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We publish original research by undergraduate students in microbiology. This includes works in all microbiological specialties and microbiology education.

SCOPE

We are an international journal dedicated to showcasing undergraduate research in all fields of microbiology. *Fine Focus* is managed entirely by undergraduate students from production to print but utilizes an External Editorial Board of experts for double-blind peer review of manuscripts.

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TABLE OF CONTENTS

PERSPECTIVE

OBJECTIVE LENS

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Our second issue will end up in the hands of many first-time readers as more of you in the undergraduate microbiology research community learn of *Fine Focus*. We are pleased to call the American Society for Microbiology (ASM) a community partner, and acknowledge the kind assistance and support we have received from ASM, and many others, below. *Fine Focus* has been busy this year attending a variety of regional and national conferences, including the ASM General Meeting, ASMCUE, NCUR, the American Dairy Science Association (ADSA) national conference in Orlando, FL, as well as the Butler University Undergraduate Research Conference, the Indiana Academy of Science, and the Indiana Branch of ASM meetings. Many of you have visited us at one of these events and have received your copy of our first issue, and if so, we would love to hear from you about how you like *Fine Focus*.

Perhaps most noteworthy, we are excited to announce that for four months this Fall and Winter, *Fine Focus* will be (temporarily) managed by a group of biotechnology students at the University of Akureyri in Iceland. I will oversee these efforts while on my sabbatical leave this Fall term, and will assist these students garner a wider array of international submissions which should appear in the January 2016 issue.

As you look through this, our Vol. 1, issue 2, you will notice a contribution from Antony Schwartz, a new partner at the National Institutes of Health (NIH), discussing relevant biosafety issues in undergraduate microbiology research. We trust this information will be useful and interesting for you and your students, and we plan on including this as a regular feature into the foreseeable future.

Enjoy this issue, as it represents an extraordinary team effort on the part of a wonderful group of committed students. Be sure to read through this roster of *Fine Focus* participants, as well as our still-growing Editorial Board of experts, listed at the end of this issue. Please consider submitting your undergraduate manuscript to *Fine Focus*, at finefocussubmissions.org or request a free print copy online at finefocus.org.
We always welcome your suggestions and feedback by email at finefocus@bsu.edu
Enjoy your summer and best wishes for a productive Fall semester.

-JLM

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RHIZOFILTRATION OF LEAD CONTAMINATED SOIL BY *HELIANTHUS ANNUUS* AMENDED WITH *BACILLUS MEGATERIUM* AND EDTA

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- Rhizofiltration
- Lead
- Phylogenetics

ABSTRACT

Heavy metal contamination causes numerous adverse effects to public health and the environment. Sources of heavy metal contamination are widespread, especially in urban environments. Certain plants such as sunflower *(Helianthus annuus)* have been shown to sequester heavy metals in their root systems, thus filtering contaminants such as lead (Pb) from soil, a process termed rhizofiltration. In the present study, *Bacillus megaterium* was applied to the root system of sunflowers growing in Pb–contaminated soil and the efficiency of rhizofiltration was examined. Lead levels in the rhizosphere of the *Bacillus megaterium* and EDTA amended plants were almost 100 mg/kg soil higher than those without treatment, suggesting the amendment may have been effective in augmenting lead sequestration. In order to further elucidate these lead–sequestering communities, preliminary phylogenetic assays were conducted on the soil with and without the presence of the plant. Although complete coverage of the community phylogeny was not possible, there was evidence indicating that the rhizosphere may have induced changes in the composition of the bacterial community. These studies offer simple methods for enhancing bioremediation in agriculture.

INTRODUCTION

Heavy metals are defined as metals that have a specific density > 5g/cm^3 (17). Elevated concentrations of heavy metals in soil can have a devastating effect on human health and the environment. This is especially true for gardens located in urban areas. Human health is most adversely affected by certain heavy metals, namely cadmium, mercury, arsenic, and lead (17). Excess Pb is especially toxic to humans because of the effects it has on kidneys and the nervous system, which can cause headaches, weakness, cramps, anemia, and may lead to mental health disorders (1). According to the U.S. Environmental Protection Agency (EPA), lead can be found in the air, soil, water, and in homes because of its presence in gasoline, industrial facilities, paint, ceramics, pipes, batteries, cosmetics, ammunition, and even food (30).These make lead a priority heavy metal for study in urban environments.

The problem of heavy metal soil contamination can be addressed using a technique known as bioremediation. Bioremediation is the process of introducing organisms, such as bacteria, into a contaminated environment in order to

remove the pollutants. Phytoremediation is a subset of bioremediation that exploits plants in order to clean up contamination. Certain plants termed hyperaccumulators can store the heavy metals in tissues or the rhizosphere (root system and surrounding soil), therefore taking it out of biological circulation. This process is termed rhizofiltration. This study was conducted to quicken the remediation process and increase its efficiency without hampering cost effectiveness. Previous studies (13, 15, 18, 20) have indicated that adding chemicals or biological elements to the soil may help plants sequester metals in the tissues. This, in turn, reverses some of the effects of the metals by lowering the concentration in the surrounding area, allowing more plants that are less metal-tolerant to grow. After a rhizosphere absorbs as much metal as it can, the plant can be uprooted, allowing for removal of contaminants from the soil. This can alleviate some of the harmful impacts of metals on the environment. However, one of the problems with phytoremediation is that it is a slow process that produces a low yield (10).

One plant that has been shown to sequester heavy metals in the rhizosphere is the sunflower *(Helianthus annuus)* (18). Sunflowers are considered hyperaccumulators and have been used for various environmental cleanup projects, (7). Sunflowers also are more tolerant to pH variation than many other common plants, capable of growing in soil ranging from pH 5.7 to over 8.0, while optimal soil pH for other plants is 6.4 (24). Sunflowers also produce more roots, shoots, and total biomass than many other common plants. This means that they can potentially hold larger amounts of pollutants and fewer plants are required to recover the same amount of pollutants, thereby becoming economically sustainable.

Bioavailability of phosphate also has an influence on the effectiveness of phytoremediation. Addition of ethylenediaminetetraacetic acid (EDTA) has shown an increased Pb uptake by almost 20% in previous studies, and has also been shown to facilitate phytoremediation in plants (13, 20). EDTA is a common and powerful chelating agent that has been added recently into heavy metal treatment systems and works especially well with Pb and copper (4). This demonstrates that certain chemical additives can help sunflowers absorb higher levels of Pb, therefore ultimately reducing the cost, amount of land used, and amount of plants needed to grow in a particular plot of land when used for bioremediation.

Bacteria that reside in rhizospheres of plants can play a role in reducing the toxic effects of heavy metals on the plants (15). These microorganisms can protect the plant from damage and, in return, benefit from living in the rhizosphere systems, therefore creating a mutualistic relationship with the plant. In this study, *Bacillus megaterium* was used because this bacterium has been shown to absorb and store Pb intracellularly, therefore making it resistant to elevated levels of Pb (25). This species is also a common soil bacteria that is considered part of plant growth promoting rhizobacteria (PGPR), which helps improve growth by releasing a key auxin (indole-3-acetic acid) to encourage cell proliferation (3). Bacterial cultures were added to germinating seedlings to help improve the health of the plants for rhizofiltration and increase the concentration of Pb in the rhizosphere through the intracellular sequestration used by *B. megaterium*. The purpose of this experiment was to analyze the impact of the addition of bioavailable phosphate and heavy-metal tolerant bacteria on the Pb concentrations in highly contaminated soil.

MATERIALS AND METHODS

COLLECTION, SETUP, AND MAINTENANCE

For this experiment, three five-gallon plastic containers of soil were collected from two sites in an urban garden in Atlanta, Georgia, USA. These samples were then transported to the laboratory at Georgia Gwinnett College. The soil was characterized as being a crumbly, fine soil. The soil (2.5 grams, manually homogenized) was put into 500 mL conical Falcon tubes with 7.5 mL distilled H_2O and stored at -80˚C for DNA extraction.

A sample was taken from the middle of each container prior to planting seedlings, homogenized, and sent to the University of Georgia Soil and Water Analysis Lab for determination of Pb concentration. A Teddy Bear sunflower *(Helianthus annuus)* seed was planted one inch deep in nine 10" x 12" containers with each weighing approximately 0.95 kg per container. The samples were watered with 40 mL of tap water on alternate days for three months. After the seeds sprouted, a 40 Watt Growlux, wide-spectrum grow light (Grower's Supply, Dexter, MI) was placed on a timer for eight hours daily. Unplanted soil was maintained as the control experiment.

Sterile Luria broth (LB, 100 mL) was inoculated with *Bacillus megaterium* ATCC14581 and placed in a shaker for 24 h at 37°C. The nine containers of soil were then separated into three different categories. Three control containers were watered with 40 mL tap water on alternate days. Three EDTA containers were watered with 80µL EDTA once then 40 mL tap water on alternate days. Three EDTA plus *B. megaterium* containers were watered with 80 µL 0.1 M EDTA once, 1 mL of freshly made bacterial culture once, and then watered with 40 mL tap water on alternate days. The bacterial culture was then serially diluted onto LB agar plates to determine the original cell count. Sunflower height was then measured seven times for a total of three months and recorded. The measurements were taken from the base of the stem to the tip of the tallest leaf.

SOIL FILTRATION

Tubes with the most contaminated soil, according to the analysis results, were thawed, and 1 mL 0.1 M EDTA was added, along with 200 µL Tris buffer and distilled water to balance the tubes. They were centrifuged at 1000 rcf for 1 min. at 25˚C, then the supernatant was removed and 10 mL of Tris buffer was added. After thorough mixing with a vortex mixer, the tubes were weighed and water was added to balance them within 0.1 g. This was repeated three times. After the fourth centrifugation, the liquid was poured through muslin to filter out particles and 1 mL 1X Tris Borate EDTA (TBE) was added to the soil, weighed out in tubes, and centrifuged one more time at the same settings. The supernatant was transferred to two microcentrifuge tubes for storage at -80˚C for five days.

DNA EXTRACTION AND CLONING

The method of Tsai and Olson (29) was used for DNA extraction in the present study with the following modifications: Tris EDTA was used in place of Tris HCl, the tubes were stored in a -80˚C freezer instead of in dry ice, and the tubes were centrifuged at 6000 rcf for 10 min. before starting the protocol. Ten new PCR

samples were made with Cetyltrimethyl ammonium bromide (CTAB) and 5 g of soil was mixed with 3 mL distilled water and 5 mg/mL lysozyme. The mixture was placed in a shaker for 2 h. Four samples were used to proceed to the transformation and cloning steps. Ammonium acetate (5 mg/mL) was used instead of magnesium acetate. The phenol chloroform extraction was a 1:1 ratio of the working solution to phenol: chloroform: isoamyl alcohol (Sigma, St. Louis, MO). The mixture was centrifuged at maximum speed (13,000 rcf) for 10 min. The top layer was then removed using a pipette and 100 µL of ammonium acetate was subsequently added. Of this volume, 350 µL were transferred into four test tubes. An ethanol precipitation was carried out, after which a Tris–EDTA (TE) suspension and DNA samples were stored at –20°C.

For the Polymerase Chain Reaction (PCR) amplification, the primers used were 8F (5'–AGAGTTTGATCCTGGCTCAG–3') and 1492R (5'–GGTTACCTTGTTACGACTT–3') (Integrated DNA Technologies, Coralville, IA). PCR samples were prepared as follows: 1 µL of each primer (Integrated DNA Technologies, Coralville, IA), 2 µL of DNA, 46 µL distilled water (48 µL for the controls), and 50 µL of Master Mix (New England Biosystems, Ipswich, MA). Twenty PCR cycles were used as follows: 94°C for 30 seconds as a hot start and denaturation, 53° C for 30 seconds for annealing, 68°C for 1 minute for elongation, and 68°C for 5 minutes for final extension.

The samples were analyzed using Nanodrop 2000 by Thermo Scientific and Polymerase Chain Reaction was again conducted as the above paragraph described with the following modifications: 50°C for 30 seconds for annealing, 72°C for 45 seconds for elongation, and the positive control used *Bacillus subtilis* DNA (48 µL).

Upon maturation of the remaining plants, each was removed at the base of the stem and 700 g of soil was collected from the middle of the container and manually homogenized. One gram was taken from the homogenized soil and put into a test tube with 5 mL distilled water. They were each mixed and stored at –80°C for 30 min. Lysozyme (75 mg) was then added to each tube after being thawed in a bead bath at 65°C for 3 min., after which the tubes were stored at 37°C for 24 h.

Gene cloning of PCR products was done through a Tri–N–Octylphosphine Oxide (TOPO) cloning reaction with standard procedures. A series of minipreps were done from a QIAprep spin miniprep kit (from Qiagen, Germantown, MD) using manufacturer's instructions. PCR and agarose gel electrophoresis were completed (29). A Nanodrop analysis was then conducted for all 95 control samples. After testing, the data was analyzed using FinchTV, Seaview, BLAST, ClustalW, and Geneious and the samples were transferred to the University of Georgia Genomics Center to be sequenced.

SEQUENCING AND PHYLOGENETICS

Sequences were manually analyzed for quality using FinchTV. Following BLAST searches, the most similar sequences were selected and aligned *via* Seaview. ClustalW was used to make a phylogenetic tree including all of the samples. Geneious was used to make an outgroup phylogenetic tree using *Methanococcus voltae* as the outgroup. Two known organisms (16S genes of *Bacillus subtilis* and *Escherichia coli*) were also added to the tree.

RESULTS

Some of the original plants did not survive, leaving only control samples and amended samples. The original cell count was determined to be 2.21 x 10^7 cfu/mL. The initial Pb concentration was 542 mg/kg (Table 1). The final concentrations of Pb for the "control" plants was 531.2 ppm, while the final concentrations for the "non-vegetated" and "*B. megaterium* plus EDTA" samples were 567.0 mg/kg and 613.7 mg/kg, respectively.

The phylogenetic tree data for all samples from the non-vegetated and control treatment indicate that the samples were dominated by a Gram-negative rhizosphere-associated phylotype (Fig. 1). Figs. 2 and 3 show the phylogenetic trees for the control or non-vegetated samples, analyzed separately. The two known bacterial operational taxonomic units (OTUs) were included in each tree for comparison purposes. Table 2 includes five different classes of bacteria in the four clades in the total phylogenetic tree. Fig. 4 is a representation of the percent breakdown of each class that was discovered in the non-vegetated and control samples. These data reiterate the difference in *Proteobacteria* between the control and non-vegetated samples.

DISCUSSION

The control plant did not appear to effectively sequester Pb compared to the non-vegetated soil (Table 1). Explanations for this could include that earlier data for rhizofiltration used a different subspecies, or natural variability that could diminish with a larger sample size. However, the amended rhizosphere shows sequestration outside the range of this hypothetical variation. The final concentrations of the control and non-vegetated samples are within a 30 mg/kg range, so that variation is unlikely to be a determinant of Pb sequestration, seen in the *B. megaterium* plus EDTA samples (Table 1). However, the *Bacillus megaterium* plus EDTA sample had sequestered almost 75 mg/kg more than the initial concentration, or more than a 13% increase in mg/kg, as compared to the control (-1.9%) and non-vegetated (4.6%) samples. This suggests that *Bacillus megaterium* and EDTA may have helped the sunflower sequester more Pb as compared to the other samples.

The EDTA-alone samples were not viable, likely due to too high of a concentration of EDTA, and therefore Pb sequestration cannot be evaluated. For future experiments, the concentration of EDTA may be varied and optimized for the best results.

The non-vegetated and control sequences were used because studies have shown that non-amended sunflowers can carry out rhizofiltration and by using these sequences, the variety in the bacterial community could be analyzed (1, 2, 18). The phylogenetic tree (Fig. 1) is split into four clades with five distinct classes of bacteria. The first contains *Alphaproteobacteria*, which were only found in the control samples and had four different OTUs (Fig. 2). The *Alphaproteobacteria* also were the main bacterial class found in the control soil (Table 2). This could be because *Alphaproteobacteria* are the common inhabitants of the rhizosphere and therefore absent in soil without plants.

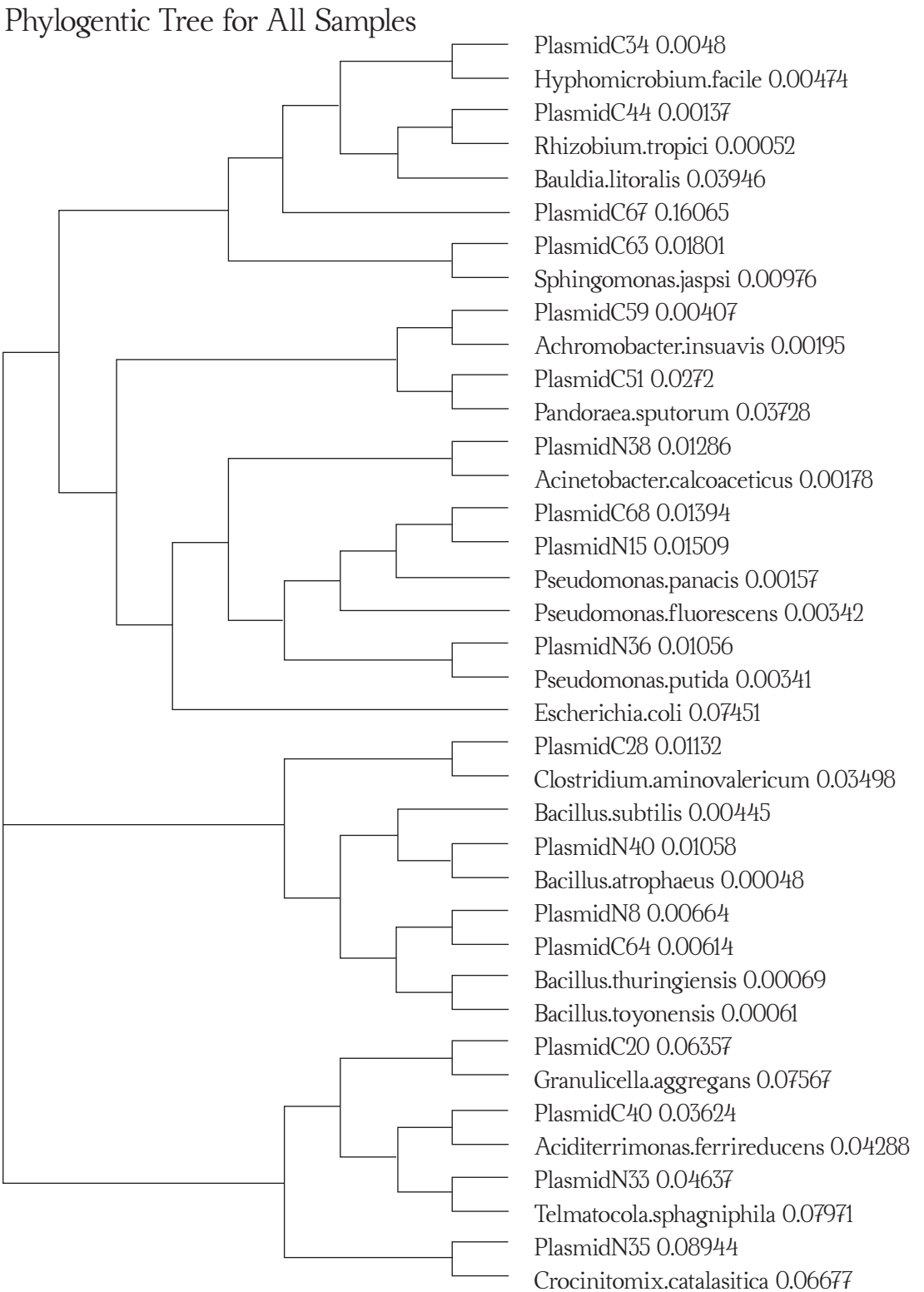

Figure 1. Phylogenetic Tree for all Non-vegetated (N) and Control (C) Samples.

