.36
Till	Dry Wet	1	14	0.111	3.94
Till	Dry Wet	2	14	0.192	6.85
Till	Dry Wet	3	14	0.037	1.32
Till	Dry Wet	1	17	0.088	3.15
Till	Dry Wet	2	17	0.032	1.13
Till	Dry Wet	3	17	0.053	1.89
Till	Dry Wet	1	31	0.205	7.31
Till	Dry Wet	2	31	0.203	7.23
Till	Dry Wet	3	31	0.145	5.15
Till	Dry Wet	1	34	0.044	1.57
Till	Dry Wet	2	34	0.053	1.87
Till	Dry Wet	3	34	0.017	0.60
Till	Dry Wet	1	48	0.070	2.51
Till	Dry Wet	2	48	0.055	1.94
Till	Dry Wet	3	48	0.009	0.33
Till	Dry Wet	1	51	0.050	1.78
Till	Dry Wet	2	51	0.044	1.57
Till	Dry Wet	3	51	0.015	0.53
Till	Dry Wet	1	65	0.023	0.81
Till	Dry Wet	2	65	0.008	0.30
Till	Dry Wet	3	65	0.002	0.08
Till	Dry Wet	1	68	0.002	0.06
Till	Dry Wet	2	68	0.002	0.05
Till	Dry Wet	3	68	0.002	0.06
Till	Wet	1	0	0.248	8.82
Till	Wet	2	0	0.230	8.19
Till	Wet	3	0	0.293	10.44
Till	Wet	1	14	0.001	0.03
Till	Wet	2	14	0.001	0.05
Till	Wet	3	14	0.006	0.20
Till	Wet	1	17	0.060	2.13
Till	Wet	2	17	0.073	2.61
Till	Wet	3	17	0.035	1.25

Table 18 continued.

Management	Treatment	Sample	Time (days)	%G1G3	Corrected %G1G3
Till	Wet	1	31	0.036	1.27
Till	Wet	2	31	0.031	1.09
Till	Wet	3	31	0.045	1.61
Till	Wet	1	34	0.08	2.86
Till	Wet	2	34	0.056	2.00
Till	Wet	3	34	0.051	1.80
Till	Wet	1	48	0.112	3.99
Till	Wet	2	48	0.065	2.33
Till	Wet	3	48	0.13	4.63
Till	Wet	1	51	0.142	5.07
Till	Wet	2	51	0.156	5.54
Till	Wet	3	51	0.127	4.51
Till	Wet	1	65	0.132	4.71
Till	Wet	2	65	0.17	6.04
Till	Wet	3	65	0.131	4.65
Till	Wet	1	68	0.155	5.50
Till	Wet	2	68	0.118	4.19
Till	Wet	3	68	0.176	6.27
Till	Dry	1	0	0.134	4.77
Till	Dry	2	0	0.062	2.19
Till	Dry	3	0	0.116	4.14
Till	Dry	1	14	0.123	4.40
Till	Dry	2	14	0.124	4.42
Till	Dry	3	14	0.085	3.02
Till	Dry	1	17	0.126	4.48
Till	Dry	2	17	0.131	4.68
Till	Dry	3	17	0.159	5.68
Till	Dry	1	31	0.148	5.29
Till	Dry	2	31	0.135	4.80
Till	Dry	3	31	0.127	4.51
Till	Dry	1	34	0.074	2.65
Till	Dry	2	34	0.071	2.51

Table 18 continued.

Management	Treatment	Sample	Time (days)	%G1G3	Corrected %G1G3
Till	Dry	3	34	0.227	8.09
Till	Dry	1	48	0.13	4.63
Till	Dry	2	48	0.158	5.64
Till	Dry	3	48	0.183	6.52
Till	Dry	1	51	0.093	3.32
Till	Dry	2	51	0.154	5.48
Till	Dry	3	51	0.136	4.86
Till	Dry	1	65	0.092	3.28
Till	Dry	2	65	0.116	4.11
Till	Dry	3	65	0.162	5.75
Till	Dry	1	68	0.282	10.05
Till	Dry	2	68	0.138	4.93
Till	Dry	3	68	0.139	4.96
No-Till	Dry Wet	1	0	0.151	5.38
No-Till	Dry Wet	2	0	0.085	3.02
No-Till	Dry Wet	3	0	0.14	4.98
No-Till	Dry Wet	1	14	0.368	13.11
No-Till	Dry Wet	2	14	0.495	17.63
No-Till	Dry Wet	3	14	0.328	11.69
No-Till	Dry Wet	1	17	0.188	6.69
No-Till	Dry Wet	2	17	0.084	2.99
No-Till	Dry Wet	3	17	0.056	1.99
No-Till	Dry Wet	1	31	0.032	1.14
No-Till	Dry Wet	2	31	0.01	0.37
No-Till	Dry Wet	3	31	0.045	1.61
No-Till	Dry Wet	1	34	0.019	0.66
No-Till	Dry Wet	2	34	0.007	0.26
No-Till	Dry Wet	3	34	0.02	0.71
No-Till	Dry Wet	1	48	0.034	1.21
No-Till	Dry Wet	2	48	0.023	0.83
No-Till	Dry Wet	3	48	0.028	0.99
No-Till	Dry Wet	1	51	0.003	0.11

