FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ENHANCED GROWTH OF TETRACYCLINE-SUSCEPTIBLE SOIL BACTERIA TREATED WITH IRON AND OXYTETRACYCLINE

An Undergraduate Honors Thesis submitted in partial fulfillment of the

requirements for the degree of Bachelor of Science

in

BIOLOGICAL SCIENCES

WITH HONORS

by

Kevin Matthew Casin

To: Dr. Timothy Collins, Chairperson

Department of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Kevin Matthew Casin, and entitled "Enhanced Growth of Tetracycline-susceptible Soil Bacteria Treated with Iron and Oxytetracycline", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor(s) have read this thesis. We recommend that it be approved.

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This thesis by Kevin Matthew Casin is approved.

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ABSTRACT OF THE THESIS

ENHANCED GROWTH OF TETRACYCLINE-SUSCEPTIBLE SOIL BACTERIA TREATED WITH IRON AND OXYTETRACYCLINE

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Dr. DeEtta Mills, Honors Research Supervisor

Antibiotic resistance has become an important area of research because of the excessive use of antibiotics in clinical and agricultural settings that are driving the evolution of antibiotic resistant bacteria. However, drug tolerance is a naturally occurring phenomenon in soil communities, and is often linked to those soils that are exposed to heavy metals as well as antibiotics. Resistance to antibiotics maybe coupled with resistance to heavy metals in soil bacteria through efflux pumps that can be regulated by iron. Although considered s a heavy metal, iron is an essential component of life that regulates gene expression through the Ferric Uptake Regulator (Fur) protein. This master regulator protein is known to control siderophore production, and other biological pathways. As a suspected controller of biofilm formation, the role of Fur in environmental antibiotic resistance may be greater than is currently realized. In this study, we sought to explore a potential Fur-regulated drug tolerance pathway by understanding the response of soil bacteria when stressed with oxytetracycline and iron. Bacteria were collected from two locations in Miami Dade County. Isolates were first tested using Kirby-Bauer Disk Diffusion tests for antibiotic resistance/susceptibility and identified by 16S rDNA sequencing. A 96-well growth assay was developed to measure planktonic cell growth with 3 mM $FeCl_3$,

Oxytetracycline HCl, and the combination treatments. A Microtiter Dish Biofilm Formation Assay was employed and Fur diversity was evaluated. Tetracycline-susceptible bacterial isolates developed drug resistance with iron supplementation, but iron did not enhance biofilm formation. Development of a Fur-dependent drug resistance may be selected for, but further study is required to evaluate Fur evolution in the studied isolates. Gene expression analysis is also needed to further understand the ecological role of Fur and antibiotic resistance.

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ACRONYMS AND ABBREVIATIONS

Fur	Ferric Uptake Regulator
CH2	Soil classification by USDA 2012: Chekika National Park (25°34’46” N, 80°29’50” W)
KK1	Soil classification by USDA 2012: Krome RD. and Kendall AVE. (25°42’15” N, 80°28’11” W)
ROS	Reactive Oxygen Species

INTRODUCTION

Antibiotic resistance is a global health concern that has been exacerbated by the superfluous use of antibiotics in clinical and agricultural practices (Kümmerer 2003; Kim and Aga 2007; Martinez 2012). Although excessive use has driven the development of different mechanisms of drug tolerance, it did not originate from anthropogenic practices. It is well know that antibiotic resistance is common in soil environments because of the presence of antibiotic-producing bacteria and fungi and the need to compete for limited nutrients (D'Costa et al. 2007; Allen et al. 2010; Davies and Davis 2010). Soil can serve as resistance reservoirs and forms a soil resistome. Bacteria within the soil can use mechanisms such as horizontal gene transfer to exchange antibiotic resistance genes between other microorganisms in the environment (Radhouani et al. 2014). Environmental concentrations of antibiotics produced by bacteria are often sublethal and are not necessarily used for killing but for communication, such as stimulating biofilm formation (Ratcliff and Denison, 2011). Consequently, the presence of antibiotics, particularly tetracycline, has had the capacity to shape the microbial diversity in soil (Baguer et al. 2000; Hund-Rinke et al. 2004; Ding and He 2010).

Antibiotics are naturally produced and can be divided into different classes. A well-known drug is penicillin, produced by a soil fungus belongs to the family of β-lactams that target the enzyme used to synthesize the bacterial peptidoglycan wall (Hartmann 1972). Aminoglycosides originate from *Streptomyces* sp. and bind to the 30S ribosomal subunit to inhibit protein synthesis via a little known mechanism (Vakulenko and Mobashery 2003). Another class of antibiotics is also isolated from *Streptomyces* sp. and has been named chloramphenicol, which prevents the peptide chain elongation through inhibition of peptidyltransferase on the 50S subunit of the 70S ribosomal subunit (Schwarz et al 2004).

Vancomycin is a member of the glycopeptides family and targets the D-alanyl-D-alanine segment of the peptidoglycan precursor and destabilized the bacterial cell wall (Gao 2002). In this study, we focused on one antibiotic commonly used in agriculture.

Tetracycline is naturally produced by *Streptomyces* spp. But in recent years, tetracycline environmental concentrations have changed because of increased use in agriculture (Moats 2000; Schlusener et al. 2003; Huang et al. 2013). In the mid-1990s, farm animals in the United States consumed, as a feed additive, almost 3.5 million kg/yr, because it is used as an animal growth promoter. Approval of tetracycline use in animal feed was granted by the Food and Drug Administration in 1951, for chlortetracycline, and 1953, for oxytetracycline (Chopra 2001). Tetracycline can accumulate in the muscle of pork (Granelli and Branzell 2007), beef, and poultry (Moats 2000; Mayrhofer et al. 2004). These animals then excrete excess tetracycline that can remain active for approximately two months (Hamscher et al. 2002; Wang and Yates 2008). Also, depending on the soil quality, such as soil type, or the availability of divalent cations like aluminum or iron, soil sorption of oxytetracycline can be increased (Figueroa and Mackay 2005; Bao et al. 2009, 2010). Even if the chemical is bound to soil oxytetracycline can retain antimicrobial activity (Chander et al. 2005). The increased soil concentrations of tetracycline can select for resistant bacteria.

The soil resistome, or antibiotic-resistance gene reservoir, has a natural and extensive tetracycline resistance component, with a pool of genes tailored to hinder the chemical's lethal activity. The target of tetracycline is the ribosome, specifically the small subunit, and prevents protein synthesis by inhibiting recognition of the tRNA anticodons (Nguyen et al. 2013). As a result of this mode of action, bacteria have developed countermeasures such as ribosomal protection, enzymatic inactivation, and up-regulation of efflux pumps (Thaker et al. 2010).

Operons of tetracycline resistance genes, or *tet* genes, and are controlled by *tet*(B) and *tet*(R) genes that encode for the repressor protein TetR(B). This protein recognizes the operator sequences, dimerizes, and restricts the RNA polymerase interaction with DNA to express tetracycline resistance genes. But when drug tolerance is needed, the high affinity of TetR(B) to tetracycline will induce binding and inactivate the protein to allow transcription of *tet* genes. Tetracycline cannot enter the cell without becoming charged by a divalent cation, which allows it to traverse OmpC and OmpF porins (Speer et al. 1992; Ramos et al. 2005). Although Mg^{2+} is typically used (Schnappinger and Hillen 1996), iron can also support tetracycline diffusion. Iron can induced tetracycline resistance by proteolysis of TetR(B) (Ettner et al. 1993), to which iron has a greater affinity to than Mg^{2+} by least 1000-fold (Ettner et al. 1995). Tetracycline efficacy is known to be negatively impacted by iron because of its ability to form a stable complex that often prevents tetracycline, especially oxytetracycline, from entering the cell (Campbell and Hasinoff 1991; Avery et al. 2004; Gu and Karthikeyan 2005). These previous studies have shown that heavy metals and antibiotics can interact to help bacteria develop drug resistance.