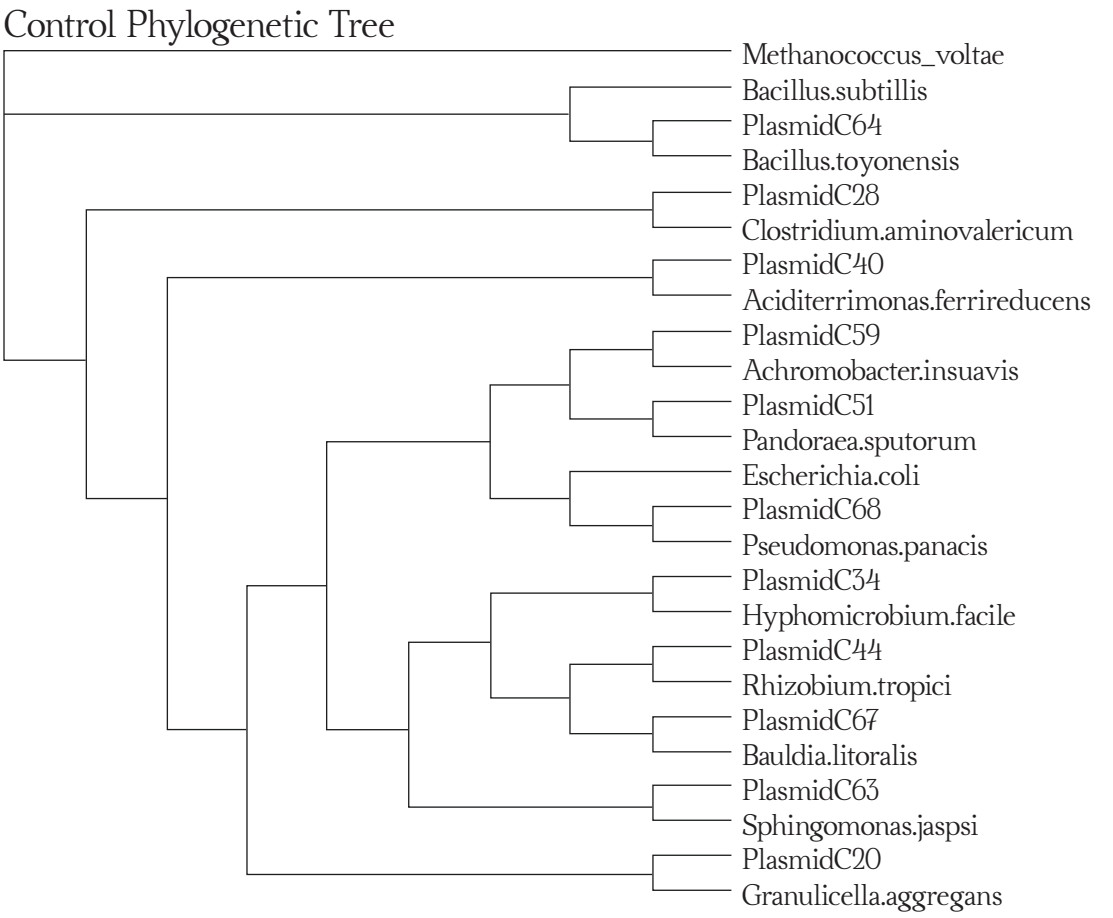

Figure 2. Outgroup Phylogenetic Tree for Control Samples.

This class of bacteria is important because they are a large and diverse group often symbiotically associated with plants. There are also species that can fix nitrogen and can be found in legumes and other plants. Legumes are hypogenous and these bacteria have adapted to create various classes of relationships with the root system, which supports the argument that they are an expected component of the rhizosphere. One OTU was found in the control samples that may correspond to bacteria such as *Rhizobium tropici*, which has been shown to fix nitrogen and be associated with legumes (22). This suggests that the rhizosphere may have recruited nitrogen-fixing bacteria to soil without the help of surrounding plants.

In the second clade, two classes are found: *Betaproteobacteria* and *Gammaproteobacteria*. Two OTUs which affiliate with *Betaproteobacteria* are only found in the control samples (Fig. 2). *Betaproteobacteria* share the same relative community composition in control soils with both *Firmicutes* and *Acidobacteria* (18%, Fig. 4). This class of bacteria consists of aerobic or facultative bacteria; they can be found in waste water and other environments and some can fix nitrogen like *Alphaproteobacteria*. One OTU found

Table 1. Initial and Final Lead Concentrations of Soil for Controls (C), Non-vegetated (NV), and *Bacillus megaterium* plus EDTA (BE) samples.

Initial and Final Lead Concentrations for Samples

Soil Type	Initial Pb Concentration	Final Pb Concentration	Total Percent Change
C	542.0	531.2	-1.992%
NV	542.0	567.0	4.613%
BE	542.0	613.7	13.229%

in these samples was related to *Pandoraea sputorum*. The genus *Pandoraea* is important to environmental health because of its known use of bio-catalytic activities, such as biodegradation (14). It is also closely related to species belonging to the *Ralstonia* lineage, which encompasses many heavy metal-resistant *Betaproteobacteria* (12). The OTUs found in these samples correlate with other samples that are found in highly polluted environments, which suggest an environmental selection for heavy metal resistance (28).

Gammaproteobacteria are a class of bacteria that contain environmentally important species, some of which are human pathogens. *Gammaproteobacteria* were found in both the control and non-vegetated samples, with a more plentiful amount found in the soil without plants (Table 2). For the control soil, this class is the least abundant (Fig. 4). However, in the non-vegetated soil, this class was the most abundant of all the other classes. This, along with previously stated data, suggests differences between the composition between the non-vegetated and control soil communities. It also suggests that the addition of the rhizosphere enriched certain types of bacteria suggesting that certain species may be integral to the observed augmented Pb sequestration (18, 26). A total of three different OTUs of *Pseudomonas* were detected in the samples and *Pseudomonas panacis* was detected in both the control and non-vegetated soil. *P. panacis* is root-associated and has been identified in root lesions of various plants (23). *Pseudomonas* spp. are known for aerobic growth and association with plants. *Pseudomonas putida* is an environmentally important species because it has been shown effective in improving the chemical and physical properties of polluted soil, bioremediating substances such as crude oil and naphthalene (11,21). *Pseudomonas fluorescens* is also an important species that inhabits the rhizosphere and is effective in bioremediation (31). This is a highly metal-resistant species that can tolerate millimolar concentrations of selected metals (5). The possibility of an enrichment of organisms with known association to polluted environments suggests that they are adapted to it and can help plants tolerate such environments, thereby supporting the previous studies of the possible use of

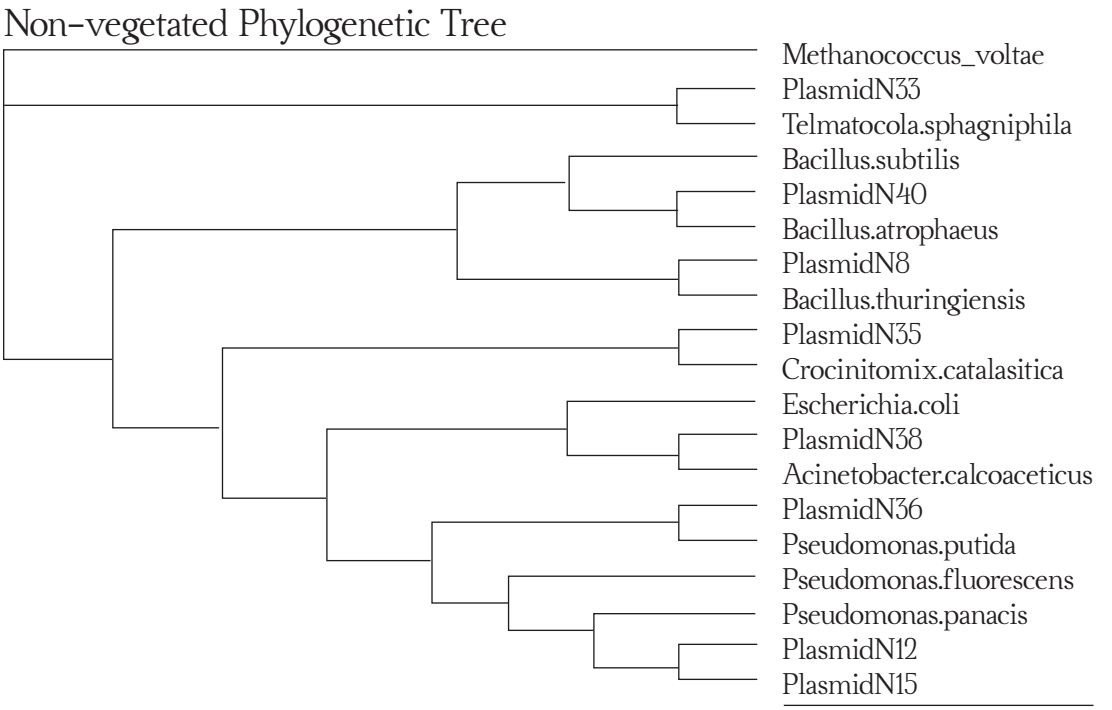

Figure 3. Outgroup Phylogenetic Tree for Non-vegetated Samples.

bacteria in bioremediation.

The third clade from Fig. 1 consists of a class of bacteria known as *Firmicutes*. Many *Firmicutes* produce endospores, have a low G+C content, and are known for carbohydrate degradation. If stress is sensed in soil, *Firmicutes* produce endospores, which can help them survive and resist desiccation. They also are abundant in root exudates with sugars and organic acids, specifically the genera *Bacillus* and *Clostridium* (27). *Bacillus* and *Clostridium* were both detected in this experiment in both types of soil at 50% (Table 2). *Clostridium*, particularly *Clostridium acetobutylicum* (a close relative to the obtained OTU related to *Clostridium aminovalericum*), is environmentally important, as previous studies have indicated its usefulness for degradation of large biological molecules, toxic organic molecules, and metals (8). This species has also been used in the bioremediation of soil and toxic sludge by chemically reducing and solubilizing the amount of radionuclides and toxic heavy metals (such as uranium (U), iron (Fe), magnesium (Mg), and (Pb)) (6). This species may help in remediation of soils, particularly those in urban locations, because of its potential use around industrial sites, buildings, and waste disposal sites.

Three different OTUs of *Bacillus* were also discovered in the samples. *Bacillus* may be a normal part of these ecosystems, suggesting that amendment with *B. megaterium* would not cause serious disruption of the bacterial community. This effect may be due to the ability of *Bacillus* spp. to survive and flourish in more hostile environments. One OTU that was found, associated with *Bacillus atrophaeus*, is a non-pathogenic, aerobic spore-forming

Table 2. Tables comparing percentage and species of bacteria found in each class according to the phylogenetic tree.

Comparison of Classes Found in Control and Non-vegetated Samples

Control Soil		
Class	Percent of Bacteria in Class	Number of Different Bacterial Species in Class
Alphaproteobacteria	100%	4
Betaproteobacteria	100%	2
Gammaproteobacteria	25%	1
Firmicutes	50%	2
Acidobacteria	50%	2

Non-vegetated Soil		
Class	Percent of Bacteria in Class	Number of Different Bacterial Species in Class
Alphaproteobacteria	0%	0
Betaproteobacteria	0%	0
Gammaproteobacteria	75%	3
Firmicutes	50%	2
Acidobacteria	50%	2

type of bacteria related to *B. subtilis* (9) (Fig. 3). *Bacillus thuringiensis*, known as the source for the entomopathogenic crystalline endotoxin for insect control, was found in the non-vegetative treatments. The last clade consists of *Acidobacteria*, a newer phylum comprising a diverse range of bacteria, especially soil-inhabiting species. *Acidobacteria* include species known to be Pb-tolerant and also sometimes acidophilic. These, like the *Firmicutes*, were found in equal proportions in the control and non-vegetated samples (Table 2). One OTU found, *Aciditerrimonas ferrireducens*, has been reported to be iron-reducing, which could have impacts on other heavy metals, such as Pb (16).

Phytoremediation, including rhizofiltration, is a vital area of inquiry because it is more cost effective and has fewer negative impacts on public health and the environment (19). These preliminary data suggest that there is a biologically helpful as well as economically viable method for increasing

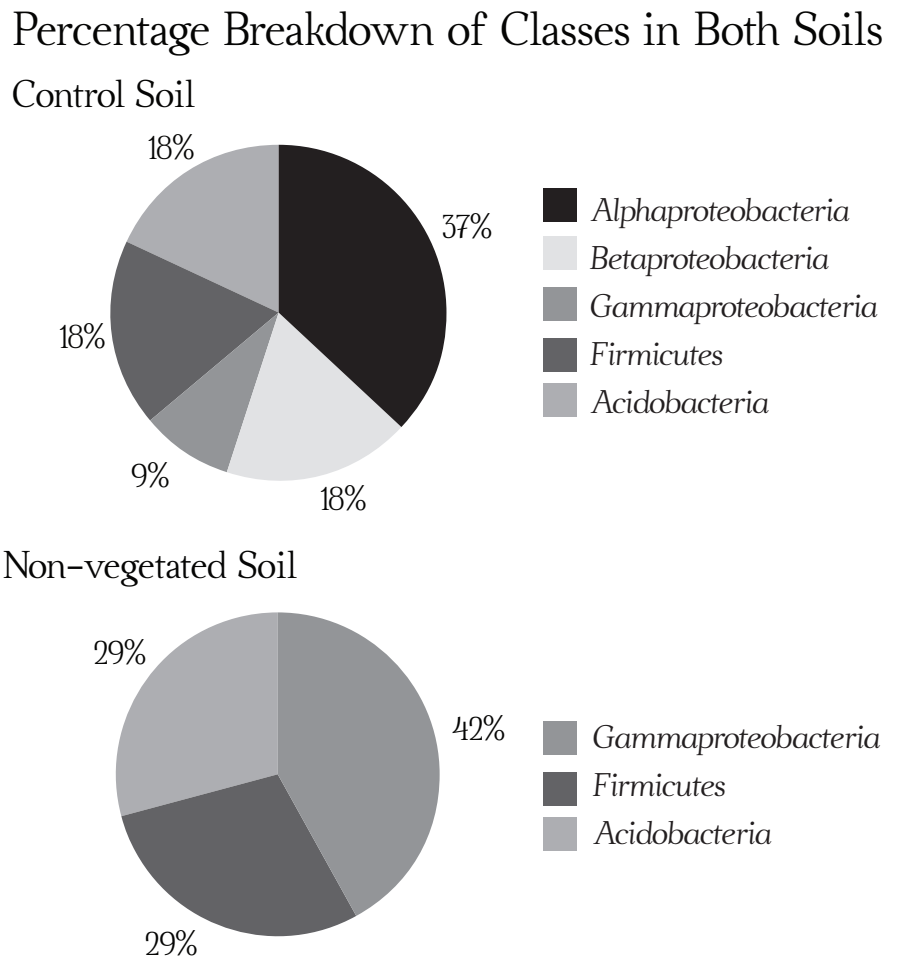

Figure 4. Pie charts representing the class diversity in the control soil and non-vegetated soil.

the efficiency of phytoextraction of Pb from soil. For example, the flower could be cut and sold to sustain the removal of the Pb-contaminated rhizosphere. It also could be used to address the problems of so-called 'food deserts' by eventually detoxifying soil in order to grow fresh produce in urban gardens. This investigation into the rhizosphere community without amendment suggests that *Acidobacteria* and *Firmicutes* may be a common component of Pb-contaminated soils but that the presence of the rhizosphere may have shifted the relative abundance of the *Proteobacteria* away from *Gammaproteobacteria* and towards *Alphaproteobacteria* and *Betaproteobacteria*. This may help elucidate the mechanism through which rhizofiltration occurs. These data aim to contribute to the ongoing process of understanding and improving on methods for removing hazardous pollutants from the environment. For future studies, a larger sample size could be used, as well as a lesser concentration of EDTA in order to see if the sunflowers remain alive for a longer period of time.