Table 18 continued.

Management	Treatment	Sample	Time (days)	%G1G3	Corrected %G1G3
No-Till	Dry Wet	2	51	0.001	0.02
No-Till	Dry Wet	3	51	0.018	0.64
No-Till	Dry Wet	1	65	0.153	5.46
No-Till	Dry Wet	2	65	0.111	3.95
No-Till	Dry Wet	3	65	0.2	7.11
No-Till	Dry Wet	1	68	0	0.02
No-Till	Dry Wet	2	68	0.001	0.04
No-Till	Dry Wet	3	68	0	0.01
No-Till	Wet	1	0	0.227	8.07
No-Till	Wet	2	0	0.128	4.54
No-Till	Wet	3	0	0.082	2.93
No-Till	Wet	1	14	0.049	1.76
No-Till	Wet	2	14	0.107	3.81
No-Till	Wet	3	14	0.076	2.72
No-Till	Wet	1	17	0.234	8.32
No-Till	Wet	2	17	0.218	7.78
No-Till	Wet	3	17	0.131	4.66
No-Till	Wet	1	31	0.148	5.28
No-Till	Wet	2	31	0.29	10.34
No-Till	Wet	3	31	0.159	5.68
No-Till	Wet	1	34	0.096	3.42
No-Till	Wet	2	34	0.109	3.89
No-Till	Wet	3	34	0.073	2.60
No-Till	Wet	1	48	0.089	3.18
No-Till	Wet	2	48	0.093	3.30
No-Till	Wet	3	48	0.133	4.74
No-Till	Wet	1	51	0.219	7.81
No-Till	Wet	2	51	0.17	6.04
No-Till	Wet	3	51	0.245	8.71
No-Till	Wet	1	65	0.243	8.66
No-Till	Wet	2	65	0.167	5.94
No-Till	Wet	3	65	0.272	9.69

Table 18 continued.

Management	Treatment	Sample	Time (days)	%G1G3	Corrected %G1G3
No-Till	Wet	1	68	0.01	0.36
No-Till	Wet	2	68	0.012	0.42
No-Till	Wet	3	68	0.001	0.04
No-Till	Dry	1	0	0.001	0.05
No-Till	Dry	2	0	0.008	0.28
No-Till	Dry	3	0	0.004	0.14
No-Till	Dry	1	14	0.131	4.68
No-Till	Dry	2	14	0.007	0.23
No-Till	Dry	3	14	0.009	0.32
No-Till	Dry	1	17	0.006	0.20
No-Till	Dry	2	17	0.003	0.12
No-Till	Dry	3	17	0.005	0.19
No-Till	Dry	1	31	0.014	0.51
No-Till	Dry	2	31	0.015	0.55
No-Till	Dry	3	31	0.574	20.45
No-Till	Dry	1	34	0.164	5.84
No-Till	Dry	2	34	0.236	8.41
No-Till	Dry	3	34	0.222	7.92
No-Till	Dry	1	48	0.976	34.75
No-Till	Dry	2	48	0.484	17.24
No-Till	Dry	3	48	0.478	17.04
No-Till	Dry	1	51	0.187	6.66
No-Till	Dry	2	51	0.228	8.11
No-Till	Dry	3	51	0.305	10.86
No-Till	Dry	1	65	0.363	12.92
No-Till	Dry	2	65	0.391	13.92
No-Till	Dry	3	65	0.561	19.98
No-Till	Dry	1	68	0.248	8.84
No-Till	Dry	2	68	0.472	16.83
No-Till	Dry	3	68	0.227	8.10

Table 19 Correction to moisture study standard curve.