Heavy metals are categorized as a metal with a density greater than 5 g/cm^3, and most transitions are considered heavy metals with a few exceptions, such Sc and Ti (Nies 1999). With a density of 7.9 g/cm^3, iron is considered a heavy metal and like most metals, it is an essential nutrient for life often used in metabolism, respiration, and cell signaling. As important as iron is, it is not biologically available. Bacteria require ferrous ion (Fe^{2+}) in a soluble state, therefore bacteria require a soil of pH 7 for iron to be optimally active. Yet, ferrous is spontaneously oxidized by oxygen to form a stable ferric hydroxide complex that results in low, environmental free-iron concentrations of about 10^{-18} M (Raymond et al. 2003; Ballouche et al. 2009). Low iron availability has also been shown to inhibit biofilm formation by preventing cell adherence

(Moreira et al. 2003) and has been shown to effect *Mycobacterium tuberculosis* survival (Jones et al. 2014). Iron can even play a role in barotolerance with high concentrations of intracellular iron having a negative effect on *Escherichia coli* growth when exposed to high pressure (Yan et al. 2013). Drug development has looked at iron assimilation as a potential target for pharmaceutical antibiotics (Foley and Simeonov 2012) and has been explored as a possible mechanism for drug delivery (McKee et al. 1991; Mislin and Schalk 2014; Tavares et al. 2013). Iron is important for bacteria growth, therefore restriction of this nutrient is not taken lightly and bacteria need to develop a mechanism for maintaining iron homeostasis.

Iron-limited environments induce bacterial gene expression of virulence factors, such as low molecular weight ferric chelators known as siderophores that are responsible for sequestering iron in the environment and transporting iron in to the cell (Neilands 1995; Smith 1998; Chu et al. 2010). These proteins are considered virulence factors because of their ability to not only regulate of other virulence factors, but they can also be responsible for the development of disease (Lamont et al. 2002). The Fur protein is transcribed from the *fur* gene, and under iron-limited conditions, siderophore production is activated. Three broad categories can be used to organize siderophores: catecholates, hydroxymates, and mixed. The catecholate group contains enterobactin, vibriobactin, and pyrochelin. In the hydroxymate category, the siderophores alcaligin, and staphyloferrin are found, and the mixed group includes mycobactin and petrobactin (Miethke and Marahiel 2007; Saha et al. 2013). These compounds are produced by several organisms, such as *Alcaligenes faecalis* (Sayyed and Chincholkar 2006), *Ochrobactrum* spp. (Martin et al. 2006), *Stenotrophomonas maltophilia* (Ryan et al. 2009), and *Bacillus cereus* (Zawadzka et al. 2009). Conditioning of bacteria in iron-restricted media induces siderophore production in an effort to facilitate iron uptake (Hennecke 1990; McHugh et al. 2003). As an

essential nutrient, bacteria need to find a way to sequester any free-iron from the environment, or enzymatically transform ferric (Fe^{3+}) into ferrous (Fe^{2+}). The presence of these naturally occurring antibiotics and, in the presence of heavy metals normally found in soil, can concurrently create stress on microbial communities and affect their ability to survive if homeostasis is not maintained.

Iron homeostasis is regulated by the Ferric Uptake Regulator (Fur) protein and has been characterized as a global regulator responsible for iron-uptake processes such as siderophore production, and may also regulate efflux pump expression (Zhang et al. 2011; González et al 2012; Yu and Genco 2012; Porcheron et al. 2013). As an iron-dependent protein, the divalent ion is necessary to induce a conformation change that allows for the binding to a particular DNA consensus sequences (GATAATGATAATCATTATC) known as the Fur Box (Escolar et al. 1999; Hantke 2001; Baichoo and Helmann 2002; Grifantini et al. 2003). Regulation of gene expression by Fur is extensive and it usually acts as a repressor for siderophore production and Reactive Oxygen Species (ROS) resistance (Lee et al. 1998; Wessling-Resnick et al. 1999; Zheng et al. 1999). It has also been suggested that Fur can act as an activator, but the activation of genes is the results of repressed transcription factors by Fur (Hall and Foster 1996; Hantke 2001; Campoy et al. 2002; Troxell et al. 2013). Under iron-limited conditions, equilibrium causes the displacement of iron and the expression of genes repressed by Fur (Escobar et al. 1999). These conditions stimulate production of siderophores and other virulence factors in *Pseudomonas aeruginosa* (Ochsner et al. 1996; Cornelis et al. 2004). Virulence control has also been reported in other microorganisms (Mekalanos 1992), such as *Neisseria meningitidis* (Delany et al. 2004), *Listeria monocytogenes* (Rea et al. 2004), and *Vibrio cholera* (Mey et al. 2005). Treatment of iron allows for the Fur repression of genes, yet few studies have been found

that report Fur-regulated efflux pumps for antibiotic resistance (Deng et al. 2012; Ding et al. 2012).

In order to explore the environmental behavior of Fur and its potential in regulating antibiotic resistance, the growth response of bacteria when stressed with tetracycline and iron must be observed. To examine this response, soil samples were collected from two locations in Miami Dade Country. Bacteria were then isolated and tested for antibiotic susceptibility before the bacteria were identified with 16S rDNA sequencing. Once the closest relative was determined for the isolates with a BLAST comparison to known GenBank sequences, planktonic growth was examined. With treatment to different concentrations of $FeCl_3$ and oxytetracycline, a biofilm formation assay was used to determine if resistance could develop as a result of a biofilm. Antibiotic resistance is a known to be conferred with the formation of biofilms; therefore observed resistance may be the result of biofilms, especially since iron is necessary for biofilm development (Singh et al. 2002; Weinberg 2004; Banin 2005; Oglesby-Sherrouse et al. 2014). The evolution of the *fur* gene and Fur protein was also explored to examine if this repressor protein can change as a result of environmental selective pressures. Growth response of soil bacteria to dosages of iron and oxytetracycline must be evaluated before a regulation of antibiotic resistance mechanisms by Fur can be determined.

MATERIALS AND METHODS

Bacteria Isolation from Miami Dade County Soils

Soil samples were collected from two locations in Miami-Dade County: one location was on Krome Avenue and SW 172nd Avenue (labeled CH2), and the other on SW 200th Street and SW 187th Avenue beside B&L Farms Airport (labeled KK1). Each soil location was selected based on low disturbance and no proximity to residential areas as determined through soil surveys conducted by the US Department of Agriculture (USDA 2012). Soil aliquots were sprinkled onto 1/20% dilute nutrient agar (BD Difco, Sparks, MD) plates and designated for each location. Once growth was present, five randomly selected bacterial colonies from each plate were streaked onto 100% nutrient agar plates and were evaluated for purity with a standard Gram staining protocol. Labels were given to the five bacteria and indicate the soil locations where it was collected: 1_CH2, 2_CH2, 3_CH2, 4_CH2, 1_KK1, and 2_KK1.