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DETECTION OF *BORRELIA* AND *EHRLICHIA* IN *RHIPICEPHALUS SANGUINEUS*

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- Borrelia
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- STARI

ABSTRACT

Rhipicephalus sanguineus, the brown dog tick, is endemic throughout the world wherever domestic dogs are present. It has been recently reported by some veterinarians in the city of Laredo, Texas, USA, that Lyme disease, the most common tick-borne disease in the Northern United States, is present in local domestic dogs. Fully engorged *R. sanguineus* ticks were collected and their DNA was purified. The ticks were screened to determine the prevalence of *Borrelia*, *Rickettsia* and *Ehrlichia* species. Sequences related to *Borrelia burgdorferi* in 9.8% (n=11/112), "*Candidatus* Borrelia lonestari" in 16.9% (n=19/112) and *Ehrlichia canis* in 12.5% (n=14/112) were detected by PCR. Sequencing has confirmed the presence of DNA from *Ehrlichia canis* and "*Candidatus* B. lonestari", corroborating that *Borrelia* and *Ehrlichia* are present in domestic dogs in South Texas.

INTRODUCTION

Rhipicephalus sanguineus, known as the brown dog tick, is the most widely distributed tick in the world (13). This tick is a known vector of *Ehrlichia canis* (23), the causative agent of canine ehrlichiosis (1,31). The symptoms of chronic *E. canis* infection in domestic dogs may include thrombocytopenia, anemia, weight loss, bleeding, fever, inflammation of the eye, and anoxic hepatitis (35). Acute ehrlichiosis in domestic dogs may result in loss of appetite, lethargy, shortness of breath, bruises, joint pain and depression (25). *R. sanguineus* is also thought to be a vector of *Rickettsia rickettsii* (13,22), the cause of the Rocky Mountain spotted fever (14). Although *R. sanguineus* ticks typically prefer to feed on domestic dogs, they have been reported to parasitize humans as well (20,33). The presence of a disease agent in the domestic dog population can indicate that the disease could also be present in humans (29).

B. burgdorferi sensu stricto (39) has been identified as the sole etiologic agent of Lyme disease in North America (24,38). Lyme disease is the most common vector-borne illness in the Northern United States (8) and is considered an emerging infectious disease (32). The main vectors are *Ixodes scapularis* (17,18,37) and *Ixodes pacificus* (34). The agent has also been detected at a lower incidence in *R. sanguineus* (9,21,42) and *Amblyomma inornatum* ticks (30). However, the vector potential of these ticks has not yet been characterized. It has been previously reported that *B. burgdorferi* in domestic dogs may result in arthritis, similar to

humans suffering from Lyme disease (27). Symptoms in domestic dogs from the acute form of Lyme disease may include fever, swelling, pain, lameness, lymphadenopathy and malaise (12). Acute renal failure, myocarditis, cardiac arrhythmia, peripheral edema, neurological syndrome and arthritis have been described as clinical signs found in the chronic form of Lyme disease in domestic dogs (2). A Lyme disease–like illness has also been described in the southern United States since the 1980s (41). This condition is referred to as the Southern Tick Associated Rash Illness (STARI), and is thought to be caused by "*Candidatus* B. lonestari" (40) after being bitten by *Amblyomma americanum* (3). The symptoms of "*Candidatus* B. lonestari" infection in domestic dogs have not been determined.

A veterinarian in Laredo, Texas, USA has reported that Lyme disease is present in local domestic dogs (Dr. Sandra Leyendecker, personal communication). *B. burgdorferi* has been previously detected in coyotes in Webb County (7), Texas. However, to the extent of our knowledge it has not been detected in domestic dogs. House pets, including dogs, have an increased exposure to ticks, and can serve as sentinel organisms for some diseases that occur in humans (29). Given the limited information available on vector-borne diseases in South Texas counties, there is a need for tick and pathogen surveillance in the area. This surveillance can help define areas at high risk for transmission (26) of infection to mammals, including humans. Identifying areas at high risk of transmission can increase awareness, potentially leading to the implementation of better diagnosis and prevention methods. In addition, as Laredo is the largest land-based port of entry in the United States, there is the movement of a large number of people and animals to and from this city into the rest of United States (11). For example, truck drivers frequently travel to Laredo, TX with their domestic dogs to warehouses in the city. They may have to wait a day or two before leaving to their destination. The purpose of this study was to determine the prevalence of DNA from tick-borne disease agents in domestic dogs from Laredo, Texas, by investigating the prevalence of *Borrelia*, *Ehrlichia* and *Rickettsia* species in *R. sanguineus* ticks.

MATERIALS AND METHODS

TICK COLLECTION AND IDENTIFICATION

Fully engorged adult *R. sanguineus* ticks were collected at multiple sites in Laredo, Texas. The ticks were collected from the walls of dog kennels, or from a CO_2 trap placed in the Laredo animal shelter. Ticks that were removed while grooming dogs were also collected from animal caregivers/owners. The researchers had no contact with any animal in the study. The ticks were counted and individually examined under the microscope to identify them to the species level using a published key (10).

DNA EXTRACTION

A total of 124 *R. sanguineus* ticks (55 males and 69 females) were used for DNA extraction using the E.Z.N.A. Mollusk DNA Isolation Kits (OMEGA Bio-tek, Norcross, GA, USA). A previously reported protocol was followed and modified as previously described (30). Briefly, the tick was homogenized in 300 µl lysis buffer.

Table 1. Primers and thermal cycler settings used in this study

Primers		
Gene	Name	Sequence (5' → 3')
12S rRNA	85F	TTAAGCTTTTCAGAGGAATTTGCTC
	225R	TTTWWGCTGCACCTTGACTTAA
flaB	FlaLS	AACAGCTGAAGAGCTTGGAATG
	FlaRS	CTTTGATCACTTATCATTCTAATAGC
	BL-Fla522F	GGTACATATTCAGATGCAGACAGAGGG
	BL-Fla1182R	GCACTTGATTTGCTTGTGCAATCATAGCC
	BL-Fla662F	AACTGCTGAAGAGCTTGGAATGC
dsb	BL-Fla860R	AGCTGGTTGAACTCCTTCCTGTTGT
	Ehr-DSB-330F	GATGATGTCTGAAGATATGAAACAAAT
16S rRNA	Ehr-DSB-728R	CTGCTCGTCTATTTTACTTCTTAAAGT
	B16S-FL	GACTCGTCAAGACTGACGCTAAGTC
	B16S-R	GCACACTTAACACGTTAGCTTCGGTACTAA
	BL-16S5F	CAGTGCGTCTTAAGCATGCAAGTCAGACGG
	BL-16S486R	CTGCTGGCACGTAATTAGCCGGGG
	B16S-23S-IGSF	GTATGTTTAGTGAGGGGGGTG
	B16S-23S-IGSR	GGATCATAGCTCAGGTGGTTAG
	B16S-23S-IGSFn	AGGGGGGTGAAGTCGTAACAAG
	B16S-23S-IGSRn	GTCTGATAAACCTGAGGTCGGA
	ECAN-F	ATTTATAGCCTCTGGCTATAGGA
	HE1-F	CAATTGCTTATAACCTTTTGGTTATAAAT
rompA	HE3-R	TATAGGTACCGTCATTATCTTCCCTAT
	Rr190 70P	ATGGCGAATATTTCTCCAAAA
	Rr190 602N	AGTGCAGCATTCGCTCCCCCT

The tick was crushed for 5 minutes using a sterile microtube and pestle. After adding proteinase K, the samples were incubated at 55°C for 3h. The sample purification was then completed following the manufacturer's protocol.

POLYMERASE CHAIN REACTION (PCR)

The samples were screened using PCR for the tick 12S rRNA gene as previously described (30). Samples positive for tick rDNA (n=112) were subjected to PCR for amplification of *Borrelia*, "*Candidatus* B. lonestari", *Ehrlichia* and *Rickettsia* bacteria species (43,44).

Table 1 *(ctd.)*. Primers and thermal cycler settings used in this study

Amplicon length	PCR conditions			
Specificity	Denaturing	Annealing	Extension	Cycles
140 bp	95°C, 30sec	45°C, 30sec	72°C, 1min	40
Not Reported				
353 bp	95°C, 1min	55°C, 1min	72°C, 1min	36
Borrelia genus				
660 bp	95°C, 1min	55°C, 1min	72°C, 1min	46
"Candidatus B. lonestari"				
198 bp	95°C, 1min	55°C, 1min	72°C, 1min	36
"Candidatus B. lonestari"				
398 bp	95°C, 1min	55°C, 1min	72°C, 1min	46
Ehrlichia genus				
131 bp	95°C, 15sec	58°C, 30sec	72°C,30sec	40
Borrelia genus				
481 bp	95°C, 1min	60°C, 1min	72°C, 1min	36
"Candidatus B. lonestari"				
Variable	94°C, 30sec	56°C, 30sec	74°C, 1min	35
Borrelia genus				
Variable	94°C, 30sec	60°C, 30sec	74°C, 1min	40
Borrelia genus				
383 bp	94°C, 30sec	52°C, 30sec	72°C, 1min	36
E. canis				
383 bp	94°C, 30sec	52°C, 30sec	72°C, 1min	36
E. chaffeensis				
Ehrlichia genus	94°C, 30sec	52°C, 30sec	72°C, 1min	36
532 bp	95°C, 1min	55°C, 1min	72°C, 1min	46
Rickettsia genus				

The PCR mixture was a 25 µL reaction volume containing 0.25 µL of GoTaq polymerase (Promega, Madison, WI), 1X GoTaq Buffer, 160 ng/µL bovine serum albumin, 1.0 mM $MgCl_2$, 200 µM of each dNTP, 2 pmol primers, and 5µL of template (1 µL for nested reactions). Amplifications were performed on a Bio-Rad MyCycler thermal cycler (Bio-Rad, Carlsbad, CA) (30). Different thermal cycler settings, as indicated in Table 1, were used for different primers due to different optimal annealing temperatures. An initial denaturation step (95°C) of 5 min. and a final extension step (72°C) of 5 min. were used.

Table 2. Detection of bacterial DNA in adult *R. sanguineus* ticks by PCR

	B. burgdorferi–like species	"*Candidatus* B. lonestari"	*E. canis*	*Rickettsia*	*B. burgdorferi*–like species +"*Candidatus* B. lonestari"	*E. canis* + *B. burgdorferi*–like species
Male	12.2% (6/49)	16.3% (8/49)	8.2% (4/49)	0% (0/49)	2.0%(1/49)	4.1% (2/49)
Female	7.9% (5/63)	17.5%(11/63)	15.9% (10/63)	0% (0/63)	1.6%(1/63)	0% (0/63)
Total	9.8% (11/112)	16.9% (19/112)	12.5% (14/112)	0% (0/112)	1.8% (2/112)	1.8% (2/112)
2005	9/85	16/85	11/85	0/85	2/85	2/85
2006	1/7	1/7	0/7	0/7	0/7	0/7
2009	1/11	2/11	1/11	0/11	0/11	0/11
2010	0/4	0/4	1/4	0/4	0/4	0/4
2011	0/5	0/5	1/5	0/5	0/5	0/5
Total	11 of 112	19 of 112	14 of 112	0 of 112	2 of 112	2 of 112

Nested PCR procedures were performed for *Borrelia* and "*Candidatus* B. lonestari" using 1µl from the initial reaction as a template. Amplification of target sequences was performed in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Carlsbad, CA) with several denaturing, annealing, and extension times and temperatures (Table 1). For each PCR assay, 5µL of sterile distilled water was used instead of template DNA as a negative control. The positive controls, when used, were added using separate hoods and pipettors to reduce the risk of cross contamination.

VISUALIZATION AND SEQUENCING OF PCR PRODUCTS

Five microliters of each PCR reaction was subjected to gel electrophoresis, using 2% agarose gels stained with ethidium bromide in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM disodium ethylene diamine tetraacetic acid) with 0.00005% ethidium bromide. The gels were run at 100 V for 40min.. After electrophoresis, the gels were examined under UV light. Positive samples were purified using SpinPrep PCR Clean-UP Kits (Novagen, La Jolla CA, USA) following the manufacturer's protocols. Each purified PCR product was sent for Sanger sequencing at Eurofins (Alabama, USA) or MCLab (San Francisco, CA) using the primers used for PCR.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The DNA sequences were visualized using Finch TV, Geospiza, Inc software version 1.5.0 and compared to reported sequences in the NCBI GenBank using BLAST. The assigned GenBank accession numbers are: KR183798-KR183823.

PHYLOGENETIC ANALYSIS

Initial alignments for *Borrelia* and *Ehrlichia* genes were executed using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (16) as performed by the European Bioinformatics Institute's

Fig. 1. Baysian inference consensus tree inferred from *flaB* of *Borrelia* species. Node support is indicated by the posterior probabilities at the node. The name of the species is followed by the GenBank accession number. A25B–F5, A25B–M1, A25B–M2, A25B–M11, A25B–M16, A25B–M17, A25B–M18, A25B–M19, A32B–F2, A32C–F5, A35A–M3, A46BB–F1, A46BB–F3, A46BB–F4, B17Z09–F2, B17Z10–F1 and E49Z02–M1 represent amplicons from *R. sanguineus* and are underlined. The scale bar indicates the mean number of nucleotide substitutions per site.

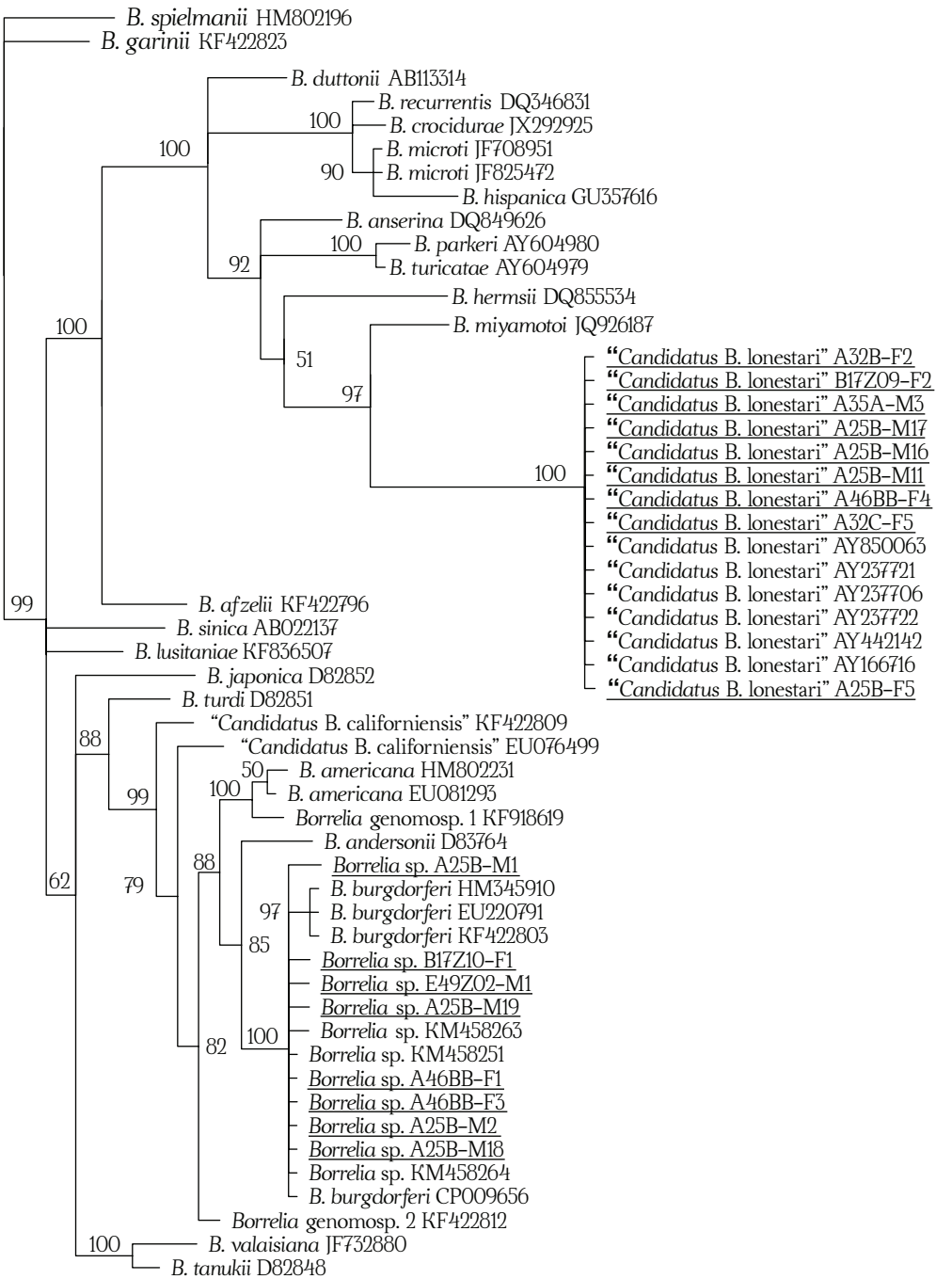
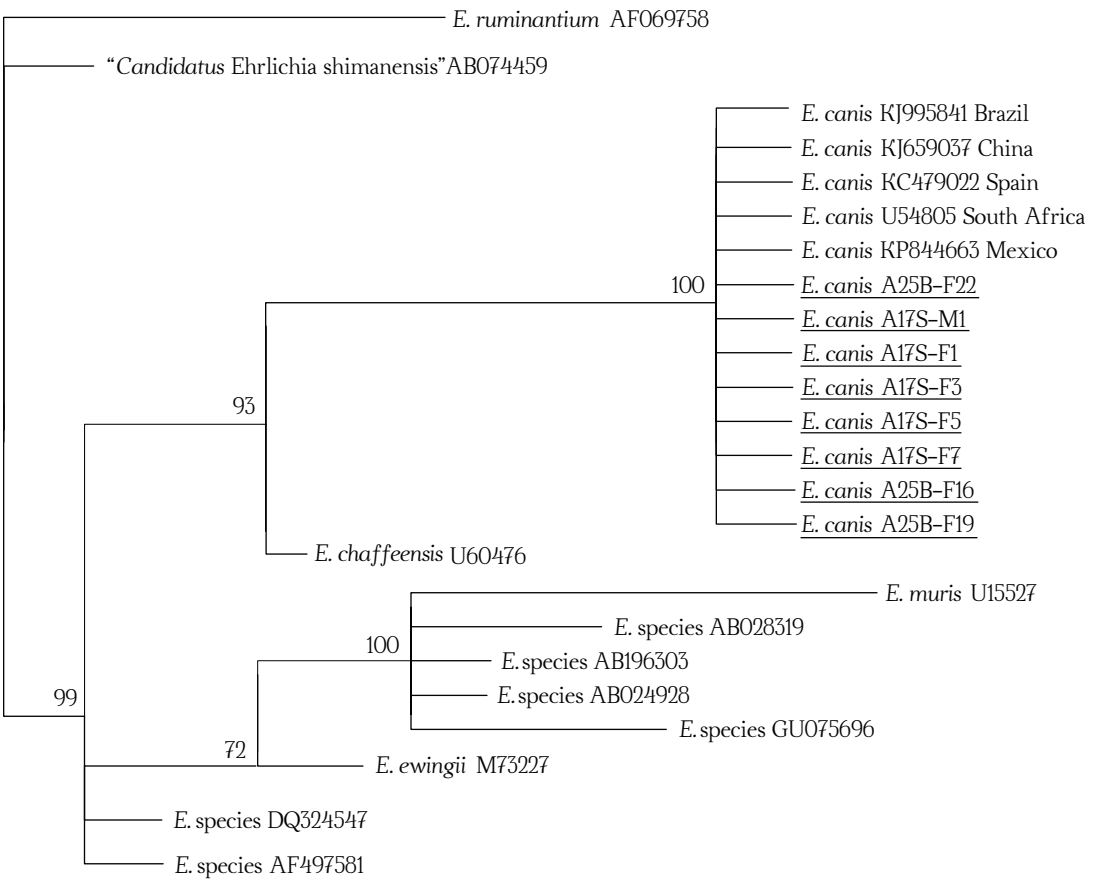


Fig. 2. Baysian inference consensus tree inferred from 16S rDNA of *Erhlichia* species. Node support is indicated by the posterior probabilities at the node. The name of the species is followed by the GenBank accession number. A17S–M1, A17S–F1, A17S–F3, A17S–F5, A17S–F7, A25B–F16, A25B–F19 and A25B–F22 represent amplicons from R. sanguineus and are underlined. The scale bar indicates the mean number of nucleotide substitutions per site.