Management	Treatment	Time (days)	Sample	G1G3 SQ Mean	G1G3 SQ Std. Dev	16S SQ Mean	16S SQ Std. Dev	Actual %G1G3	Experimental %G1G3	% Error
Till	Dry Wet	0	2	31725	1459	895042	43481	3.54	0.094	-97.35
Till	Dry Wet	31	3	24077	2134	346412	137841	6.95	0.145	-97.91
Till	Dry Wet	65	1	53570	3196	1687869	103637	3.17	0.023	-99.28
Till	Wet	0	3	47780	1351	917153	121951	5.21	0.293	-94.38
Till	Wet	14	1	91370	15382	4488399	2313236	2.04	0.001	-99.95
Till	Dry	0	3	184774	42261	4805708	538217	3.84	0.116	-96.98
Till	Dry	14	1	47694	10449	849581	362620	5.61	0.123	-97.81
Till	Dry	17	1	42808	5555	1139931	319667	3.76	0.126	-96.64
Till	Dry	31	2	49666	2834	1195265	34617	4.16	0.135	-96.75
Till	Dry	65	2	14557	600	257556	13756	5.65	0.116	-97.95
No-Till	Dry Wet	14	2	46924	16569	657630	235388	7.14	0.495	-93.06
No-Till	Dry Wet	17	2	18811	3165	237913	38023	7.91	0.084	-98.94
No-Till	Dry Wet	31	2	32806	9771	1139307	479819	2.88	0.010	-99.65
No-Till	Dry Wet	31	3	80350	7302	1562670	295671	5.14	0.045	-99.12
No-Till	Wet	17	2	8627	1419	137899	28305	6.26	0.218	-96.52
No-Till	Wet	17	3	37637	12347	836965	140084	4.50	0.131	-97.09
No-Till	Wet	34	2	201371	31378	3449556	993368	5.84	0.109	-98.13
No-Till	Dry	0	3	23482	8762	505834	78557	4.64	0.004	-99.91
No-Till	Dry	14	2	78045	24458	2318113	784430	3.37	0.007	-99.79
No-Till	Dry	17	2	692684	153370	8207670 4	7039423 6	0.84	0.003	-99.64
No-Till	Dry	31	1	30624	9615	830041	42120	3.69	0.014	-99.62
No-Till	Dry	34	2	83963	21459	2015562	38430	4.17	0.236	-94.33
No-Till	Dry	51	3	57362	4172	1032504	57889	5.56	0.305	-94.51
No-Till	Dry	65	1	49556	6554	1807062	51474	2.74	0.363	-86.76

Table 20 Moisture study Type 3 Test of Fixed Effects.

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Management	1	16	3.46	0.0813
Treatment	2	16	4.10	0.0365
Management*Treatment	2	16	0.55	0.5894
Time	8	16	0.55	0.8030
Management*Time	8	16	0.74	0.6596
Treatment*Time	16	16	1.52	0.2071

Table 21 Moisture study Effect=Management Method=LSD(P<.05) Set=1

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
1	No-Till		—	5.5856	0.7526	A
2	Till		—	3.6052	0.7526	A

Table 22 Moisture study Effect=Treatment Method=LSD(P<.05) Set=2

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
3		Dry	—	6.5605	0.9218	A
4		Dry Wet	—	2.8460	0.9218	B
5		Wet	—	4.3796	0.9218	AB

Table 23 Moisture Study Effect=Management*Treatment Method=LSD(P<.05) Set=3

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
6	No-Till	Dry	—	8.3375	1.3036	A
7	No-Till	Dry Wet	—	3.4303	1.3036	B
8	No-Till	Wet	—	4.9889	1.3036	AB
9	Till	Dry	—	4.7836	1.3036	AB
10	Till	Dry Wet	—	2.2618	1.3036	B
11	Till	Wet	—	3.7702	1.3036	B

Table 24 Moisture study Effect=Time Method=LSD(P<.05) Set=4

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
12			0	4.2765	1.5966	A
13			14	4.4543	1.5966	A
14			17	3.3291	1.5966	A
15			31	4.6769	1.5966	A
16			34	3.2021	1.5966	A
17			48	6.4337	1.5966	A
18			51	4.5349	1.5966	A
19			65	6.5201	1.5966	A
20			68	3.9307	1.5966	A