Kirby Bauer Disk Diffusion Antibiotic Susceptibility Test

Five soil bacteria were tested for antibiotic resistance using the Kirby Bauer Disk Diffusion Susceptibility Test (Bauer et al. 1966) modified with nutrient agar instead of Mueller-Hinton agar. Four antibiotics were used to test susceptibility of each isolate: Tetracycline (30 μg), Chloramphenicol (30 μg), Erythromycin (15 μg), and Penicillin (10 μg) (BD, Sparks, MD). The diameters of the zones of inhibition were directly measured and susceptibility was determined to be clear zones of >20 mm. Mild susceptibility was determined as a zone between 10-20 mm and low susceptibility required a zone of <10 mm.

Bacteria Identification Based on the 16S rRNA Gene

Five unknown isolates were randomly selected and DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA). Amplification of the 16S rDNA was achieved with PCR, which is conventionally used to genetically identify bacteria (Woese 1987; Chen et al. 2014). A 1 µL aliquot of undiluted genomic DNA was incorporated into a 20 µL solution with the following reagents: 1X PCR buffer (pH 8.5), 2.5 mM $MgCl_2$, 250 µM dNTPs, 0.1% BSA, fraction V, and 25 mU/µL GoTaq Flexi DNA polymerase (Promega, Madison, WI). A 0.5 mM concentration of a universal primer pair commonly used to examine soil bacteria was used in this study: 27F (5'-AGAGTTTGATCCTTGGCTCAG-3'), and 1492R (5'-TACGGCTTACCTTGTTACGACTT-3') (Dojka et al. 1998; Dees and Ghiorse 2001; Guo 2007; Tian et al. 2013). The PCR parameters were as follows: 95°C for 2 minutes, 30 cycles of 95°C denaturing step for 1 minute, 55°C annealing step for 1 minute, and 72°C extension step for 1 minute, concluding with the enzyme inactivation step at 72°C for 10 minutes. Amplified DNA products were evaluated for quality using a 1% agarose gel stained with GelRed (Biotium Inc., Hayward, CA) and visualized using a UV transilluminator. Before quantifying the DNA, it was purified according to the manufacturer's protocol with ExoSAP-IT (Affymetrix, Santa Clara, CA). Quantification of DNA was performed with the Qubit dsDNA HS Assay Kit and analyzed using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY). Sanger Sequencing was carried out with the BigDye Terminator v.3.1 Cycle Sequencing Kit, a pGEM control DNA, M13 primers, and BigDye Terminator v.3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA). Purification of DNA for sequencing was achieved with 70% isopropanol clean up, dried down, and then HiDi Formamide was added (Applied Biosystems,

Foster City, CA). Samples were sequenced by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the same PCR universal primers. The raw sequence data were evaluated with Sequence Analysis v.5.2, Patch 2 software (Applied Biosystems, Foster City, CA). Pairwise and multiple alignment on 400 bp long DNA fragments for soil isolates and GenBank sequences was executed by ClustalW v.2. A Neighbor-Joining dendrogram bootstrapped 1000 times was constructed with *MEGA* v.6.06 software.

Planktonic Cell Growth with Iron and Oxytetracycline Treatment

Using a clear, flat-bottom, 96-well microtiter plate, the isolates 1_CH2, 2_CH2, and 3_CH2 were exposed to 1, 2, 3 mM $FeCl_3$ and Oxytetracycline HCl (Sigma-Aldrich, Saint Louis, MO), separate and in combination, for 24-hours in unchelated Luria-Bertani (LB) broth (Acros, Geel, Belgium). As a complex media, LB media has an iron concentration of 17 µM (Abdul-Tehrani et al. 1999; Semsey et al. 2006). Growth was monitored using Optical Density (OD_{600}), also known as absorbance, which follows the Beer-Lambert law that dictates absorbance is the logarithmic ratio of light intensity before and after transmission through a material (Ricci 1994). Cells were cultured for 24 hours in LB Broth and OD_{600} measurements of 500 µL of sample were calculated using the BioPhotometer Plus (Eppendorf, Hamburg, Germany). Approximately equal cell density for the bacteria were achieved with serial dilutions to obtain OD_{600} measurements <1 OD_{600}, or about 10^8 *Escherichia coli* cells/mL (1 $OD_{600} \approx 10^9$ *E. coli* cells/mL). Aggregation and the growth media can affect OD_{600} readings (Sezonov et al. 2007). Therefore, to minimize the variation, the 96-well plate was inoculated and scanned in triplicates to produce nine replicates. Each well had a volume of 150 µL, with the untreated controls at full volume. Treatment wells were given 100 µL of culture and 50 µL of their respective $FeCl_3$ or oxytetracycline

concentrations. Samples with a combined regimen were supplied with 25 µL of $FeCl_3$ and oxytetracycline to maintain an equal concentration. A well containing only LB broth served as a negative control. Measurements of OD_{600} were taken with a Modulus II Microplate Multimode Reader (Turner Biosystems, Sunnyvale, CA). The initial measurement was marked as 0 hours and all subsequent time points were normalized by subtracting them from 0 hours. Statistical difference between treatments and controls was determined using Student's t-Test, and growth curves were developed with the average and standard error at each time point. Significance was determined with $p<0.05$.

Microtiter Dish Biofilm Formation Assay

Biofilm development at 24 hours was evaluated with a Microtiter Dish Biofilm Formation Assay (O'Toole 2011). As a positive and negative control, biofilm hyper-producer *P. aeruginosa* Δ*retS* (PAO1 +) (Goodman et al. 2004) and biofilm non-producer *P. aeruginosa* Δ*psl*Δ*pel*Δ*algD* (PAO1 -) (S. Lory lab collection) were used respectively (these isolates were generously donated by the laboratory of Dr. Kalai Mathee). The assay began with a 24 hour culture of isolates 1_CH2, 2_CH2, 3_CH2, and two controls, and cell density was equalized with serial dilutions to obtain OD_{600} measurements <0.1 OD_{600}. A clear, flat-bottom, 96 well microplate was used with the same treatment protocol as previously described. A solution of crystal violet was diluted in de-ionized water, and 125 µL was added after the plate was carefully washed with 100 µL de-ionized water using a multi-channel pipettor. Biofilm formation was quantified by adding 30% acetic acid to each well and the OD_{600} was measured using the Modulus® II Microplate Multimode Reader (Turner Biosystems, Sunnyvale, CA) (O'Toole and Kolter 1998). To determine if a biofilm had formed quantitatively, the treated isolates were

statistically compared to the positive control (PA +) and a bar graphs was constructed with the mean and standard error for three replicates. Significance was calculated using a Student's t-Test, and significance was determined as $p<0.05$.

Evolution of *fur* Gene and Fur Protein

Sequence conservation was gauge by assessing known *fur* gene and Fur amino acid sequences from GenBank. After a search using the phrase "soil Fur", 24 sequences were collected depending on if the bacteria were isolated from soil. Sequences were then trimmed to 600 bp and 200 amino acids. Pairwise and multiple alignment was performed by ClustalW v. 2, and a Maximum Parsimony phylogenetics tree bootstrapped 1000 times was developed by the *MEGA* v. 6.06 software.