MUSCLE server (http://www.ebi.ac.uk/Tools/muscle/). Default settings were used, with posterior manual adjustments if needed. Bayesian inference phylogenetic analyses were performed using MrBayes v.3.2.5 (36) using two runs, for 10,000,000 generations each, using eight chains and a temperature coefficient of 0.1, and trees sampled every 5,000 generations. Determination of the appropriate model for each genus was completed via jModelTest 2 (15): GTR + I + Γ for *Borrelia* and *Ehrlichia*. The gamma distribution included six categories for all models obtained. After analysis was completed, the first 25% of trees from each run were discarded as burnin. The consensus trees were observed in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

PCR DETECTION OF *BORRELIA* AND *EHRLICHIA*

Of the 112 positive samples for tick rDNA, 44 ticks (18 males and 26 females) were positive for *B. burgdorferi*–like species, "*Candidatus* B. lonestari" or *Ehrlichia* bacteria species (Table 2). "*Candidatus* B. lonestari", the most commonly detected tick-borne pathogen, was detected in 16.9% (n=19, 16.3% of males (8/49) and 17.5% (11/63) of females) of all ticks.

B. burgdorferi–like species were detected in 9.8% (n=11, 12.2% (6/49) of males and 7.9% (5/63) of females) of all ticks. *E. canis* was detected in 12.5% (n= 14, 8.2% (4/49) of males and 15.9% (10/63) of females) of all samples. Two male ticks (4.1%, n=2 of 49) were positive for both *E. canis* and *B. burgdorferi*–like species, indicating a 1.8% co-infection rate (2 of 112) of all ticks. One male and one female tick (male: 2.0%, n=1 of 49; female: 1.6%, n=1 of 63) were positive for both "*Candidatus* B. lonestari" and *B. burgdorferi*–like species, indicating a 1.8% co-infection rate (2 of 112) of all ticks. No tick was positive for the spotted fever group rickettsial *rompA* gene.

PHYLOGENETIC ANALYSIS

Samples with strong bands by gel electrophoresis were prepared for sequencing. Sequencing confirmed the PCR results. A Bayesian inference tree including these sequences and other published sequences are shown in Figs. 1 and 2. Phylogenetic analysis showed that "*Candidatus* B. lonestari" amplicons were in the same clade as known sequences of "*Candidatus* B. lonestari" and *B. burgdorferi*–like species amplicons clustered with *B. burgdorferi* species complex (Fig. 1). "*Candidatus* B. lonestari" sequences were all identical except A25B-F5, which was polymorphic at one position. Three of the *B. burgdorferi*–like species sequences (B17Z10-F1, A25B-M1 and E49Z02-M1) were different at one position. *Ehrlichia* amplicons were in the same clade as known sequences of *E. canis* (Fig. 2).

DISCUSSION

The ticks used for this study were fully engorged. Thus, the detection of pathogen DNA either represents the most recent blood meal or potentially a prior infection of the tick. We detected the presence of *B. burgdorferi*–like species, "*Candidatus* B. lonestari" and *E. canis* DNA in *R. sanguineus* ticks from Laredo, Texas. Our study does not address the issue of vector competency of *R. sanguineus* ticks in regard to *B. burgdorferi*–like species and "*Candidatus* B. lonestari".

The detection of pathogens in canines can indicate a potential risk for infection of humans (28,29). We have detected *B. burgdorferi*–like species DNA in 9.8% of ticks collected from the local animal shelter, as well as ticks submitted by pet caregivers. The main vector for *B. burgdorferi* in the Northeastern United States is *I. scapularis* (17,18,37). This tick is present throughout much of Texas and Northern Mexico. *B. burgdorferi* was previously detected in 45% of tested *I. scapularis* ticks (19). However, in Webb County TX, no *I. scapularis* were identified in the combined collection of over 70,000 ticks (5). *B. burgdorferi*–like

species have been previously reported in *A. inornatum* from Webb County (30) and *A. mixtum* from Northeastern Mexico (21). We also detected "*Candidatus* B. lonestari", which is thought to be the cause of STARI (40), in 16.9% of ticks. Cohen *et al.* (1990) reported a 5.5% seroprevalence for *Borrelia* in domestic dogs from Texas. However, of the dogs in their study 0 of 5 dogs that came from Webb County were seropositive (9). Likewise, Bowman *et al.* (2009) reported *B. burgdorferi* in Central and Northern Texas, but did not have any results for South Texas. This is the first report of *Borrelia* from *R. sanguineus* ticks and domestic dogs in Webb County, Texas.

R. sanguineus is the only known vector of *E. canis* (23), and is widely distributed throughout the United States (6). We detected *E. canis* in 12.5% of ticks. However, all of the ticks that were positive for *E. canis* were collected from the Laredo animal shelter. Many pet owners acquire their pet from the animal shelter. These dogs are at a high risk for acquiring *E. canis* at this location. However, domestic dogs in the city of Laredo appear to be at a low risk for acquiring *E. canis*. The previously reported seroprevalence for *E. canis* (2.0%) was higher in Texas than in much of the United States (4). We did not detect *E. chaffeensis* and *E. ewingii* in *R. sanguineus*, even though they are present in the area (30). We also did not detect any spotted fever group *Rickettsia*. This would suggest that any spotted fever group *Rickettsia* are either absent from the area or were present at a very low prevalence.

Further research on *R. sanguineus* distribution and the prevalence of *B. burgdorferi*–like species, "*Candidatus* B. lonestari" and *Ehrlichia* species in South Texas is needed. Further research will help elucidate if *R. sanguineus* is a vector of a *B. burgdorferi*–like species. This additional research would allow for better and more accurate diagnosis of tick-borne illnesses, ultimately leading to better treatment and health care for domestic dogs and humans. This research supports the observation of Dr. Sandra Leyendecker and suggests that domestic dogs should be screened for Lyme disease if they present with appropriate symptoms.

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CHARACTERIZATION OF A MUCOID-LIKE *PSEUDOMONAS AERUGINOSA* BIOFILM

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ABSTRACT

Pseudomonas aeruginosa biofilms are implicated in chronic infections. A key element of *P. aeruginosa* pathogenicity is the formation of a biofilm, a community of bacteria encased in an exopolymeric substance (EPS) that shields the bacteria from the host immune response and antibiotic treatment. A crucial step in biofilm production is a switch in motility from freely swimming, planktonic bacteria to twitching movement and then to attached and sedentary bacteria that develop into a mature pillar-shaped biofilm. A mucoid biofilm produces an excess of alginate and is clinically the most pathogenic and the most resistant to antibiotics. Biofilms from patients exhibit a wide variety of structure, motility, and levels of attachment. *In vitro* biofilms do not exhibit such a wide variety of structure and physiology. The difference between *in vivo* and *in vitro* biofilms has made the translation of *in vitro* studies into *in vivo* treatments difficult. Under different growth conditions in our lab, the *P. aeruginosa* strain PAO1 demonstrates two phenotypes: a non-mucoid and a mucoid-like phenotype. Confocal laser scanning microscopy (CLSM) indicates the mucoid-like phenotype is intermediate in height to the non-mucoid phenotype and biofilms formed in a once-flow-through chamber. Both mucoid-like and non-mucoid phenotypes exhibit a significant increase in twitching between 24 and 72 hours of development. The mucoid-like phenotype had greater attachment at 72 hours compared to non-mucoid phenotype. Therefore, the two phenotypes observed in our lab may represent the effect of environment to stimulate development of two types of biofilms by PAO1.

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KEYWORDS

- Psl
- Pel
- alginate
- type IV pili
- flagella

INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen implicated in chronic infections in cystic fibrosis (CF) (15, 17, 33) and chronic wounds (4, 8, 17). Its virulence is due, in part, to formation of biofilms which confer resistance to both the innate immune system and antibiotics. Biofilms recovered from the sputum of CF patients

can exhibit a widespread variability in biofilm formation both in structure and virulence (1, 10, 27, 33).

BIOFILM FORMATION

Biofilms are understood to have several steps in their formation (8, 33). There is an initial attachment to a surface followed by microcolony formation. The biofilm may then mature to a pillar-shaped structure which can release planktonic (freely moving) bacteria to colonize new areas. Inside biofilms, cells in different regions exhibit differences in gene expression as well as functional heterogeneity, indicating a complex community of cells within biofilms (8). The time span for the formation of a pillar-shaped biofilm depends on many factors, but has been estimated to take 5–7 days at minimum (33).

The triggers for biofilm formation are complex, but one trigger is the formation of a quorum sensing (QS) signaling cascade. One QS molecule, N-(3-oxodo-decanoyl)-L-homoserine lactone (3OC12-HSL) is constitutively released from *P. aeruginosa*. When *P. aeruginosa* has multiplied and reached a large enough population (i.e. a "quorum"), 3OC12-HSL levels reach a threshold and signal extensive changes in gene expression through the LasR-LasI system (8, 9, 28, 33). One of these changes includes the formation of a second QS molecule, N-butyryl-L-homoserine lactone (C4-HSL). C4-HSL acts through the RhlR-RhlI system independently and in a coordinated fashion with 3OC12-HSL further influences gene expression. In fact, blocking QS molecule action has been one target of therapeutic interventions, but with limited success (2).

EXOPOLYMERIC SUBSTANCE (EPS)

Biofilm maturation requires the formation of an exopolymeric substance (EPS) which confers antibiotic resistance and protection from host immunity by the biofilm (4, 8, 33). The EPS in a *P. aeruginosa* biofilm is composed of three components: alginate, Psl, and Pel, although other polymers such as certain proteins, lipids, and extracellular DNA also contribute to biofilm structure (12, 24, 25, 36).

Psl, composed of rhamnose, mannose, and glucose, is required for maintenance of any biofilm structure, and for biofilm construction (31, 36). Overproduction of Psl promotes mushroom-shaped biofilms (36). Pel, a glucose-rich component of the EPS, is required for initiation of any biofilm, as well as for the growth of air-liquid interface biofilms (31, 36).

The final EPS component, alginate, is a complex polysaccharide composed of two different sugars, ß-D-mannuronate and L-glucuronate, and is one of the most studied and well understood portions of the matrix. Over-expression of alginate causes what has been termed the mucoid biofilm phenotype for its mucus-like appearance (31). Mucoid biofilms have been shown to be more virulent and are often found in chronic infections (11, 23). Mutant strains of *P. aeruginosa* which produce mucoid biofilms can be studied *in vitro*; however, the transformation of wild-type *P. aeruginosa* into a mucoid biofilm is not well understood (7). Because of the homogeneity of *in vitro* biofilms grown in the laboratory setting and the heterogeneity of *in vivo* biofilms as recovered from CF patients, it has been difficult to study these pathogenic mucoid biofilms within the lab.

This heterogeneity of biofilms recovered from CF patients is well documented. They differ structurally as demonstrated

in scanning electron microscopy as pillar-shaped, knobby, or flat (10). The PAO1 within biofilms are also functionally distinct as seen in the QS molecules released, their virulence, the types of motility they exhibit, their ability to attach to a surface, and their response to treatment (1, 27, 33). In recent years several strategies have been employed to characterize *P. aeruginosa* isolates from patients in order to identify the most effective treatment. Virulent strains of *P. aeruginosa* form biofilms, but also have decreased motility and decreased production of virulence factors such as pyocyanin (1, 27). It has been hypothesized that a lack of production of flagella or type IV pili might help *P. aeruginosa* evade the host immune system since both innate and adaptive immune systems respond to epitopes on flagella and type IV pili (1, 27). A lack of motility may also increase the ability of *P. aeruginosa* to attach to a surface and produce a biofilm. These studies also demonstrated a decrease in production of virulence factors such as pyocyanin (1, 27). Virulence factors are common in acute infection and may spur the immune system response. Therefore, eradicating or decreasing the production of virulence factors may decrease stimulation of the host immune system by *P. aeruginosa*, increasing the ability of *P. aeruginosa* to evade the host immune response. Finally, the production of biofilms increases the ability of *P. aeruginosa* to evade the host immune system by creating the exopolysaccharide (EPS) matrix that limits the ability of the host immune system to reach *P. aeruginosa*.

Clinical isolates of *P. aeruginosa* biofilms can be classified by their motility patterns and biofilm structure (1, 10, 27). Mulet *et al.* (2013) demonstrated that high risk clones of *P. aeruginosa* from CF sputum contained greater drug resistance as well as increased biofilm production, decreased twitching, and decreased production of pyoverdine and pyocyanin, two virulence factors. Therefore, changes in motility from swimming to swarming to twitching are a standard part of the formation of biofilms. These changes allow *P. aeruginosa* to attach to a surface, form a microcolony, and finally, form a biofilm. However, the emerging clinical picture indicates that motility patterns are being used to classify and characterize clinical isolates based on their virulence and ability to develop chronic infections (10, 27). In the clinical setting then, motility may not only serve a role in biofilm formation, but may also serve as an additional marker that may lead to identification of the most effective methods for treatment of chronic *P. aeruginosa* infections.

MOTILITY

P. aeruginosa exhibits swimming, swarming, and twitching types of motility. Twitching is mediated by type IV pili which act as retractable arms that can pull or "slingshot" cells across a surface (5, 19). Twitching is usually seen in more viscous types of media or environments. Swimming through a liquid medium is performed by the use of flagella (18, 36). Swarming movements use both type IV pili and flagella resulting in a movement in between swimming and twitching (14, 36). To test motility, assays are used measuring distance displaced by *P. aeruginosa* on plates of media with different fluidity (5, 10).

As previously stated, the motility of *P. aeruginosa* within a biofilm can be used to identify virulent strains that are more likely to cause chronic infection. However, the correlation between motility and its role in biofilm formation is not entirely clear. Swimming motility may not be required

for biofilm formation, but may be important in allowing *P. aeruginosa* to reach an environment with greater nutrients or to colonize a new area (36). Therefore, although it may not be directly involved in the stage of biofilm formation, swimming motility may play a role in determining sites where *P. aeruginosa* can travel to form a biofilm.

Perhaps the most critical type of motility for *P. aeruginosa* during biofilm formation is the ability to twitch. This is how bacteria move on a solid surface, perhaps as they are attaching to a surface to form a microcolony (10, 36, 37). Psl may be secreted to coat a solid surface as bacteria twitch in a spider web fashion. Other bacteria then attach to this surface to form a microcolony, which is one of the first steps in biofilm formation (36, 37). There is evidence that bacteria favor the use of flagella to swim or type IV pili to twitch; once there is a switch to a different type of movement, it may be difficult for the bacteria to revert to the previous type of motility (35).

Swarming, which uses both flagella and type IV pili, may represent *P. aeruginosa* in transition from swimming to attachment in which twitching will then predominate. Originally, research indicated that swarming is only mediated by flagella (10); however, more recent data indicate the use of both flagella and type IV pili are required (5, 36). Therefore, swarming may be an intermediate form of motility for *P. aeruginosa* between swimming which requires only flagella and twitching which requires only type IV pili. In fact, data indicate that bacteria can twitch or swim, but rarely do both well (35), indicating that modes of motility vary according to stimuli.

TWO PHENOTYPES OF PAO1 DEVELOP UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

PAO1 is a strain of *P. aeruginosa* commonly used in laboratory experiments. We have been able to grow PAO1 in our lab with two distinct phenotypes, which have named non-mucoid and "mucoid-like" phenotypes. We wanted to investigate the differences in structure, motility, and surface attachment between these two phenotypes. It is our hypothesis that PAO1 in the mucoid-like phenotype produce a biofilm closer to a "mucoid" biofilm with greater twitching motility, greater attachment to a surface, and greater height and pillar-shaped structure, when compared to the non-mucoid phenotype of PAO1.

To investigate these two phenotypes we used confocal scanning laser microscopy, motility assays, and a surface attachment assay. If the mucoid-like phenotype exhibits greater twitching behavior, surface attachment, and more pillar-shaped morphology than the non-mucoid biofilm, these environmental conditions may be replicated to study different phenotypes of PAO1 biofilms.