Table 25 Moisture study Effect=Management*Time Method=LSD(P<.05) Set=5

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
21	No-Till		0	3.2644	2.2579	AB
22	No-Till		14	6.2170	2.2579	AB
23	No-Till		17	3.6597	2.2579	AB
24	No-Till		31	5.1024	2.2579	AB
25	No-Till		34	3.7439	2.2579	AB
26	No-Till		48	9.2532	2.2579	AB
27	No-Till		51	5.4413	2.2579	AB
28	No-Till		65	9.7366	2.2579	A
29	No-Till		68	3.8515	2.2579	AB
30	Till		0	5.2886	2.2579	AB
31	Till		14	2.6917	2.2579	B
32	Till		17	2.9986	2.2579	AB
33	Till		31	4.2514	2.2579	AB
34	Till		34	2.6603	2.2579	B
35	Till		48	3.6142	2.2579	AB
36	Till		51	3.6284	2.2579	AB
37	Till		65	3.3035	2.2579	AB
38	Till		68	4.0100	2.2579	AB

Table 26 Moisture study Effect=Treatment*Time Method=LSD(P<.05) Set=6

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
39		Dry	0	1.9270	2.7653	BCDE
40		Dry	14	2.8437	2.7653	BCDE
41		Dry	17	2.5572	2.7653	BCDE
42		Dry	31	6.0176	2.7653	ABCDE
43		Dry	34	5.9004	2.7653	BCDE
44		Dry	48	14.3041	2.7653	A
45		Dry	51	6.5487	2.7653	ABCDE
46		Dry	65	9.9937	2.7653	AB
47		Dry	68	8.9524	2.7653	ABCD
48		Dry Wet	0	3.7370	2.7653	BCDE
49		Dry Wet	14	9.0905	2.7653	ABC
50		Dry Wet	17	2.9730	2.7653	BCDE
51		Dry Wet	31	3.8003	2.7653	BCDE
52		Dry Wet	34	0.9444	2.7653	CDE
53		Dry Wet	48	1.3016	2.7653	CDE
54		Dry Wet	51	0.7743	2.7653	DE
55		Dry Wet	65	2.9517	2.7653	BCDE
56		Dry Wet	68	0.04163	2.7653	E
57		Wet	0	7.1656	2.7653	ABCDE
58		Wet	14	1.4288	2.7653	CDE
59		Wet	17	4.4573	2.7653	BCDE
60		Wet	31	4.2129	2.7653	BCDE
61		Wet	34	2.7616	2.7653	BCDE

Table 26 continued.

Obs	Management	Treatment	Time	Estimate	Standard Error	Letter Group
62		Wet	48	3.6954	2.7653	BCDE
63		Wet	51	6.2816	2.7653	ABCDE
64		Wet	65	6.6148	2.7653	ABCDE
65		Wet	68	2.7982	2.7653	BCDE

VITA

Mariam Fawaz was born March 21, 1987 in Blacksburg, Virginia to parents, Annette Elaine and Hassan Zaki, and welcomed by brother, Zaki Hassan. The Fawaz's moved to Gahanna, Ohio in 1989 shortly after the birth of her sister, Samar Tamara. Mariam's younger brother, Ahmad Hassan, was born in 1991. Mariam attended elementary school at Royal Manner Elementary until the fourth grade. Often during summer vacations Mariam and her siblings traveled to Beirut and Jowaya, Lebanon and once to Kano, Nigeria to live with her grandparents Ezza Abdallah and Zaki Abdulghani Fawaz. At the age of 8 Mariam was diagnosed with scoliosis. In 1997, Mariam's family moved to Riyadh, Saudi Arabia following her father's work. Mariam's family moved back to the United States of America and took up residence in Knoxville, Tennessee. Due to the rapid and severe progression of her scoliosis Mariam underwent a spinal fusion to correct her scoliosis in 2000 at Vanderbilt Children's Hospital. Mariam attended Farragut Middle School and graduated from Farragut High School in 2005. Mariam received her Bachelor of Science degree in Environmental and Soil Science with a concentration in Soil Science and a minor in Chemistry from the University of Tennessee in December 2010. Following her graduation she retained her position as a research assistant in a soil microbiology laboratory and continued her research of a novel isolate from the phylum *Gemmatimonadetes*. During her hiatus Mariam and her sister traveled to Australia and New Zealand. Mariam was accepted into the Environmental and Soil Science Master of Science program at the University of Tennessee where

she furthered her work with bacteria from the phylum *Gemmatimonadetes*. She graduated with a Master of Science from the University of Tennessee in 2013.