RESULTS

Kirby Bauer Disk Diffusion Susceptibility Test

Six isolates were selected based on Gram stain and soil location, and were evaluated for antibiotic susceptibility by direct measurement of zones of inhibition (Table 1). Of the four antibiotics tested, penicillin did not illicit a highly susceptible response and three of the six bacteria were actually resistant. Susceptibility to chloramphenicol and erythromycin was mixed, with some isolates seemingly resistant while others were strongly affected. Although susceptibility varied among the isolates, growth was stymied when treated with 30 µg, or 450 µM, of tetracycline. Tetracycline susceptibility was observed for three isolates, yet for the other three, the antibiotic was still mildly effective.

Table 1: Kirby Bauer Disk Diffusion Susceptibility Test. Zone of inhibition diameters are reported for isolates treated with four antibiotic disks. Concentrations of each disk are presented in parentheses. High susceptibility was determined if diameters were >20 mm, mild susceptibility diameters were between 10 to 20 mm, and low susceptibility (or resistance) diameters were <10 mm. Of the four antibiotics tested, tetracycline susceptibility was exhibited by all isolates. N=4, mean with ±SE.

	Gram Stain	Tetracycline (30 µg)	Chloramphenicol (30 µg)	Erythromycin (15 µg)	Penicillin (10 µg)
1_CH2	Negative	27 (±0) mm	27 (±0) mm	18 (±1) mm	8 (±3) mm
2_CH2	Negative	24 (±0) mm	16 (±0) mm	18 (±0) mm	10 (±0) mm
3_CH2	Positive	16 (±3) mm	16 (±0) mm	9 (±5) mm	0 mm
4_CH2	Positive	28 (±1) mm	29 (±0) mm	25 (±0) mm	13 (±0) mm
1_KK1	Negative	17 (±0) mm	0 mm	16 (±0) mm	0 mm
2_KK1	Negative	17 (±7) mm	30 (±2) mm	6 (±6) mm	13 (±0) mm

Genetic Diversity in Miami Dade County Soil

Bacteria collected from soil locations needed to be identified, so a 400 bp fragment of the 16S rRNA gene was sequenced and compared to known soil bacteria on GenBank using BLAST. Only a 400 bp fragment sequence could be obtain because of the length of the capillary used for the Genetic Analyzer. In order to assess the accuracy of the comparison, sequences from GenBank were trimmed to 400 bp, aligned and a Neighbor-Joining tree was developed (Figure 1 and Table 2). Three of the six isolates were identified as a particular species according to a BLAST and tree comparison. The identities of the closest relatives of three were *S. maltophilia*, *Rhodobacter sphaerodes*, and *Bacillus thuringiensis*. All three were found in the same soil location, but isolates from the other soil location were ambiguous. Isolate 1_KK1 grouped as an outlier on the phylogenetic tree (Figure 1). The tree did not group 1_KK1 with any of these organisms despite a 98% identity (Table 2). In reference to 2_KK1, a 91% identity was observed, yet genus identification was possible with 96% bootstrap value that grouped this isolates with members of the *Alcaligenes* spp. and *Rhodobacter* spp. (Figure 1 and Table 2).

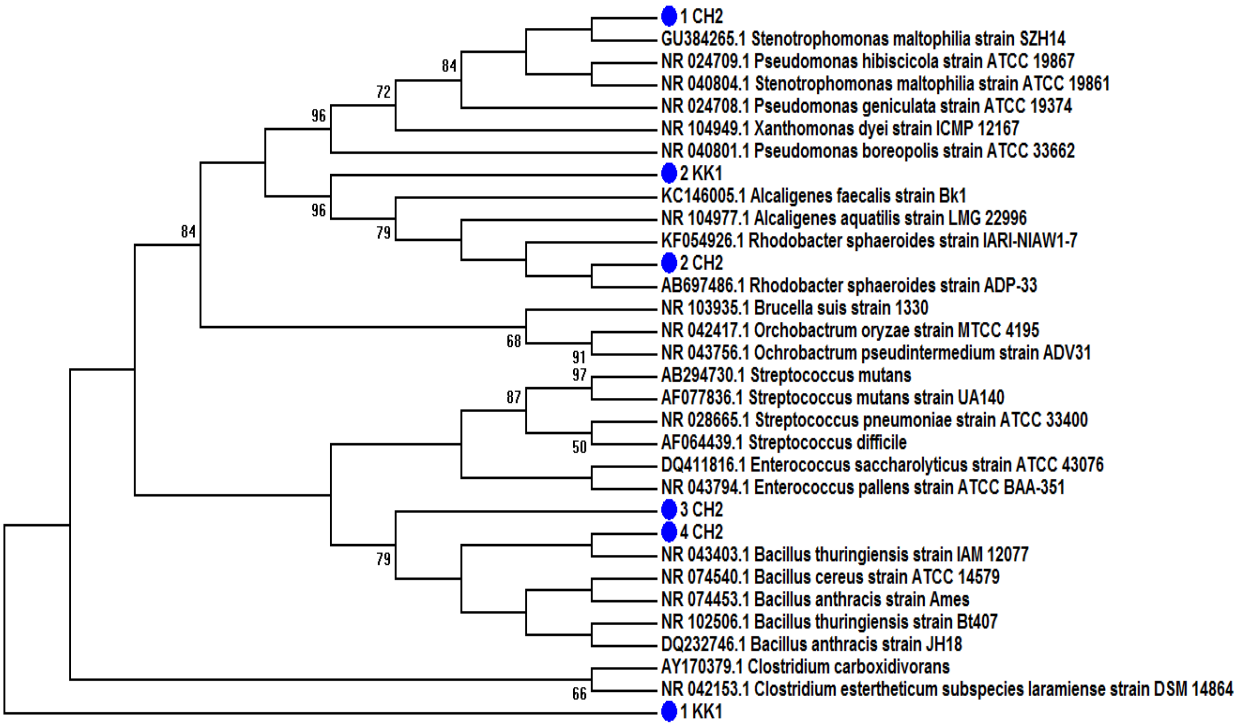

Figure 1. Neighbor-joining 16S rDNA phylogenetic tree constructed from isolated soil bacteria collected in Miami Dade County. Displayed at each node are bootstrap percentage values with 1000 replicates. Pairwise and Multiple Sequence Alignments were done with ClustalW and the phylogenetic tree was generated using *MEGA* v.6.06.

Table 2. The closest relatives for each isolate based on a 16S rRNA GenBank-BLAST comparisons of a 400 bp DNA fragment for each putative isolate.

ID	Closest Relative	Accession Number	Gram Stain	Percent Identity	Percent Query Cover
1_CH2	*Stenotrophomonas maltophilia*	GU384265.1	Negative	99%	98%
2_CH2	*Rhodobacter sphaeroides*	KC146005.1	Negative	98%	99%
3_CH2	*Bacillus spp.*	NR_074540.1	Positive	100%	100%
4_CH2	*Bacillus thuringiensis*	DQ232746.1	Positive	99%	99%
1_KK1	Uncultured bacterium	AB841138.1	Negative	98%	99%
2_KK1	*Alcaligenes spp./Rhodobacter spp.*	AB697486.1	Negative	91%	97%

Planktonic Cell Growth Curves

Three soil isolates were exposed to varying concentrations of iron and oxytetracycline to observe their growth response over a 24 hour time period. Two Gram negative (1_CH2, 2_CH2) and one Gram positive (3_CH2) bacteria were treated with separate and concurrent doses of 1, 2, and 3 mM of iron and oxytetracycline. Regimens of 1 and 2 mM showed a slight depression of growth when compared to the control (data not shown). With a 3 mM supplementation of $FeCl_3$, growth was augmented at two hours by at least two-fold (Student's t-Test, $p<0.05$), but only isolates 1_CH2 and 3_CH2 continued to grow until 0.5 OD_{600} was reached at five hours (Student's t-Test, $p<0.05$) (Table 3). Possible toxicity to iron could be seen for 2_CH2 because cell stationary phase began at five hours and continued for the remainder of the study (Figure 2).