MATERIALS AND METHODS

GROWTH OF MUCOID-LIKE AND NON-MUCOID CULTURES:

PAO1 (ATTC BAA-47) cultures were grown overnight in 10 ml tryptic soy broth (TSB) (Difco Laboratories, Sparks, MD) in standard 16 ml glass test tubes with a plastic cap (VWR, 89000-482) before being streaked for isolation on tryptic soy agar (TSA) plates (Difco Laboratories, Sparks, MD). Single colonies were used to inoculate TSB under two environmental conditions to produce biofilms with either mucoid-like or non-mucoid phenotypes. Mucoid-like phenotype PAO1 biofilms were grown in 10 ml TSB in standard 16 ml glass test tubes with a plastic cap (VWR, 89000-482) on a shaker incubator at 37°C and 225 RPM. Non-mucoid phenotype PAO1 biofilms were grown in a 250 ml Erlenmeyer flask (VWR, 29136-060) with 100 ml TSB and a foil cap at the same conditions. The shaking was set so that the biofilms did not grow on the surface of the culture or on a solid surface, but throughout the media. The two phenotypes were easily distinguished. The mucoid-like phenotype developed strings of a mucous-like substance that spun down toward the bottom of the test tube. The shaking was necessary to keep the mucoid-like strings suspended in the TSB and maintain oxygenation of the TSB. Without shaking, the mucoid-like strings of biofilm sank to the bottom of the test tube and died (data not shown). The non-mucoid biofilms developed a thick, consistent composition throughout the TSB. Previous studies have implicated nutrient or oxygen deprivation in initiating biofilm formation (13, 16, 32, 34). Samples were grown for 24, 48, or 72-hour time periods.

GROWTH OF BIOFILMS USING ONCE FLOW THROUGH CHAMBERS:

Mature biofilms are most fully developed *in vitro* in a flow cell apparatus with a constant flow of media over the biofilm. As a positive control for biofilm development, a three-chambered flow cell apparatus (IBI Scientific, Peosta, IA) was set up as previously described (6, 24). TSB at room temperature was pumped through the individual chambers at a rate of 2.5 ml/minute. Prior to inoculation of the apparatus chambers, a single colony of PAO1 was inoculated into 10ml TSB overnight at 37°C. The 10 ml sample was diluted until an absorbance of 0.5 at OD600 was reached using a protocol outlined in Deligianni *et al.* (10). Each chamber was inoculated with 0.5 ml of the diluted sample. During the inoculation period, chambers were inverted and flow was stopped for one hour while bacterial cells were given time to attach (6). Each of the three chambers was assigned to a 24, 48, or 72 hour sample. One chamber was inoculated each day for three days. All three chambers were stopped on the same day, 24 hours after the inoculation of the final chamber. Biofilms were first fixed with 100 ml of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 minutes. Flow chambers were then rinsed with 50 ml PBS for approximately 30 minutes before embedding. The embedding gel was a 20% 200:1 acrylamide:bis-acrylamide polyacrylamide gel that was prepared with 20 µl of AP S (10 mg/ml), 8 µl TEMED, and 1 ml of 20% polyacrylamide 200:1 acrylamide:Bis solution. The gel was injected into each flow chamber using a 1ml tuberculin syringe. (Bio-Rad, Hercules, CA) (6, 26). Flow direction was marked before the glass which covered each chamber was carefully removed and the fixed biofilms embedded in the polyacrylaminde gel were kept in PBS until stained for microscopy.

STAINING SAMPLES FOR EXAMINATION BY CONFOCAL LIGHT SCANNING MICROSCOPY:

Cultures of mucoid-like or non-mucoid bacterial cells were allowed one hour to attach to the surface of poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated cover slips before being fixed with 4% paraformaldehyde. Biofilms grown in the flow cell apparatus were stained through the polyacrylamide gel. All biofilms were stained with a 1:300 dilution of DAPI (Invitrogen, Grand Island, NY), which was used as a counterstain to label nucleic acids. A FITC conjugated antibody to lectin from *Hippeastrum amaryllis* (HHA) (EY Laboratories, San Mateo, CA, F-8008-1), which binds to 1,3- or 1,6- mannosyl units in polysaccharides of the Psl component of the biofilms (25), was then applied at 0.2 mg/ml. Slides were rinsed, and mounted using an anti-fade reagent (ProLong, Life Technologies, Grand Island, NY). Flow cell samples were too thick to add a coverslip and were maintained under wet conditions for microscopy.

IMAGING OF BIOFILMS USING CONFOCAL LASER SCANNING MICROSCOPY

A Zeiss LSM 700 confocal laser scanning microscope was used to image the previously stained bacterial samples for DAPI label of nucleic acids or FITC-HHA label of the Psl component of PAO1 biofilms. Single images of the biofilms were taken for DAPI or FITC and Image J was used to generate composite images (30). Z-stacks were also taken (data not shown) and used to give an estimate of biofilm height (20). As a negative control coverslips with mucoid-like PAO1 were examined without any stain or FITC-HHA label. These were viewed through the DAPI filter or the FITC filter to check for autofluorescence of *P. aeruginosa*.

MOTILITY ASSAY

Motility assays were conducted as previously described (5, 10). Briefly, swim medium contained 0.982% tryptone, 0.295% agarose, and 0.491% sodium chloride. This is the most fluid medium that allows the easiest movement. Swarm medium contained 0.786% nutrient broth, 0.491% agar, and 0.491% glucose. This is a thicker medium that restricts movement. Twitch plates contained 0.976% tryptone, 0.9763% agarose, and 0.976% sodium chloride. These plates have the highest concentration of agarose and the most restricted movement.

Non-mucoid and mucoid-like samples for the motility assay were prepared as described above and given 72 hours to develop. At 72 hours, an inoculating needle was sterilized in alcohol and flame, inserted into the non-mucoid or mucoid-like sample just beneath the surface of the biofilm, and each motility plate was stab inoculated to the bottom of the plate, according to Deligianni *et al.* (10). The point of inoculation was marked on the bottom of the plate and the plates were incubated at 37°C. Growth of colonies was measured 24, 48, or 72 hours after inoculation; images were taken after 48 hours. The sample size was three to six plates per time point and plate type. Data were analyzed using two comparisons. The purpose of the first analysis is to evaluate changes in motility over the 72 hour period of the motility assay. To do this, values for each type of motility (swimming, swarming, or twitching), were statistically compared to the 24 hour mucoid-like phenotype with an unpaired t-test using GraphPad. The second analysis determines if there is a difference in motility between the two phenotypes at each time point. In this analysis, an unpaired t-test compared mucoid-like to non-mucoid samples at each time point.

SURFACE ATTACHMENT ASSAY

Surface attachment assays were conducted as previously described (21). Briefly, bacterial cultures were grown under either mucoid-like or non-mucoid conditions for 24, 48, or 72 hours and then diluted down to an absorbance of OD600 of 0.5. The cultures were then diluted 1:2 in TSB and 0.5 ml of each diluted culture was placed in an individual well of a poly-L-lysine coated 24 well plate for 1.5 hours. Culture fluid was removed before the cultures were fixed with 70% methanol for 30 minutes and stained with 0.5% crystal violet for 30 minutes. Excess crystal violet was then washed from the wells using PBS. Digital images of the plates were obtained.

RESULTS

NON-MUCOID AND MUCOID-LIKE PHENOTYPES

An example of non-mucoid and mucoid-like biofilms developed in our lab is shown in Fig. 1. As can be seen in Fig. 1A, the non-mucoid phenotype grown for 72 hours is concentrated with a consistent composition. The mucoid-like biofilm seen in Fig. 1B has thick, mucous-like strings that emanate from the surface and spread interior into the TSB. If re-

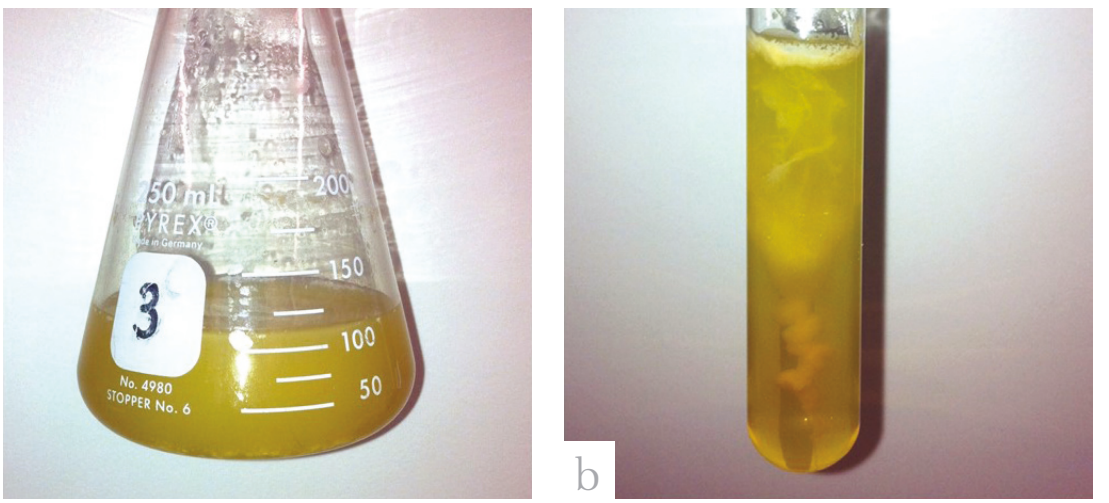

Figure 1: Non-mucoid and mucoid-like *Pseudomonas aeruginosa* biofilm. The non-mucoid biofilm (A) was grown in 100 ml TSB in a 250 ml Erlenmeyer flask and has a consistent composition. The mucoid-like biofilm (B) was grown in 10 ml TSB in a 16 ml test tube and demonstrates strings of mucoid-like biofilm. All samples were incubated at 37°C on a rotary shaker at 225 rpm for 72 hours. The specific differences in environment conditions leading to the formation of two phenotypes was not investigated. However, studies have shown that oxygen deprivation can induce biofilm formation; thus, it is possible the low surface-to-volume ratio in the test tube may lead to favorable conditions for biofilm development.

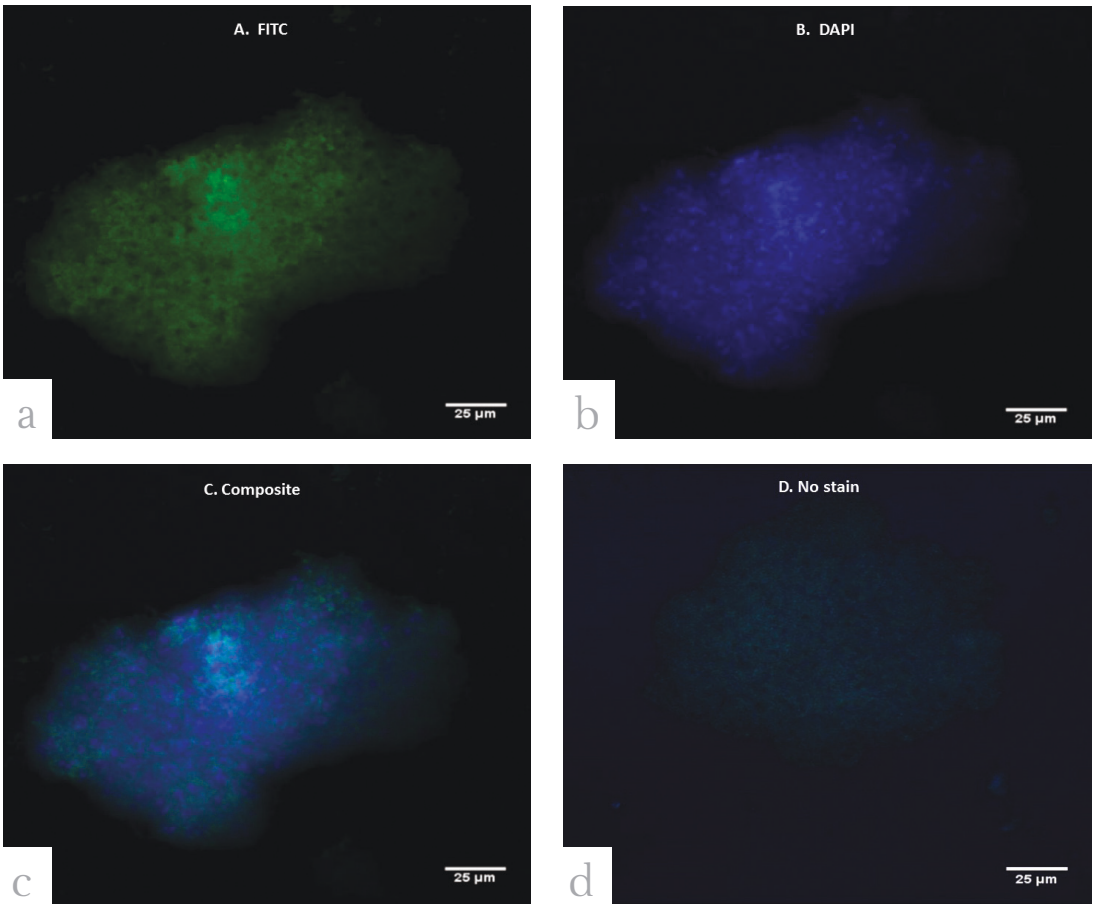

Figure 2. A–D is an example of a 72 hour mucoid-like biofilm stained with DAPI and HHA-FITC shown with the FITC filter (A), the DAPI filter (B) and the composite image (C). D is the corresponding negative control without DAPI or HHA-FITC antibody. Pockets, indicated by dark "holes" within the image, appear to be formed throughout the EPS matrix of the biofilm. At these pockets a higher concentration of nucleic acids are present as seen by the DAPI fluorescence. These "pockets" filled with PA may be the beginning of pillar-shaped biofilms. Scale bars are 25µm.

moved from the test tubes, these mucous-like strings are easily disrupted and disseminated. However, in the test tube, they are discrete from the surrounding media. The observation of these two phenotypes is the basis of our examination. When cultured for longer than 72 hours, PAO1 in the mucoid-like phenotype died, perhaps from a lack of nutrients.

CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

Fig. 2 A–D demonstrates a composite image of a 72 hour mucoid-like biofilm labeled with FITC-HHA (A), DAPI (B), and the composite image (C). In confocal images of pillar-shaped biofilms, it is common to view the EPS matrix

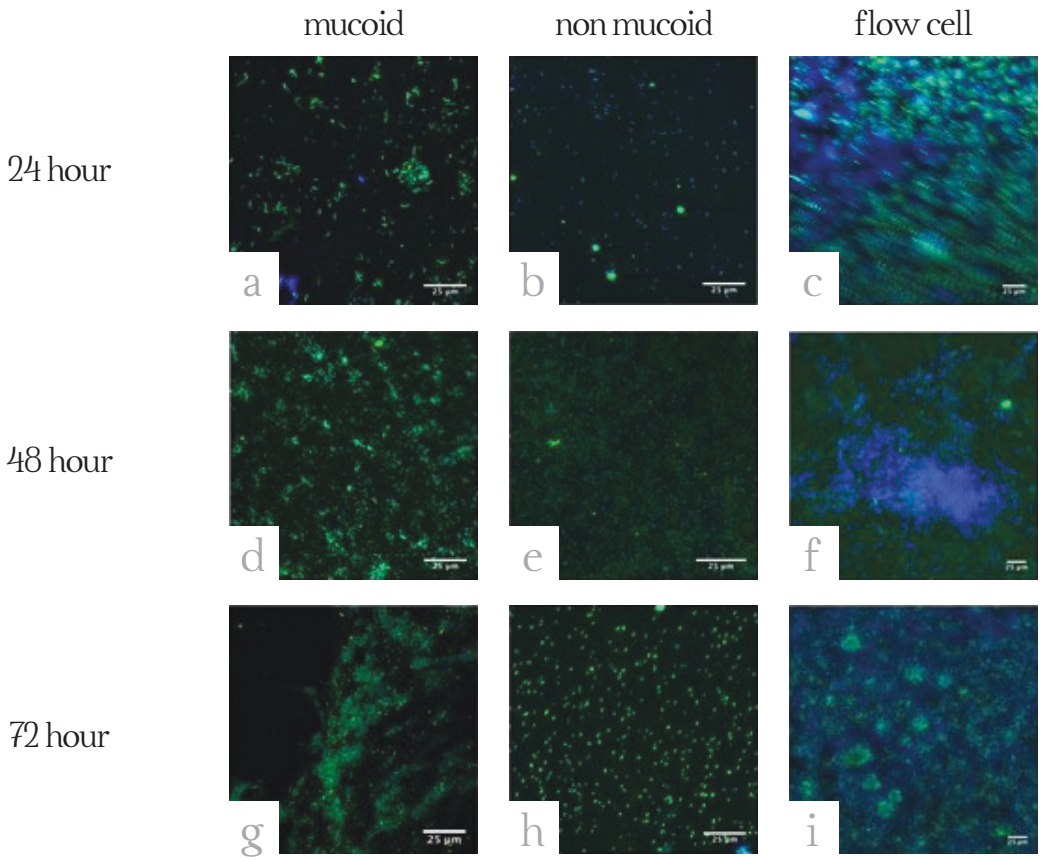

Figure 3. Confocal microscopy of PA biofilms. PA samples were prepared as described in the methods. At 24 hours, all samples demonstrated biofilm growth (A–C). The flow cell samples (C) are slanted in the direction of media flow through the chamber and difficult to obtain focused images. At 48 hours, the mucoid-like and flow cell samples demonstrate greater growth (D, F) compared to the non-mucoid sample (E). The spherical structures in D could be the beginning of pillar-shaped biofilms. In the 72 hour samples (G–I), the mucoid-like sample (G) shows string-like biofilms structures typical of the mucoid-type phenotype compared to the punctate labeling seen in the non-mucoid sample (H). The flow cell sample (I) has circular structures indicative of pillar-shaped biofilms. Calibration bars are 25µm.

surrounding a "hollow-like" center that may contain bacteria (8). Our biofilms contain similar structures. Fig. 2A demonstrates "holes", black spaces in the biofilm surrounded by FITC-labeled matrix. These "holes" are, however, "filled in" with DAPI label in Fig. 2B, indicating that bacteria are within this structure. The composite image, Fig. 2C demonstrates the separation of the matrix from the bacteria with the pattern of blue DAPI stain surrounded by the FITC-HHA label for the matrix. Fig. 2D is a negative control of mucoid-like PAO1 biofilm indicating some background label; however, it is much less than the positive label seen in Fig. 2A–C.

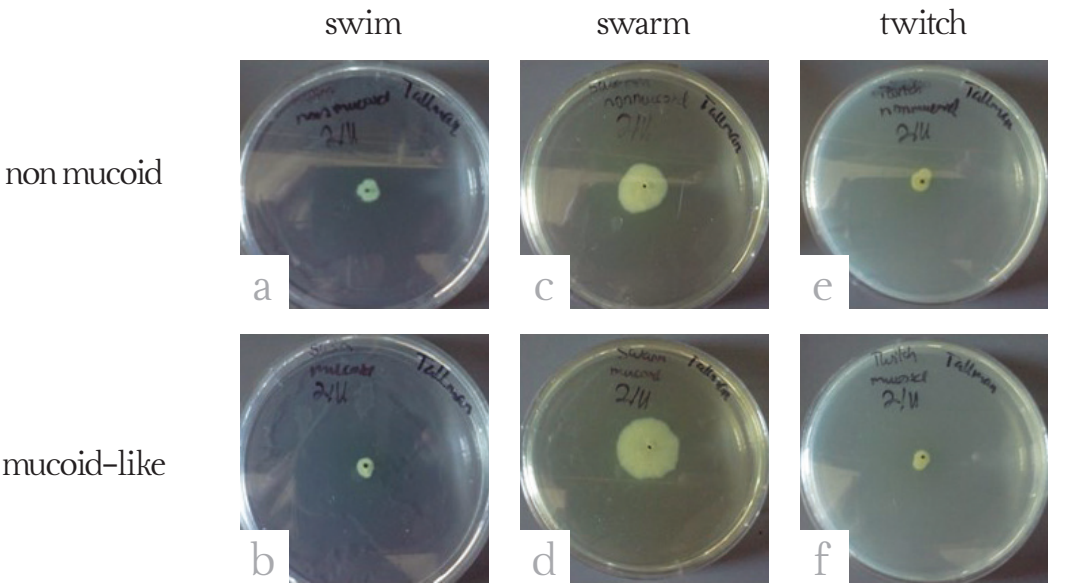

Figure 4. Motility assay. Shown are images of swim (A–B), swarm (C–D), and twitch plates (E–F) for non-mucoid and mucoid phenotypes. All plates were inoculated with 72 hour samples and allowed to develop for 48 hours. Swarming showed the greatest motility of the three types of movement as indicated by the increased diameter. All three types of movement demonstrated similar diameters, and therefore similar amounts of movement, between the non-mucoid and mucoid-like phenotypes.

In order to compare biofilm development between the non-mucoid and mucoid-like phenotypes generated in this lab, biofilms were also grown in once-through flow cell chambers. These chambers have been shown in the past to provide optimal conditions for biofilm development (3, 29). Fig. 3 compares non-mucoid, mucoid-like, and flow cell biofilms 24, 48, and 72 hours after inoculation of a chamber. Biofilm development was greatest in the mucoid-like and flow cell samples at 72 hours (Fig. 3G and I). There was some labeling in the 72 hour non-mucoid sample (Fig. 3H), but it is much more punctate and does not show the elaborate Psl network seen in the mucoid-like or flow-cell apparatus biofilms.

In Fig. 3I, the 72 hour flow-cell biofilm sample demonstrates circular structures similar to the pillar shaped biofilms shown in Fig. 2C. The mucoid-like biofilms (Fig. 3D, 3G) demonstrate biofilm structure that has the Psl matrix as observed by the FITC-HHA label, but is not always a discrete or circular structure (Fig. 3G). However, there is much greater development of the Psl matrix than in the non-mucoid biofilm which is a more consistent, punctate FITC-HHA label indicating a much less complex and fully formed biofilm matrix in the non-mucoid sample.

To measure biofilm height, z-stacks of 72 hour samples were used since biofilms at this point are the most developed (data not shown). Non-mucoid phenotype biofilms were smaller and flatter with an average height about 6 μm. Mucoid-like phenotype biofilms were taller with an average height of 6–12 μm. Flow

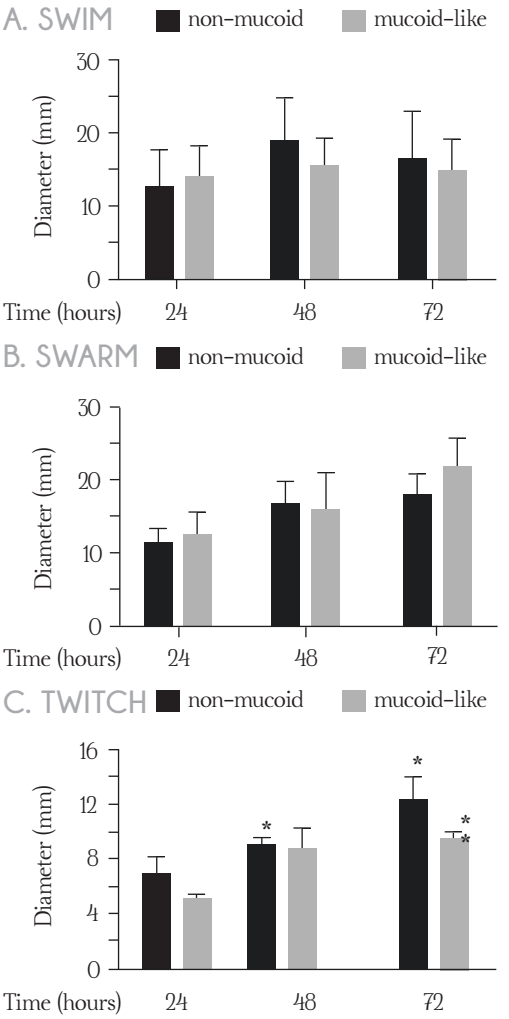

Figure 5. Graphs of motility data. A–C are graphs of the average diameter for each assay. Data were compared to the 24 hour mucoid sample for each motility using an unpaired t-test (GraphPad). Twitch motility demonstrated a significant increase from the 24 hour sample for the 72 hour mucoid, 48 hour non-mucoid, and 72 hour non-mucoid. This indicates that over time, both mucoid and non-mucoid phenotypes exhibited a significant increase in twitching. This indicates that both phenotypes are exhibiting characteristics of biofilm formation; however, the biofilm structure between the two phenotypes remains different as observed by a flatter biofilm structure for non-mucoid biofilms using microscopy. Non-mucoid p values: $p = 0.0019$, 48 hours; $p = 0.0031$, 72 hours. Mucoid-like phenotype p value at 72 hours, $p = 0.0001$).

cell biofilms were the largest biofilms with average heights between 30–40 µm. Biofilms grown in a flow cell chamber were slanted in the direction of the flow of media, making it difficult to focus the image.

MOTILITY ASSAY

Fig. 4 A–F demonstrates images of the motility assays. As can be seen swarming motility demonstrated the greatest diameters (Fig. 4 C, D). Visual inspection alone indicates that motility is very similar between PAO1 in each phenotype.

The average movements of swarm motilities at 72 hours as shown on the column graphs (Fig. 5B) were 18.1 mm (non-mucoid) and 21.8 mm (mucoid-like) as compared to the swim plate values (Fig. 5A) of 16.3 mm (non-mucoid) 14.6 mm (mucoid-like). Swarming is the only motility in which the 72 hour mucoid-like biofilm exhibited greater motility as compared to the non-mucoid sample (Fig. 5B) although there was no statistical difference. It was hypothesized that twitching movement would be greater in the mucoid-like sample (Fig. 5C); however, it was slightly greater in the 72 hour non-mucoid sample (12.2 mm vs. 9.5 mm). Twitching had the only statistically significant data with both the 48 hour non-mucoid and 72 hour non-mucoid being significantly higher in twitching ($p = 0.0019$ for 48 hour; $p = 0.0031$ for 72 hour) than the 24 hour mucoid-like phenotype. The 72 hour mucoid-like phenotype also demonstrated significantly more twitching than the 24 hour mucoid-like sample ($p = 0.0001$). Changes in motility are a part of biofilm formation as *P. aeruginosa* moves from swimming to swarming to twitching, eventually attaching to a surface and forming a microcolony and then a biofilm. Clinical data from CF patient sputum indicate that clinical isolates containing *P. aeruginosa* exhibit widely varying patterns of motility (10, 27). These differences in motility may indicate differences in virulence and ability to develop chronic

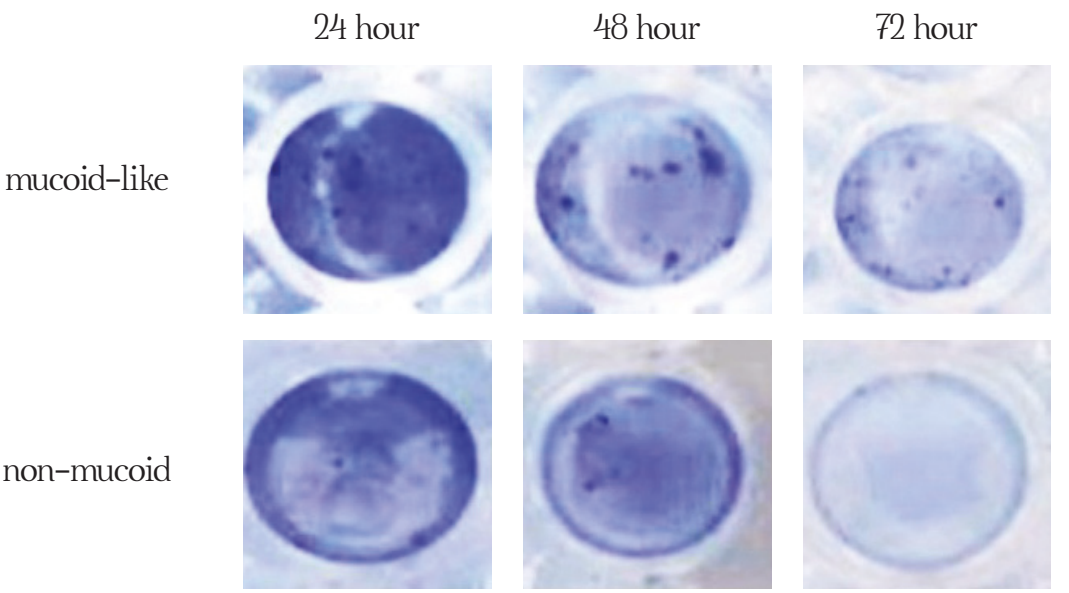

Figure 6. Surface attachment assay. All samples were given 1.5 hours to attach to the well before being fixed and stained with crystal violet. As can be seen, attachment decreased for both mucoid-like and non-mucoid samples between 24 and 72 hours. Biofilm formation is usually associated with an increase in attachment. While the trend in attachment was not as expected, it can be noted that the 72 hour mucoid-like sample exhibited greater punctate staining; these punctate areas of staining could be biofilms that attached. There are clearly qualitative differences in the development of the mucoid-like phenotype as compared to the non-mucoid phenotype.

infections. Motility is now being used as a method to further identify and characterize *P. aeruginosa* in sputum from CF patients (10, 27). These motility patterns may help identify the most effective treatments (27).

SURFACE ATTACHMENT

Attachment decreased as one moved from 24 hour to 72 hour samples (Fig. 6). There was a qualitative difference in that the mucoid-like samples attached with more punctate staining indicating clusters or colonies of PAO1 whereas the non-mucoid phenotype exhibited a more diffuse, even attachment. Each sample was given 1.5 hours to attach so the punctate staining represents clusters of bacteria that existed at the time of sample attachment not colonies formed through cell division after attachment. The clusters may be similar to the circular structures observed in Fig. 2A-C in which a cluster of PAO1 is surrounded by Psl matrix. Absorbance data from the surface attachment assays did not indicate any significant difference in surface attachment when compared to the 24 hour mucoid-like sample or when comparing mucoid-like and non-mucoid samples at each time point.

DISCUSSION

Biofilm formation is a critical component of bacterial infections in a clinical setting; for example, in chronic infections in CF that increase mortality and morbidity (17). Biofilms are also implicated in chronic sinusitis and chronic wounds as well as other infections (4). It is well documented that mucoid biofilms are more pathogenic (11, 23). Alginate, one of the prime components of a mucoid biofilm elicits different responses from airway epithelium that attenuates the host response (7). It is also well documented that biofilms isolated from clinical patients exhibit widely different morphology and physiology with some forming pillar-shaped biofilms, some forming knobby shaped biofilms, and some with flat biofilms (10, 27). Motility assays are one way in which clinical strains are analyzed to determine trends in pathogenicity. Multiple studies using motility assays have found that pathogenic strains have decreased motility (1, 27).

The classic biofilm structure is that of a pillar-shaped structure containing bacteria and surrounded by an exopolymeric substance (EPS) composed of Psl, Pel, and alginate. It is hypothesized that biofilm formation may be driven by environmental conditions such as nutrient depletion, although the environmental triggers driving biofilm formation are not completely understood (1, 4, 7). A common way in which biofilms are grown within the lab is with rotation in a shaker, similar to the non-mucoid phenotype in this study using an Erlenmeyer flask. This biofilm produces different ratios of quorum-sensing molecules than biofilms formed within patient sputum (33). The environmental conditions that trigger biofilm transformation from a non-mucoid to a mucoid phenotype remain unknown (7).

Our lab was able to grow two distinct phenotypes of PAO1 biofilms under two environmental conditions that may include oxygen or nutrient deprivation. It was our hypothesis that PAO1 in the mucoid-like phenotype would produce a biofilm closer to a clinical "mucoid" biofilm with greater twitching motility, greater attachment to a surface, and greater height and pillar-shaped structure when compared to the non-mucoid phenotype of PAO1.

Based on our data, both phenotypes exhibit some markers of biofilm development such as increased twitching behavior by PAO1 as well as a clearly defined matrix, indicated by FITC-HHA labeling. However, the mucoid-like phenotype exhibits characteristics closer to the more fully formed, mature pillar-shaped biofilms described in literature (8, 10). The mucoid-like biofilm developing in our lab had evidence of small pillar-shaped structures that were beginning to form (Fig. 2 A-C), but did not reach the height or complexity of biofilms formed in a flow-through chamber (Fig. 3G and 3I).

Biofilms are associated with a decrease in motility, both swimming and swarming (10, 27). Swimming utilizes flagella to propel bacteria to a new location, possibly to obtain a better food source. Twitching utilizes type IV pili to slowly move across a surface and is associated with surface attachment and the beginning stages of biofilm formation. Swarming is one of several intermediate forms of motility that utilizes both flagella and type IV pili as well as a bacterial-secreted rhamnolipid that acts as a surfactant to create a more fluid surface for bacterial movement (5, 22, 35). PAO1 in our mucoid-like phenotype exhibited swimming and swarming at 24, 48, and 72 hour time points (Fig. 4F); however, the only significant differences

in motility when compared to the 24 hour mucoid-like phenotype were in twitching. At both 48 hours and 72 hours the non-mucoid phenotype had significantly greater twitching than the 24 hour mucoid-like phenotype (Fig. 5C, p = 0.0019, 48 hours; p = 0.0031, 72 hours). At 72 hours, the mucoid-like phenotype had significantly greater twitching than the 24 hour mucoid-like (Fig. 5C, p = 0.0001). These data signal an increase in twitching across a 72 hour period and support that both of these phenotypes may be progressing through the early stages of biofilm development.

Surface attachment is also an established and necessary component for biofilm formation (8, 17). This may be facilitated by a switch from swimming motility to swarming and then to twitching as the bacteria become sedentary. In our phenotypes, attachment consistently decreased from 24 to 72 hours in both the mucoid-like and non-mucoid phenotypes. This is counter to the generally accepted view that surface attachment of bacteria increases as biofilms form. However, there were also qualitative differences, especially in the mucoid-like phenotype which exhibited more punctate staining. These punctate spots could be small biofilms (Fig. 6) which would be consistent with biofilm formation.

In conclusion, our data indicate both phenotypes are in the process of biofilm formation due to FITC-HHA label and increased twitching. However, there are also differences between the phentoypes as shown in CLSM images in which the mucoid-like biofilm has more pillar-shaped biofilms and in the increased level of punctate staining in the surface attachment assay. Not much is known about the environmental factors that drive the formation of the wide variety of biofilms observed in clinical isolates. Nutrient and oxygen deprivation have been implicated in the formation of mucoid-like biofilms (16, 32, 37). The role of specific environmental triggers was not investigated in this study; however, knowing that environments as simple as using a test tube or an Erlenmeyer flask can produce two different phenotypes of biofilms might lead to further identification of the triggers that lead to the development of pathogenic biofilms in patients.

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STRIKING UP THE CONVERSATION: QUORUM SENSING IN FUNGI

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ABSTRACT

Quorum sensing is a form of communication observed in different species of microbes. Numerous studies have shown the ability of bacteria and fungi to carry out quorum sensing by releasing specific molecules to enable communication in a large population. Quorum sensing has been shown to influence growth, morphology, and other factors pertaining to virulence in pathogenic microbes. In this review, we address three important fungal species and explain how each fungus has a unique and dynamic way of communicating. *Candida albicans* is an opportunistic pathogen, or one that is part of the normal microbiota that can become pathogenic and cause several diseases. Here, we address two quorum sensing molecules (QSMs) identified by investigators. These chemicals are tyrosol and farnesol, which act together to control cellular growth, morphology and biofilm production. Another opportunistic fungal pathogen, *Cryptococcus neoformans*, has been shown to display quorum sensing activity by using pantothenic acid as well as a peptide called quorum sensing–like peptide 1. These molecules have both been shown to control growth rates of *C. neoformans*. *Saccharomyces cerevisiae* is another dimorphic fungus that uses QSMs, although it is nonpathogenic. Using two aromatic alcohols, phenylethanol and tryptophol, S. *cerevisiae* can alter pseudohyphal growth in diploid cells as well as invasive growth in haploid cells. By understanding more about the ways these organisms communicate, we present the potential for new and better targets for the treatment of fungal infections.