When treated with oxytetracycline at 3 mM, the results were as expected when compared to the Kirby Bauer tests, which demonstrated the isolates' susceptibility to tetracycline (Table 1). Although growth after two hours was improved by 0.3 OD_{600} (Student's t-Test, $p<0.05$), cells did not recover growth after five hours and the OD measurement was halved relative to the control (Student's t-Test, $p<0.05$) (Table 3). After five hours, the bacteria were inhibited by the antibiotic and growth did not change with entrance to stationary phase (Figure 2).

Within two hours, the cultures had increased in cell density to 0.4 OD_{600}, but after an additional three hours cell density was enhanced by less than 0.1 OD_{600} (Student's t-Test, $p<0.05$) (Table 3). Despite a slow growth rate, a 24 hour incubation allowed for treated cells to grow better than the control by reaching a 0.6 OD_{600} (Student's t-Test, $p<0.05$). Isolate 2_CH2 did not show any differences from the control during the study.

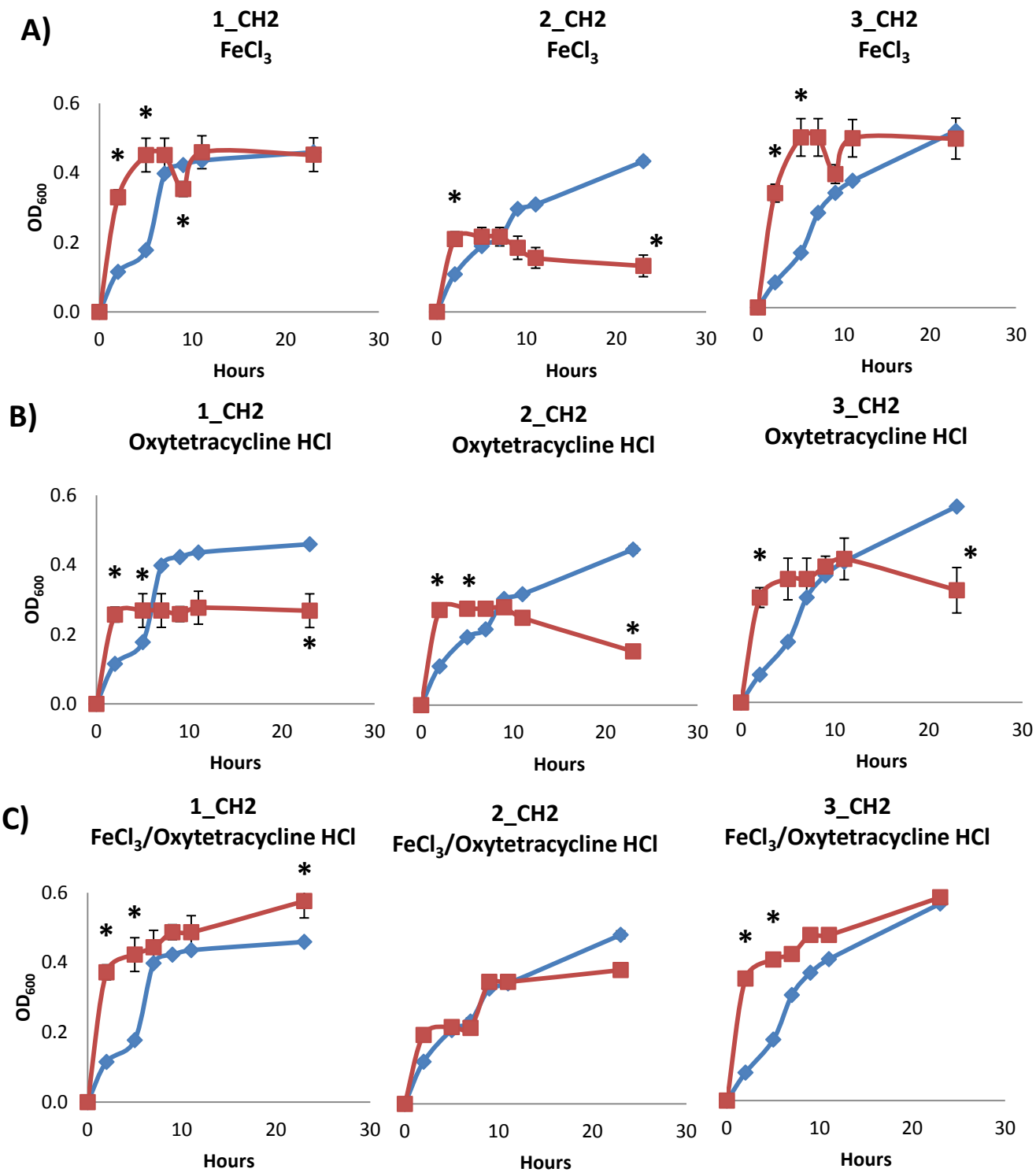

Figure 2. Planktonic cell growth treated with iron and oxytetracycline. Treatment of three isolates with 3 mM (red) $FeCl_3$ and oxytetracycline were compared to untreated controls (blue). **A) FeCl3.** An early improvement of growth and an early stationary phase was observed with 3 mM $FeCl_3$, except for 2_CH2 which responded with growth suppression. **B) Oxytetracycline.** Treatment of 3 mM oxytetracycline produced expected growth responses in the three tetracycline-susceptible soil isolates. **C) $FeCl_3$/Oxytetracycline.** Addition of $FeCl_3$ to oxytetracycline-treated isolates recovered growth. Plotted are means ± SE. Student's t-Test. *$p<0.05$. N=9.

Table 3. Mean OD_{600} measurements for each experimental treatment reported for 5 and 24 hours, and statistically compared to the control. Bolded numbers represent a significant difference from the control determined by Student's t-Test ($p<0.05$). N=9.

Samples	**Control**		**$FeCl_3$**		**Oxytetracycline**		**$FeCl_3$ + Oxytetracycline**	
	5 hr	24 hr	5	24	5	24	5	24
1_CH2	0.2 (±0.00)	0.5 (±0.00)	**0.5 (±0.05)**	0.5 (±0.05)	**0.3 (±0.03)**	**0.3 (±0.04)**	**0.4 (±0.03)**	**0.6 (±0.05)**
2_CH2	0.2 (±0.00)	0.4 (±0.01)	0.2 (±0.03)	**0.1 (±0.03)**	**0.3 (±0.02)**	**0.1 (±0.01)**	0.2 (±0.05)	0.3 (±0.08)
3_CH2	0.2 (±0.00)	0.6 (±0.01)	**0.5 (±0.06)**	0.5 (±0.06)	**0.4 (±0.08)**	**0.3 (±0.07)**	**0.4 (±0.02)**	0.6 (±0.02)

Microtiter Disk Biofilm Formation Assay

To explore if the resistance observed in Figure 2C was the result of a biofilm, a formation assay was used. The three isolates, 1_CH2 (red), 2_CH2 (blue), and 3_CH2 (green) used to examine growth response with iron and oxytetracycline, were subjected to the same experimental conditions. A positive (PAO +) and a negative (PAO -) were used to validate the assay as they respectively represent an excessive biofilm or a deficient biofilm. Each treatment isolate was compared to an untreated control and relative biofilm formation was observed. Improvement of biofilm formation resulted with 3 mM $FeCl_3$ at an almost two fold increase for 2_CH2 and 3_CH2, yet a slight gain in biofilm formation can be seen with 1_CH2 (Student's t-Test, $p<0.05$). Oxytetracycline suppressed biofilm development. The addition of iron to oxytetracycline-treated isolates did not help recover biofilm formation for any of the isolates (Student's t-Test, $p<0.05$). Treatment with oxytetracycline suppressed biofilm formation in all three isolates (Student's t-Test, $p<0.05$), and the supplementation of $FeCl_3$ did not allow isolates to recover biofilm formation.