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KEYWORDS

- quorum sensing
- fungi
- *C. albicans*
- *C. neoformans*
- S. *cerevisiae*

INTRODUCTION

Many microbes use a form of communication known as quorum sensing, which is a function of population density. This phenomenon was first discovered in the bacteria *Vibrio fischeri* and *Vibrio harveyi* in the 1970s. These bacteria were shown to luminesce only when high numbers of bacteria cells were present (30, 31). In 2001, Hornby *et al.* reported evidence of a quorum sensing mechanism in the eukaryotic yeast *Candida albicans* (15). Similar to the process used by bacteria, fungal cell density was found to play a major role in communication (18, 25). Fungi, along with

other organisms, secrete compounds known as quorum sensing molecules (QSMs) into their environment to communicate with neighboring cells. QSMs, also known as autoinducers, regulate many functions of the population as a whole. QSMs must accumulate to reach high concentrations to have an effect on the organism. A large population of microbes, therefore, must be present to produce high enough levels of QSMs to have an effect (3). The QSMs bind receptors on the microbe's cell and when enough receptors are bound they activate signaling pathways. The induction of these signaling pathways affects gene expression in the organism, which in turn controls various cellular processes, including cell growth, biofilm formation, motility, cell morphology and secretion of virulence factors (1, 2, 8, 14, 25, 27, 45).

Much work has been done to uncover the various mechanisms that regulate quorum sensing by identifying the pathways and signaling proteins involved. For example, Kruppa *et al.* were the first to report a two-component signal transduction pathway in eukaryotic cells that mediates morphology, phenotype, and biofilm formation (18). Other recent studies have determined Ras1, cyclic AMP (cAMP), and mitogen-activated protein kinase (MAPK), among others, to be important factors for quorum sensing in both *C. albicans* and *Cryptococcus neoformans* (10, 20, 22).

Although more is known about quorum sensing in bacteria, this method of communication in fungal microbes has been an area of great interest in recent years. Quorum sensing in *Candida albicans* is perhaps the best understood example of quorum sensing in fungi (3, 7, 10, 15, 35, 41, 45). In addition, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* have also been identified as fungal species that utilize this cellular communication (2, 6, 21, 22). This review will discuss the quorum sensing mechanisms that *C. albicans, C. neoformans,* and *S. cerevisiae* employ in regulating the cellular population, as these are the species in which fungal quorum sensing has been most thoroughly studied. Although other fungal species have been shown to have quorum sensing activities, the QSMs in these other species have not yet been identified. The significance of fungal quorum sensing will also be addressed as it applies to fungal signaling, the pathogenicity of fungi, and treatment of fungal infections.

CANDIDA ALBICANS

Candida albicans is a dimorphic fungus that has been extensively studied due to its unique ability to cause diseases even though it is part of the normal human microbiota. This fungus causes thrush, genital candidiasis, and bloodborne invasive candidiasis (16, 17, 20). An important attribute contributing to the virulence of *C. albicans* is its dimorphism, or the ability to switch morphological forms between mycelium (hyphae) and budding yeast. The mycelium form of fungi embeds into tissue, while the yeast form is associated with initiating infections and dissemination (32, 38). Two QSMs, farnesol and tyrosol, have been determined to have an effect on this dimorphism phenomenon as well as other cellular processes (7, 10, 15, 18, 20, 33).

Farnesol was discovered in 2001 and was the first QSM identified in a eukaryotic cell (Fig. 1) (15, 34). Farnesol, an isoprenoid, was identified as a QSM in *C. albicans* by observing the effects that supernatants from spent media (cell-free, filtered supernatant from a previously grown culture) had on the differentiation and mycelial growth of the fungi. The results of the assays showed that the highest amount of mycelial growth occurred when no supernatant was added.

Figure 1. Schematic diagram illustrating fungal quorum sensing molecules and their effects.

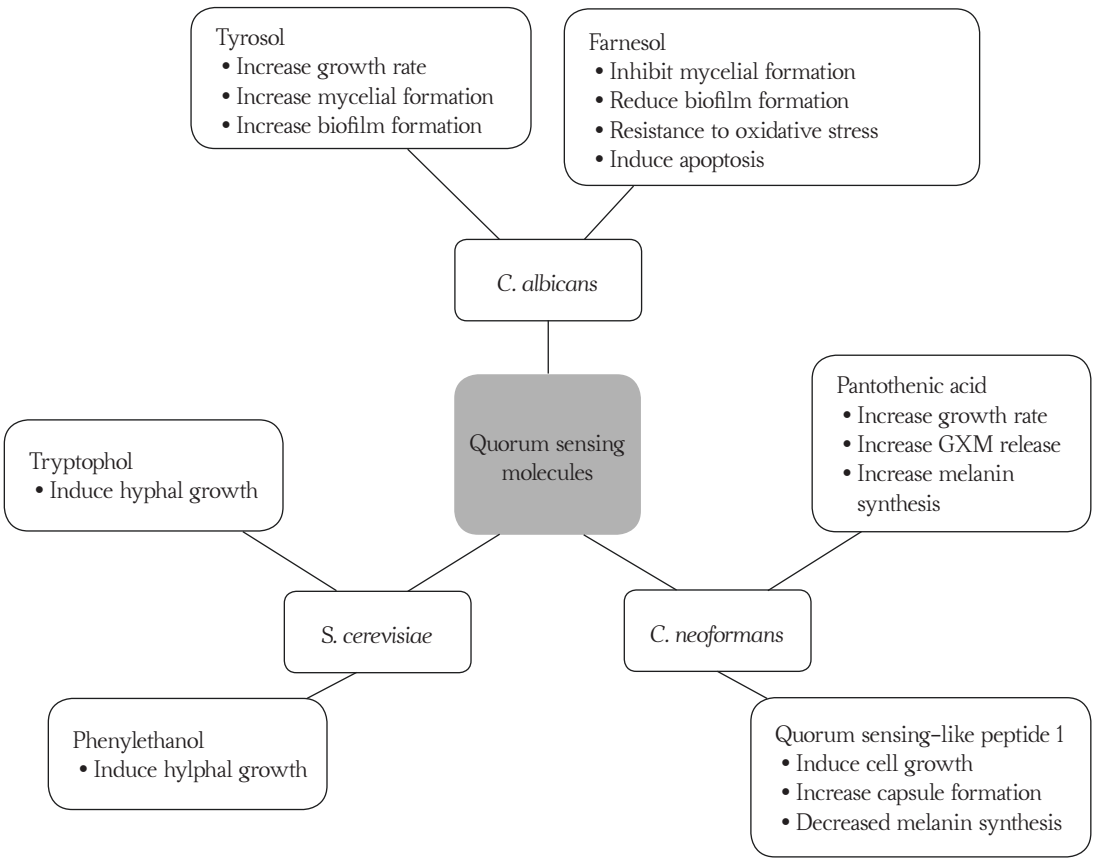


Table 1. Summary of quorum sensing in fungi

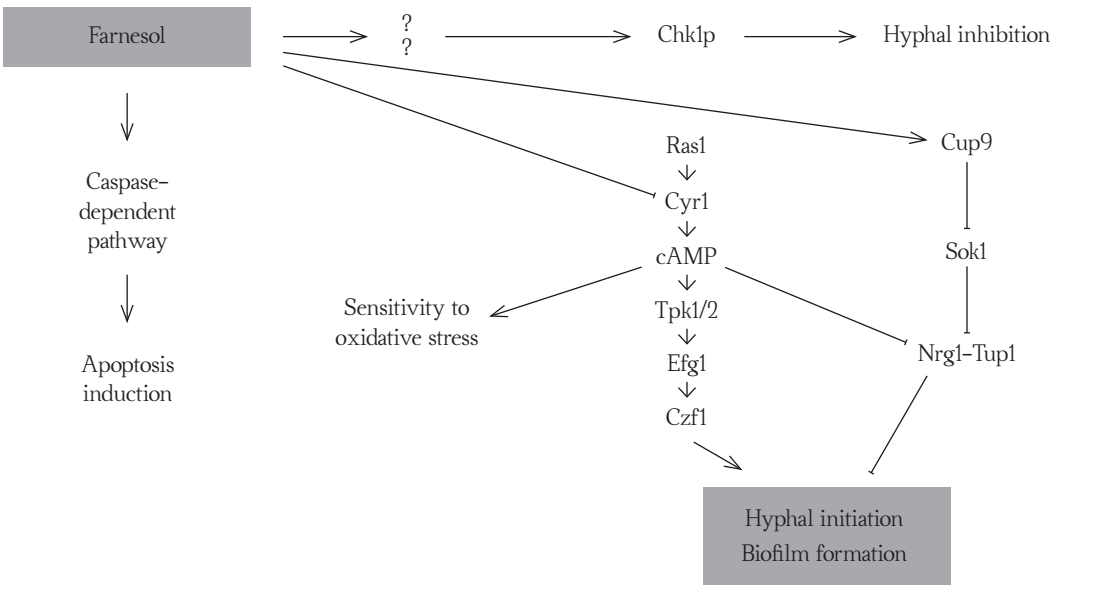
Organism	Quorum sensing molecule	Effect of quorum sensing molecule	References
Candida albicans	Farnesol	Prevent mycelial formation	15
		Reduce biofilm formation	35
		Increase resistance to oxidative stress	10,45
		Induce apoptosis	41
Candida albicans	Tyrosol	Increase growth rate	7
		Increase mycelial formation	7
		Increase biofilm formation	3
Cryptococcus neoformans	Pantothenic acid	Increase growth rate	2
		Increase GXM release	2
		Increase melanin synthesis	2
Cryptococcus neoformans	Quorum sensing-like peptide 1	Induce cell growth	21
		Increase capsule formation	22
		Decrease melanin synthesis	22
Saccharomyces cerevisiae	Phenylethanol	Induce hyphal growth	6
Saccharomyces cerevisiae	Tryptophol	Induce hyphal growth	6

When a high concentration of supernatant was added, the amount of mycelia was substantially decreased. This indicated QSMs had a dose-dependent effect on the morphology (15). To identify the QSM in the supernatant, solvent extraction of spent media was performed and coupled with thin-layer chromatography (TLC) and gas chromatography-mass spectroscopy (GC-MS). The use of these techniques combined with the elimination of other potential QSMs resulted in the identification of farnesol as the QSM responsible for the effect on mycelial growth of *C. albicans*. Commercially prepared farnesol also prevented mycelial formation; further suggesting that farnesol was the QSM responsible for the observed effect on morphology (Table 1). Growth rates were also observed when farnesol was added to *C. albicans* cultures and it was concluded that farnesol did not have an effect on yeast budding (15). The discovery of a molecule that inhibits mycelial formation is important because it is the mycelium form of the organism that allows it to enter the bloodstream, resulting in invasive candidiasis (15).

Researchers have demonstrated that farnesol inhibits the mycelial phase and promotes the yeast phase through the regulation of many different signaling pathways as depicted in Fig. 2. Chk1p was identified as a possible two-component signal transduction protein that acts with an unidentified upstream protein to mediate farnesol sensing. Authors tested a wild type strain and a Chk1 mutant strain of *C. albicans* and found that the Chk1 mutant germinated, resulting in mycelial growth, in the presence of farnesol while the others did not. This could be valuable knowledge because two-component signaling genes are not found in humans and could provide a potential therapeutic target for this fungal pathogen (18). In 2011, it was reported that farnesol suppresses hyphal formation through the inhibition of the Ras1-Cyr1-cAMP signaling pathway (10, 23, 34). In fact, cells with increased cAMP signaling are more resistant to the morphology effects of farnesol (23). Tpk1/2, Czf1, and Efg1 were discovered to be required for the morphology effects of farnesol. These findings are in line with the previous studies, as Tpk1/2, Efg1, and Czf1 are downstream of the Ras1-Cyr1-cAMP pathway (20). Farnesol signaling was discovered to lead to resistance to oxidative stress, also due to inhibition of the Ras1-Cyr1-cAMP pathway (10). The discovery of farnesol enabling the cell to resist oxidative stress provides an additional mechanism farnesol uses to aid in the pathogenicity of *C. albicans* (10, 45).

Evidence also suggests that the transcription regulator Tup1 plays a role in the inhibition of mycelial formation in response to farnesol. Although Tup1 seems to play a role in the effects of farnesol signaling, the signaling pathway linking Tup1 to farnesol-mediated morphology effects is still unclear (20). The involvement of Tup1 in quorum sensing of *C. albicans* is especially interesting because Tup1 is also involved in quorum sensing in *C. neoformans*, which will be discussed later in this review (20, 21). Furthermore, Nrg1 was discovered to have a crucial role in the farnesol-dependent inhibition of hyphal formation (25). Nrg1 inhibits hyphal formation and therefore must be absent for hyphal initiation. The absence of Nrg1 is controlled by two mechanisms: 1) The Ras1-Cyr1-cAMP pathway has a negative effect on *NRG1* transcription and 2) protein degradation of Nrg1. Farnesol inhibits the Ras1-Cyr1-cAMP pathway, resulting in the expression of *NRG1* which results in inhibition of hyphal formation. In addition, farnesol blocks Nrg1 degradation by stabilizing the Cup9 transcriptional repressor, which in turn represses *SOK1* expression.

Figure 2. A diagram depicting the pathways that have been implicated in farnesol signaling in *C. albicans*.



Sok1 leads to the degradation of Nrg1, therefore a repression of *SOK1* results in inhibition of Nrg1 degradation thus allowing Nrg1's presence to inhibit hyphal formation. Interestingly, both the Ras1-cAMP pathway and the Cup9-Sok1-Nrg1 pathway are required for farnesol to elicit its effects on the morphology of *C. albicans* (Fig. 2) (25).

Additional effects of farnesol on *C. albicans* include the induction of apoptosis *via* a caspase-dependent pathway (Table 1) (41). In addition, farnesol was shown to induce apoptosis and decrease proliferation in human oral squamous carcinoma cells (39). The ability of farnesol to affect cell death may have important implications for the development of therapies for fungal infections and for cancer (39, 41).

Tyrosol is another QSM produced by *C. albicans* that regulates growth and morphology (Table 1) (7). Tyrosol increases fungal growth by substantially decreasing the length of time the fungus is in lag phase. This was observed when Chen *et al.* (6) compared the growth of dilute overnight *C. albicans* cultures in the presence and absence of tyrosol over an 8-hour period. They also demonstrated that tyrosol is continuously released into the medium during growth and that it promotes filamentous morphology. Tyrosol was shown to promote germ tube formation, stimulating the conversion of yeast cells to hyphae (Fig. 1). The effect tyrosol has on cell growth may be due to tyrosol stabilizing transcripts that encode proteins involved in DNA synthesis and the cell cycle, therefore affecting growth by regulating the processes of DNA synthesis and the cell cycle (7). These results suggest that tyrosol and farnesol work together to control cellular growth and morphology of *C. albicans* (7).

Another important factor contributing to the virulence of some pathogens is biofilm

formation. Biofilms play an important role in natural and medical environments. Biofilms are organized microbial communities that adhere to surfaces, posing a serious health threat for patients with medical devices like stents and catheters (35). The mycelium form of fungi is crucial for biofilm development of *C. albicans*; therefore, inhibiting mycelial formation could be important for controlling fungal biofilms (35). Studies done by Ramage *et al.* and Kruppa *et al.* demonstrated that farnesol had a direct effect on biofilm formation (Table 1) (18, 35). The amount of biofilm formation was reduced as the concentration of farnesol increased. There was a 60% reduction in biofilm formation when the wild type strain of *C. albicans* was exposed to 25 μM and 250 μM of farnesol (18). Ramage *et al.* hypothesized that the reduction in biofilm formation was due to the fact that farnesol decreased mycelial formation, which is crucial for biofilm development of *C. albicans*. They concluded that this was a mechanism the organism uses to control overgrowth and limit competition (35). Microarray analysis comparing *C. albicans* biofilms inhibited by farnesol to natural *C. albicans* biofilms shows expression changes in genes controlling hyphal formation, drug resistance, cell wall maintenance and iron transport (4).

Interestingly, when tyrosol was added to *C. albicans* during different stages of biofilm formation, there was no effect observed. When farnesol and tyrosol were added together, the effect was dependent on the combination of the individual concentrations of the two QSMs. Tyrosol eliminated the inhibitory effect of low concentrations of farnesol. However, tyrosol was unable to overcome farnesol's inhibition of biofilm formation when 1 mM of farnesol was used. In addition, biofilms exposed to both farnesol and tyrosol were almost entirely in the yeast form suggesting that farnesol has a dominant effect. These discoveries could lead to farnesol as a possible treatment for fungal biofilms (3). As shown in Table 1, the QSMs produced by *C. albicans* affect multiple aspects of its pathogenicity, including: morphology, biofilm formation, and resistance to oxidative stress. These quorum sensing molecules require additional study to further investigate this common opportunistic fungus.

CRYPTOCOCCUS NEOFORMANS

Cryptococcus neoformans is another dimorphic fungal microbe and can cause life-threatening meningitis, particularly in AIDS patients (5, 19). Quorum sensing is not as well understood in *C. neoformans;* however, it appears to play a significant role in the regulation of several cellular processes of the fungus (Fig. 1). It was observed that when the gene for the transcriptional repressor *TUP1* is disrupted, *C. neoformans* colonies were less than 1% of the normal size and number compared to cells expressing *TUP1* (21). The authors also discovered that growth of *TUP1* mutants (*tup1*Δ) was dependent on the density of cells. This cell density dependent phenotype is an indication *C. neoformans* participates in quorum sensing. When a low cell number of *TUP1* mutants were plated, no growth occurred. Even though a growth or no-growth phenotype had not previously been described as an effect of quorum sensing, the effects of TUP1 on growth would seem to indicate quorum sensing is involved. The effect TUP1 has on the growth of *C. neoformans* was shown when *tup1*Δ strains were grown on conditioned medium (filtered supernatant from a previously grown culture) from cultured *tup1*Δ cells. At low cell numbers, the cells placed in conditioned medium formed colonies, while cells plated at the same low cell number,

but without conditioned medium failed to grow (21). The molecule in the conditioned medium responsible for this activity is quorum sensing–like peptide 1 (QSP1) (Table 1). The investigators artificially synthesized QSP1, placed it in media with *tup1*Δ strains and observed that growth patterns were just as strong as the *tup1*Δ strains grown in conditioned medium (21).