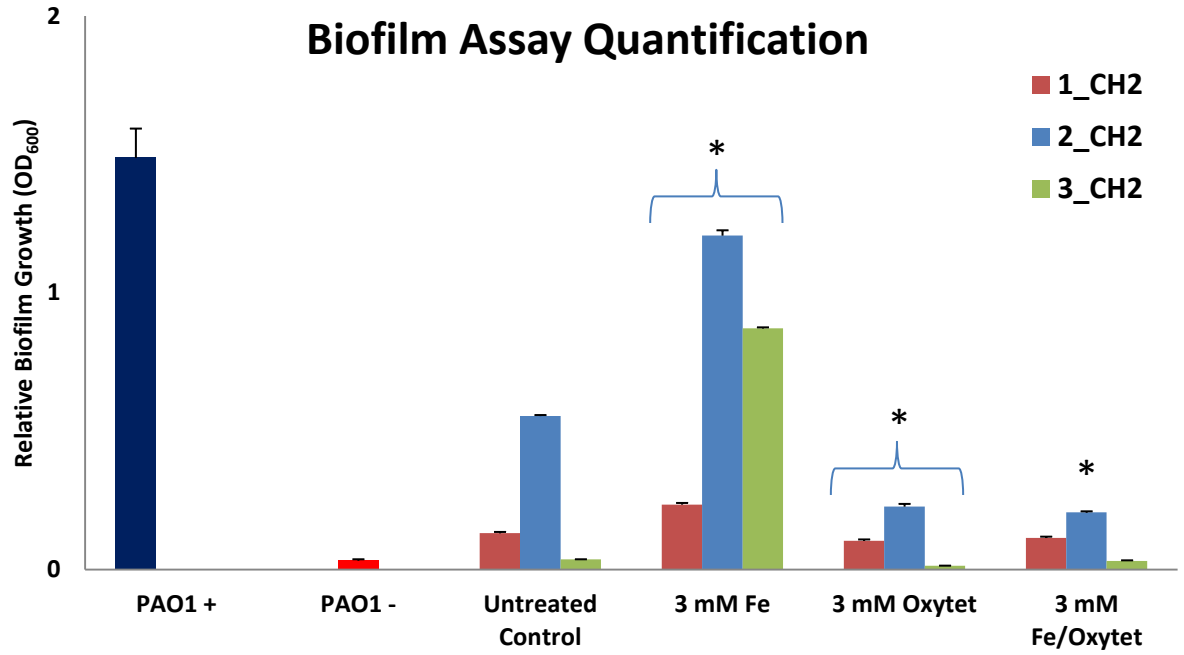

Figure 3. Microtiter Dish Biofilm Formation Assay. To evaluate if biofilm formation may be the cause of resistance to oxytetracycline, a biofilm formation assay was conducted. A hyper-biofilm producer (PAO1 +) and a non-biofilm producer (PAO1 -) *P. aeruginosa* strains were used determine if the assay was performed correctly. Biofilm formation relative to an untreated control was improved with treatment of 3 mM $FeCl_3$. A 3 mM oxytetracycline dose suppressed biofilm formation. Supplementation of 3 mM $FeCl_3$ did not recover biofilm formation for oxytetracycline-treated isolates when compared to the untreated controls. Plotted are mean ± SE. Student's t-Test. *$p<0.05$. N=3.

Genetic Diversity of *fur*

Sequences from known bacterial *fur* genes were collected from GenBank and a Maximum Parsimony tree was constructed to evaluate homology that may have developed over evolutionary time. In Figure 4, DNA sequence alignment (top) demonstrated a split in the eubacterial common ancestor that produced two lineages of proteobacteria. One with the evolution of *Pseudomonas* spp. relatives grouping as γ-proteobacteria, and with the other node, with a 57% bootstrap value, a non-class specific proteobacteria cluster, the diversity of *fur* is represented. The latter branch also contains a non-proteobacteria group, but these are shown to have a more distant relationship to the proteobacteria. Branching from the node that forms the γ-proteobacteria cluster, the *Bacillus* spp. relatives form their own grouping with a 100% bootstrap value and known soil isolated *Bacilli* and show a stronger relation than the unknown isolate *B. cereus* strain FRI-35.

Protein diversity of Fur (bottom) was not as extensive as *fur*, because the distance between nodes was not as great as with DNA sequences. Yet, the relationship that once existed amongst the genes was no longer evident when amino acid sequences were examined. The common ancestral division is still observed, and developed three groups in a node after the split. A proteobacteria group was created, along with a separate cyanobacteria and mixed group, which contains Firmicutes, Actinobacteria, and Deinococcus-Thermus taxa. In the other node, created after the eubacterial common ancestral split was similar to the event shown for *fur*. A proteobacteria cluster arose composed primarily of γ-proteobacteria, but the branch that leads to the Firmicutes node was unique to the Fur protein.

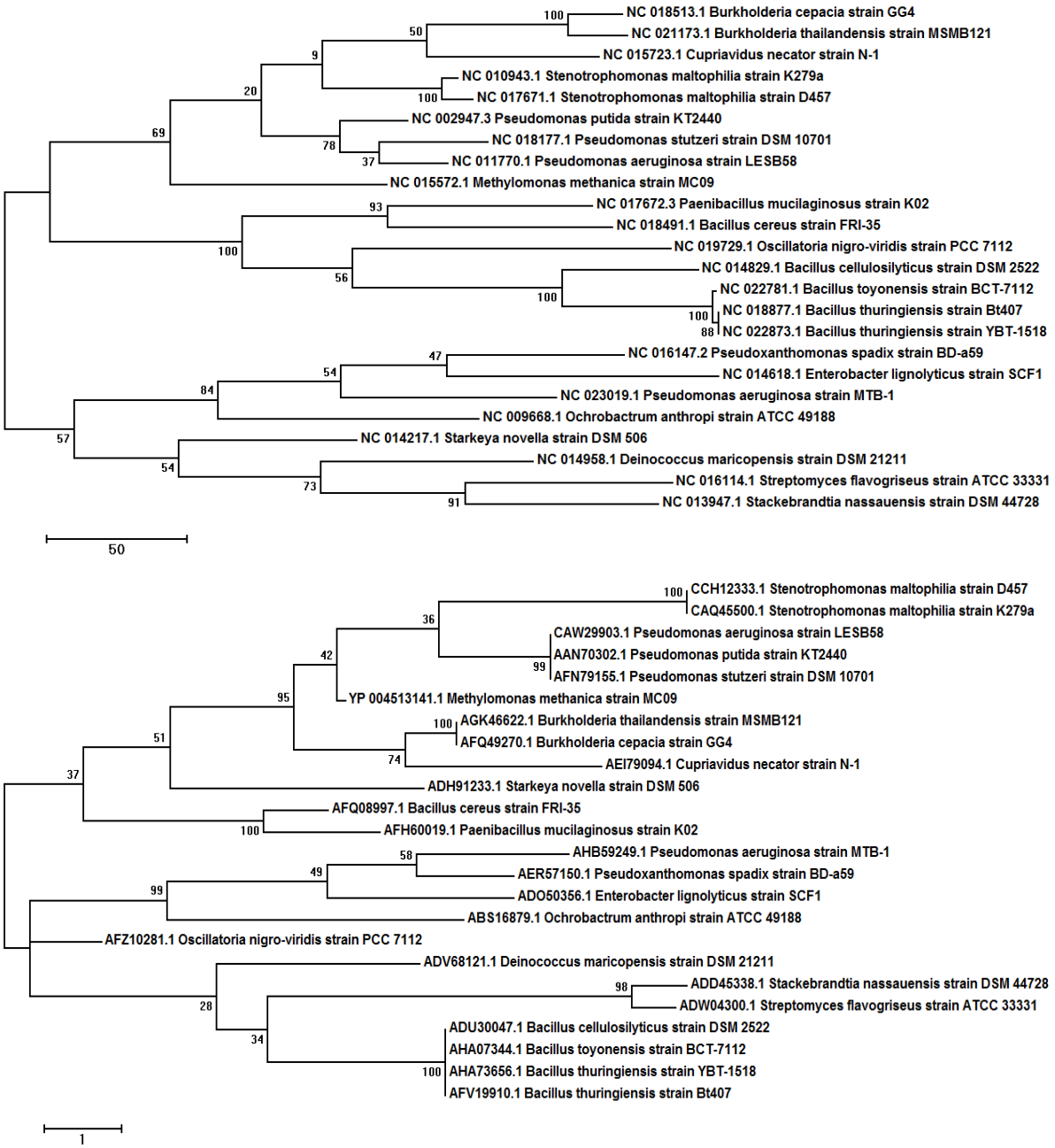

Figure 4. Maximum Parsimony phylogenetic tree for the *fur* gene and Fur protein. Fragments of 600 bp *fur* genes (top) and 200 amino-acid sequences of the Fur protein (bottom) from soil bacteria were collected from GenBank. Displayed at each node are bootstrapped values (%) with 1000 iterations. Greater genetic distance is observed with *fur* DNA sequences than with the translated protein. Variability of the *fur* gene and Fur protein were observed between the Proteobacteria class, with γ-proteobacteria evolving as a distinct group. The *Bacillus* spp. showed expected relationship to other Firmicutes and branched from the γ-proteobacteria node. Pairwise and Multiple Sequence Alignments were done with ClustalW and the phylogenetic tree was generated using *MEGA* v.6.06.

DISCUSSION

Antibiotic resistance is a phenomenon that has become a global health problem because of misuse in clinical and agricultural settings. As a natural component of soil microbial communities, there are unavoidable interactions between the organic and inorganic structure of the environment. Bacteria are constantly exposed to antibiotics and therefore must develop a tolerance for them. However, other stressors must be taken into account when considering the mechanism by which tolerance is obtained, especially stressors such as heavy metals. Iron, as well as other heavy metals, can be involved in regulating gene expression through the repressor protein, Fur. To determine a potential role for Fur in antibiotic resistance, bacterial growth must be evaluated for soil isolates exposed to doses of iron and oxytetracycline. Moreover, before this growth can be examined, the soil bacteria isolates from Miami Dade County soils needed to be identified in order to partially examine the diversity of the soil microbial community.

Although sequencing of the 16S rRNA gene provided information for the closest relative of the isolates, it did not definitively report the closest relative for all. It has been reported that the base pair length for 16S rDNA sequencing is between 1,300 bp to 1,500 (Janda and Abbott 2007), can provide an adequate description of a bacteria but it can overlook characteristic and unique sequences (Petti et al. 2005; Frank et al. 2008). Many phylogenic analyses for 16S rDNA sequences, however, are determined with about 400-500 bp. In many cases this truncated sequence is determined by the genetic analyzer's capillary length and/or capacity and cost of sequencing using Sanger sequencing technologies (Mizrahi-Man et al. 2013). This is an acceptable method of bacterial identification, despite the limitations that have been well characterized (von Wintzingerode et al. 1997; Rosselló-Mora and Amann 2001; Frank et al. 2008). The limitations of 16S rRNA gene sequencing are plentiful; a variety of factors can

influence identification accuracy, such as primer design (Frank et al. 2008; Klindworth et al. 2013), experimental design (Mizrahi-Man et al. 2013), and fragment size (Werner et al. 2012) as well as the sequence content archived in the databases. Keeping in mind that most bacteria found in nature cannot be cultured under laboratory conditions, it is not surprising that the exact identity of soil organisms is difficult to obtain.

Once the closest relatives were determined, the antibiotic susceptibility of these isolates was observed. The isolates were classified as either mildly, or highly susceptible to tetracycline (Table 1). With these results, the antibiotic-susceptible isolates were treated with iron and oxytetracycline to evaluate the growth responses. Dosages of oxytetracycline and iron yielded expected results, because these isolates were determined as tetracycline-susceptible, and the necessity of iron in bacterial growth is understood (Ballouche et al. 2009). The most interesting results were presented with the combined dosage of equal concentrations of iron and oxytetracycline, because there was a recovery of growth even in the presence of the antibiotic. With the presence of iron, isolates developed resistance to oxytetracycline, but this phenomenon has been previously observed (Moreau-Marquis et al. 2009; Oglesby-Sherrouse et al. 2014) and presented the possibility it was due to interference of biofilm formation. Although 3 mM of iron and oxytetracycline could become toxic over time, when coupled, the chemicals allowed for planktonic growth and cells maintained an exponential phase.

Consideration of iron and tetracycline interaction is necessary when discussing tetracycline resistance. Iron is a known antagonist of tetracycline because of its ability to form a stable complex and prevent diffusion across the cell membrane (Campbell and Hasinoff 1991) Sorption of iron by tetracycline to form a stable compound has been reported after eight hours of exposure (Gu and Karthikeyan 2005). Also, it has been shown to complex extracellularly with

treatment of 3 mM $FeSO_4$ to inhibit membrane passage (Avery et al. 2004). Regardless of this attribute, when iron is present it has the ability to regulate tetracycline resistance presumably via TetR(B), which controls genes necessary for drug tolerance (Ettner et al. 1993, 1995). Studies have reported that efflux pumps are responsible for intrinsic resistance in multidrug resistant pathogens such as *S. maltophilia* and these efflux pumps are actually controlled by SmeT, which is a member of the TetR family (Hernandez et al. 2009).

There is often a dynamic interaction between the organic and inorganic components of soil, which can either benefit or hinder the growth of soil bacteria (Gu and Karthikeyan 2005; Colombo et al. 2013). A study has shown that iron can influence an increase in peroxidase activity as a result of greater concentrations of ROS (Szajdak and Meysner 2013). Also, iron can accelerate and inhibit nitrate reduction in *Klebsiella pneumonia* (Liu et al. 2014) and *Alcaligenes* spp. (An et al. 2013), respectively. In this study, when isolates were treated with iron and oxytetracycline, it may be possible that the antibiotic is chelating the iron. The media was not chelated, therefore an additional 17 μM of iron was present, and if it was not chelated with the antibiotic, it may have promoted the expression of these efflux pumps in 1_CH2 (Table 1). It was reported in *S. aureus* that the *norA* genes, which is encodes for the NorA efflux pump, was controlled by Fur in iron-restricted media and with iron concentrations at 10 and 100 μM (Deng et al. 2012). The same group observed expression of NorD, another efflux pump, in *S. aureus* under iron-limited conditions (Ding et al. 2012). Considering this possibility, iron did not promote growth after five hours and stationary phase was reached earlier than the controls. The slow, yet continuous, growth rate may be indicative of biofilm formation.

A biofilm formation assay was performed to determine if 1_CH2 growth in the presence of 3 mM $FeCl_3$ is the result of a biofilm. After 24-hours of growth, a biofilm was not observed

for either the untreated control or any of the treated wells (Figure 3). It may be that during early time points, growth of 1_CH2 was possible because the iron supplementing was enough to promote growth, but within five hours, the added nutrient was exhausted (Figure 2). It has been reported that in *P. aeruginosa* high levels of iron can repress expression of genes necessary for biofilm production (Musk et al. 2005) and induces twitching motility, which eliminates the biofilm (Patriquin et al. 2008). However, iron is still needed for biofilms to prevent twitching motility in *P. aeruginosa* (Singh et al. 2002; Weinberg 2004). Iron has also been shown to be important in *Streptococcus epidermis*, a Gram positive bacterium, where low concentrations of iron were needed to promote growth (Evans et al. 1994). In recent years, the role of Fur in biofilm development has been the subject of much investigation. A link between Fur and biofilm formation has been developed in *Staphylococcus aureus* (Johnson et al. 2005) and *P. aeruginosa*, by controlling carbon metabolism (Banin et al. 2005). The molecular mechanisms of how Fur can regulate the production of biofilms remains to be understood, but this study may provide support for the importance of iron with regard to biofilms.

When isolates were presented with 3 mM $FeCl_3$, biofilm development was not inhibited for 2_CH2 and 1_KK1. There was no indication that a biofilm had formed with 1_CH2 because the untreated control did not test statistically different to the positive control (Figure 3). Actually, the data suggests that treatment with 3 mM $FeCl_3$ promoted biofilm growth in 2_CH2. Nevertheless, the biofilm was prevented in 2_CH2 with treatment of 3 mM oxytetracycline and the dosage did not induced biofilm formation in either 1_CH2 or 1_KK1. The presence of oxytetracycline may inhibit the growth of 2_CH2 even though studies have concluded that biofilms are used to confer antibiotic resistance (Burmolle et al. 2006; van Acker et al. 2014). However, biofilms are not usually visualized before six hours and can take about 24 hours to

form. With *Pseudomonas fluorescens* it was shown that by 10 hours, the biofilm is visible by crystal violet staining (O'Toole and Kolter 1998). Therefore, if biofilm construction is not immediate, then planktonic cells have time to be affected by the antibiotic before assembling the biofilm, thus preventing their development (Burnolle et al. 2006; Hindre et al. 2008).

With oxytetracycline in the media, biofilm formation may be inhibited, and a previous study reported that increasing concentrations of tetracycline antibiotics will inhibit biofilm formation (Takahashi et al. 2006). That same study also reported that tetracycline increased bioactivity and may explain the phenomenon demonstrated by 1_CH2 and 1_KK1 when exposed to a combined dosage. With iron and oxytetracycline, activation of efflux pumps may be responsible for the development of this resistance to tetracycline. Iron can facilitate tetracycline by interacting with the regulator protein TetR(B) and inducing proteolysis of that protein (Ettner et al. 1993, 1995). Once TetR(B) has been degraded, tetracycline-resistance genes are activated. Another possibility, suggested by this study is the binding of iron to Fur and repressing expression of TetR(B) to enhance tetracycline resistance. Further studies are needed examining expression of Fur and TetR(B) regulator proteins, as well as efflux pumps controlled by TetR(B) family members.

As a master regulator, many organisms use Fur family to control gene expression. The evolution of Fur, and its homologues is needed if a potential role in antibiotic resistance regulation is to be explored. It is necessary to understand if an organism's environment can manipulate the function of Fur. Homologues have been described in *Bacillus subtilis.* Three Fur-like repressors have been reported, *yqkL*, *yqfV*, and *ygaG*, that share a relationship with Gram negative bacteria such as *E. coli*, *S. epidermidis*, and *S. pyogenes*, respectively. The sequences that are conserved between the Gram positives and the Gram negatives belonged to a helix-turn-

helix motif that is responsible for DNA recognition (Bsat et al. 1998). In a study of *fur* homologues in the Actinobacteria phylum found that gene duplications of this functional gene have made them specialized (Santos et al. 2008). In Figure 4, both *P. aeruginosa* MTB-1 and *P. spudix* are γ–proteobacteria, but their Class relationship may have caused the grouping in the phylogenetics tree. However, *P. aeruginosa* MTB-1 is never grouped with the other *Pseudomonas* spp. at either the DNA or the protein level, but it may be a result of the environment where these bacteria were originally collected. As reported in GenBank, both organisms were isolated from soils contaminated with the hydrocarbons hexachlorocyclohexane and BTEX, which is an acronym for benzenes, toluenes, ethylbenzenes, and xylenes. These chemicals can provide enough stress to affect soil microbial communities (Bordenave et al. 2007) and may influence species divergence. Environmental influences are known to drive the evolution of genes and result in the specialization of certain genes. A protein like Fur, which has evolved to produce family members able to bind a myriad of divalent metals, may have experienced the same effect (Zheleznova et al. 2000; Lee and Helmann 2007). A hypothesis by Santos et al. 2008 describes a split in the Fur protein sequence with the eubacterial common ancestor that may have led to a specialization event. These data suggest that such a split occurred and the *fur* gene began to evolve, producing distant relationships according to genus, yet environmental stimulates still influence the evolution of *fur*. If specialization events occurred, then the Fur protein tree shows closer relations that may be accounted for by conserved regions such as DNA recognition motifs needed to bind to the Fur box, which is a consensus sequence (Grifantini et al. 2003), and metal-binding motifs (Lee and Helmann 2007; Jacquamet et al. 2009). Although not addressed in this study, the variability in the protein may be caused by the

metal-binding motifs that may be altered depending on the environment and availability of certain metals.

A possible involvement of Fur in the regulation of efflux pump gene expression may exist, but further inquiry is needed. With an understanding of how three soil isolates responded to treatments of iron and oxytetracycline, the interaction of Fur and antibiotic resistance genes may exist. An exploration of this potential mechanism will provide insight into the depth of Fur regulation and provide a target for drug development, especially targeting multidrug resistant microorganisms. Since drug resistant bacteria are in constant interaction with the environment and responses to different stimuli, this may result in the expression of certain genes needed for survival. Antibiotics and heavy metals are both present in soil, and each can induce toxicity, so understanding how bacteria adapt to these stressors in essential in understanding the ecology of antibiotic resistance.

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