In addition to growth regulation, TUP1 has been determined to play a role in the pathobiology of *C. neoformans*. For example, Lee *et al.* demonstrated that TUP1 regulates iron and copper utilization, which are known modulators of melanin synthesis, a pigment produced by *C. neoformans* that contributes to its pathogenicity (22). Melanin plays a role in the protection of the yeast from oxidative stress (24). Melanin production is controlled by the laccase enzyme, which requires copper ions to function. Gene mutations resulting in a loss of metal ion homeostasis can therefore result in a lack of melanin production, which can be overcome by the addition of copper. Lee *et al.* observed a reduction in melanin synthesis in the *tup1*Δ strain that was restored after the addition of 10 μM $CUSO_4$ (22). This suggests TUP1 has an effect on metal ion homeostasis and therefore melanin synthesis.

Additional studies also showed melanization was cell density–dependent, indicating a quorum sensing effect. Melanin production began when the cell density reached 4 x 10^7 CFU/ml. It was observed that the rate of melanization increased as the cell density increased (11). The capsule, which plays a role in the pathogenicity of *C. neoformans*, was also shown to be regulated by TUP1. Lee *et al.* reported a significant increase in capsule size of the *tup1*Δ strain compared to the wild-type strain (22). The capsule, composed primarily of glucuronoxylomannan (GXM), protects the yeast from phagocytosis and aids in dissemination (24). Finally, the *tup1*Δ strain of *C. neoformans* was demonstrated to be less virulent than the wild-type strain. Mice were infected with both strains and observed. Mice inoculated with the wild-type lived for 9 days while those inoculated with *tup1*Δ lived for 20 days (22). Thus, the effect on virulence is a result of the multiple effects TUP1 has on *C. neoformans* (Fig. 1).

Albuquerque *et al.* described quorum sensing in *C. neoformans* after they observed regulation of fungal activity dependent on cell density (2). They determined quorum sensing was involved in the regulation and production of several cellular components of the yeast such as the capsule and melanin. Investigators took conditioned medium from *C. neoformans* cultures and placed it in fresh *C. neoformans* cultures. Conditioned medium caused increased growth rates, GXM release, and melanin synthesis. Pantothenic acid was identified as a key component of these observed quorum sensing effects (Table 1) (2). Pantothenic acid was identified by conducting mass spectrometry and NMR. When pantothenic acid was placed in cultures, the same quorum sensing effects seen with conditioned medium were observed. It was found that higher concentrations of synthetic pantothenic acid were required to reproduce the effect seen from the concentration of pantothenic acid naturally found in conditioned medium. The authors also found the effects caused by pantothenic acid alone were not to the same extent as conditioned medium, indicating that some other molecule plays a role in the quorum sensing activity. Pantothenic acid was found to be an important bioactive molecule that most likely works in concert with one or more other molecules to produce the quorum sensing effects (2). At this time, there is not a definitive identification of the

other QSM(s) that may be utilized by *C. neoformans*. Interestingly, the growth rate of *C. neoformans* was also increased, to a lesser extent, by conditioned medium produced by *Cryptococcus albidus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Sporothrix schenckii*, and *Blastomyces dermatitidis* suggesting there may be QSMs produced by other fungi that have an effect on *C. neoformans* (2).

C. neoformans produces GXM and melanin, two important cellular components of the yeast that contribute to its pathogenicity. The QSMs addressed are capable of increasing GXM production as well as decreasing the time required for both GXM and melanin production. They have also been shown to control growth (Fig. 1) (2, 21). A virulence factor of *C. neoformans* that is known to be regulated by the environment is antiphagocytic protein 1 (App1) (46). One possible future study would be to test if quorum sensing has an effect on the expression of App1 or other factors of the yeast. *C. neoformans* appears to have a complex quorum sensing system, but more research is needed to identify all of the strategies this yeast uses to survive inside the host.

SACCHAROMYCES CEREVISIAE

Saccharomyces cerevisiae is a nonpathogenic, dimorphic fungus. Its morphology has been shown to be auto-controlled by QSMs. When *S. cerevisiae* cells were placed in conditioned medium produced from cultures in the stationary phase, the *FLO11* gene used for hyphal formation was upregulated five-fold (6). The two QSMs discovered to be causing this effect are two aromatic alcohols, phenylethanol and tryptophol (Table 1). During nitrogen starvation, phenylethanol and tryptophol induce pseudohyphal growth in diploid cells and invasive growth in haploid cells (Figure 1). Filamentous growth was observed even with low concentrations of each molecule; however, when the molecules were combined the effect on filamentation was even higher (6). Tpk2p, a component of the PKA signal transduction pathway, and Flo8p, a transcription factor, are required for the upregulation of *FLO11*. These findings indicate that *S. cerevisiae* uses the PKA pathway to control morphology.

In addition to nitrogen starvation, cell density also affects the release of QSMs. High density cultures produce more phenylethanol and tryptophol per cell than do low density cultures. In addition, this work discovered a link between quorum sensing and nutrient sensing (6). Although this yeast is non-pathogenic, it is important to learn more about how the organism communicates at a population level, to add to the body of knowledge of fungal quorum sensing.

SIGNIFICANCE AND CONCLUSION

Quorum sensing is a complex phenomenon that allows microorganisms to communicate and regulate many processes. Communication is accomplished through molecules, known as quorum sensing molecules, which are released into the extracellular environment. QSMs must accumulate to reach a concentration threshold in order to have an effect on the organism. These molecules bind to receptors on the organism's cells and once enough receptors are bound they activate signaling pathways. Many fungi utilize

quorum sensing to affect pathogenicity by controlling growth rates, morphology, gene expression, biofilm formation, and virulence factors, all at a population level. The understanding of these mechanisms is of great importance in today's world and needs extensive attention due to the prevalence of fungal infections and the need for new and better antifungal therapies. Table 1 gives a summary of the QSMs discussed in this review. In *C. albicans*, farnesol aids in altering the morphology of the fungi by inhibiting hyphal formation, while tyrosol promotes hyphal formation and increases growth rates (7, 15, 35). *C. neoformans* QSMs are not as well-known as those of other microbes. Pantothenic acid was identified as a QSM in *C. neoformans*; however, it appears other molecule(s) also play a role in the quorum sensing effects of this organism. Pantothenic acid was found to affect the growth rate, melanin production, and GXM release of *C. neoformans* (2). Peptides have been known for some time to have quorum sensing properties in bacteria; the peptide QSP1 is able to have quorum sensing-like effects on the growth of the fungal species *C. neoformans* (21). In *S. cerevisiae*, the QSMs that have been discovered are the aromatic alcohols phenylethanol and tryptophol. These molecules induce hyphal growth (6).

Quorum sensing was a significant discovery as many of the processes it affects are required for the pathogenicity of microbes. One of the most interesting hypothesized reasons bacteria use quorum sensing is to remain hidden from the host's immune system until a large population of the pathogen has grown. This allows the bacteria to grow in number and be ready to overcome the host's immune response before releasing virulence factors that activate the host's immune response (12). It has even been observed that host organisms have the ability to inhibit quorum sensing in bacteria by mimicking QSMs that the bacteria secrete. These host organisms include the macro alga *Delisea pulchra*, as well as several plant species (26, 43). Macroalgae and plants have been shown to inhibit the most well-known QSM, *N*-acyl-homoserine lactone (AHL), which is produced by many Gram negative bacterial species. The inhibitor molecules were found to compete with AHL molecules for their receptor, which is the LuxR receptor. By binding and blocking the receptor, the inhibitors prevent AHL-dependent signaling. This finding was further supported by findings that these inhibitor molecules also inhibit AHL-dependent processes in *V. fischeri*, *P. aeruginosa*, *V. harveyi*, and *E. carotovora*.

The discovery of these natural methods of inhibiting quorum sensing led to studies aimed at developing drugs that will inhibit quorum sensing (12). There are three potential mechanisms to inhibit quorum sensing systems: [a)] inhibit the production of the QSMs, [b)] degrade the QSMs, and [c)] block the QSM receptor (36). More recent studies have resulted in the identification of compounds that inhibit quorum sensing in pathogenic bacteria (9, 37, 42, 44). The use of quorum sensing inhibitors is of great interest as potential therapeutics to bacterial infections due to the increasing rate of resistance to conventional antibiotics.

In this review, we discussed the quorum sensing processes and QSMs in fungi. Knowledge of how fungi participate in quorum sensing has great potential for improved medical treatments and preventions. For example, farnesol produced by *C. albicans* shows potential as a preventative option against biofilm formation, which could be important, as biofilm formation on medical devices is an emerging problem in patient care (35). Farnesol was demonstrated to have protective effects in mice with oral candidiasis

(13). Another study showed farnesol to aid the effects of certain antifungal drugs against *C. albicans* and alter drug efflux (40). While these studies have shown farnesol to result in a less severe infection, other studies have shown farnesol increases virulence of *C. albicans*. One group discovered that farnesol given to mice with systemic candidiasis resulted in an increase in virulence (28). The effect farnesol had on the virulence of *C. albicans* was a result of modulation of the host's cytokine response (29). These contrasting studies on the effect of farnesol on pathogenicity demonstrate the complexity of the effect of QSMs on fungi and fungal infections. In particular, farnesol may have a different effect on the pathogenicity of localized *C. albicans* infections compared to systemic infections.

Due to the growing threat of fungal infections, new and better therapeutics are needed for these infections. The modulation of quorum sensing in fungi represents a promising potential mechanism of new antifungal therapies. This treatment approach is especially promising due to the ability to target fungal cells with the potential for little to no effect on mammalian cells. More research is needed to understand quorum sensing in fungi and uncover methods of regulating these processes.

Many current antifungal treatments are toxic and costly. Thus, exploration into quorum sensing may be very beneficial in this area (2). Quorum sensing in bacteria has become a growing area of research due to the increasing number of drug resistant bacteria. Inhibiting cell–to–cell communication could be beneficial by targeting one type of bacteria in a host's microbiota while not harming the whole population (12). This strategy could potentially be used to treat fungal infections as well, while potentially preventing the overwhelming side effects of the commonly used antifungals. Many QSMs overlap in different microbes. This may conceivably represent a form of competition that may be exploited to target pathogenic fungi. Insights gained from studies on fungal quorum sensing provide potential for the development of new therapeutics aimed at treating and preventing fungal infections.

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SAFE SCIENCE IS GOOD SCIENCE

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INTRODUCTION

Each year thousands of student researchers pursue honors theses and practicums at university laboratories in preparation for future careers. While many will learn the nuances of performing biomedical research, a majority will likely graduate without knowing much about biosafety. As a new generation of scientists emerge from academia, it is crucial that they learn about the value of biosafety and begin to develop safe work practices to protect themselves, others, and the environment. Researchers must practice laboratory techniques and safety skills with equal diligence. Individual responsibility is the foundation for developing a culture of safety in biomedical research.

WHAT IS BIOSAFETY?

Biosafety is a form of risk management. The ultimate goal of biosafety is to decrease the incidence of laboratory-associated infections (LAIs) by lowering the risk of accidental exposure to biological agents through a combination of risk assessment and mitigation strategies. There is never a total reduction in risk, but the probability of an undesired outcome can be reduced to almost zero with an appropriate understanding of the risks presented by the agent, the procedures involved in the research, and the facilities where the work is conducted.

The practice of biosafety has progressively become a mainstream discipline and an international profession in itself. Several national and international biosafety associations exist to enable biosafety professionals to share best practices, inform policy discussions, and provide guidance on standards. Biosafety professionals work in occupational health and safety programs at universities, corporations, nonprofits and government agencies. They perform risk assessments and establish controls on practice and procedures to protect the worker, public, and environment. Hazard analysis, applied research, engineering, program management, emergency response, microbiology, molecular biology, and even investigative work are common elements of the biosafety profession.

WHAT SHOULD BE CONSIDERED IN A RISK ASSESSMENT?

A risk assessment is a formal consideration of the potential hazards associated with a particular agent and its use. When conducting a biosafety risk assessment, one must be able to identify possible sources and routes of infection to the worker. Once the hazards are anticipated, specific controls can be put into place to reduce the risk of LAIs.

There are many factors that must be considered when conducting a standard risk assessment, but they can generally be categorized as either agent hazards or laboratory hazards. Agent hazards are the intrinsic or modified characteristics of a biologic that may have an adverse impact on health. The type of species or strain used, virulence, transmissibility, resistance, infectious dose, tropism, and other factors are crucial elements to consider when evaluating the inherent risk of an agent to cause disease. The potential outcomes of natural mutation and genetic engineering must also be considered when assessing the hazards of an agent. A determination as to whether a biological agent is harmful depends on the host as well. What may be a harmless microorganism to a healthy individual could be a formidable pathogen leading to disease in another individual who has a weakened immune system. For example, the yeast, *Candida albicans*, is an opportunistic pathogen commonly found in human flora. Individuals who are immunocompromised, such as those with autoimmune diseases or

who undergo chemotherapy treatment may develop serious disease when infected with this organism.

The risk of an LAI also depends on laboratory hazards. Laboratory hazards are vulnerabilities in the lab environment that increase the risk of injury or illness. Unsafe practices such as recapping a syringe may lead to a sharps injury. Common practices like pipetting, vortexing, sonication, and other aerosol-generating procedures may create bioaerosols leading to a potential inhalation exposure. Faulty equipment such as a cracked seal in a centrifuge cup have been known to release aerosols. Among the most common of all laboratory hazards is the failure to wear the right attire and personal protective equipment (PPE) appropriate for the laboratory procedure to be performed.

Although risk assessment often involves considering many factors at the same time, its purpose is not to confound but to clarify. Its careful application reveals potential hazards and vulnerabilities before research begins. Preventive action can be taken to protect the worker and others. No matter how virulent or transmissible the microorganism, it cannot cause disease if proper biosafety measures are implemented to prevent exposure.

HOW IS RISK REDUCED?

Once the agent and laboratory hazards are assessed, steps must be taken to reduce the likelihood of potential harm to the worker. In the biosafety field, it is standard practice to categorize research with biological hazards according to a "biosafety level". Based on the risk assessment, work with a pathogen can be assigned to Biosafety Levels (BSL) 1 through 4, with BSL-4 offering the highest protection to the worker and the environment. Each biosafety level builds upon the safety requirements of the previous level. An appropriate combination of microbiological practices, safety equipment, personal protective equipment, and facility design according to each biosafety level reduces the risk of potential biological exposures.

An undergraduate microbiology teaching laboratory may be considered a BSL-1 laboratory. BSL-1 typically involves work with agents that are not known to cause disease in healthy adults. *Bacillus subtilis, Naegleria gruberi, Escherichia coli* (K12) are examples of agents that may be used in a BSL-1 laboratory. The protection afforded by BSL-2 practices and procedures should be utilized when working with moderate-risk agents that can be acquired through mucous membrane exposure, percutaneous injury or ingestion. Examples of agents that may be used in a BSL-2 laboratory include *Staphylococcus aureus, Pseudomonas aeruginosa,* and the measles virus. In the case of measles, BSL-2 is appropriate because adequate vaccines are available. Biosafety Level 3 (BSL-3) is assigned for potentially lethal agents that are known to be transmitted *via* aerosols and may or may not be indigenous to the region. *Mycobacterium tuberculosis*, highly pathogenic avian influenza A virus, and *Francisella tularensis* are a few common examples of agents worked with in BSL-3 laboratories. When there are no treatments available for life-threatening infectious diseases caused by exotic pathogens, and there is a high individual risk to the worker, particularly through the aerosol route, these agents are handled under BSL-4 conditions. These laboratories are usually referred to as maximum-containment facilities and provide the highest levels of protection for the laboratory worker. Ebola virus, Marburg virus, Crimean-Congo Hemorrhagic Fever virus and Nipah virus

are examples of pathogens that require BSL-4 containment.

In addition to these standard biosafety levels, there are specific containment designations depending on the activity being performed. For example, infectious disease work involving animals are designated as Animal Biosafety Levels 1, 2, 3 and 4 (ABSL). The challenges presented by the animal model are taken into consideration when performing a risk assessment. Animals can be unpredictable. They can bite, scratch, kick, shed fluids, produce splashes and aerosols, and increase the risk of accidental exposure to the worker. Some animals may even be infected naturally with zoonotic agents that can cause dangerous, sometimes deadly infections. Research involving large or loose-housed animals is conducted in specially designed BSL-3-Agriculture (BSL-3 Ag) laboratories. In this scenario, the risk assessment places greater emphasis on preventing the pathogen from escaping into the environment. If there were a release of an agricultural pathogen, the economic implications could be significant. For example, an accidental release of a pathogen that infects cattle could potentially impact a $44 billion industry. A useful resource for learning more about biosafety levels is the CDC/NIH publication, *Biosafety in Microbiological and Biomedical Laboratories* 5th edition (4).

THE VALUE OF BIOSAFETY

Careful adherence to biosafety principles protects individuals, reputations, and research. Perhaps the best way to communicate the value of biosafety is through the tragic example of a laboratory-associated infection that occurred at Yerkes Regional Primate Center at Emory University. On October 29, 1997, a young researcher was conducting a routine procedure that involved moving a non-human primate in caging. The primate, a rhesus macaque (*Macaca mulatta*), was unknowingly infected with a zoologic virus known as *Macacine herpesvirus* (formerly *Cercopithecine herpesvirus* 1 [CHV-1]). The pathogen can be present in monkey saliva, urine, fecal matter, and conjunctival fluid. Although disease is usually mild in primates, it is frequently deadly in humans who are exposed to the virus. During the transfer, the researcher suffered an ocular exposure to hazardous macaque fluids. Despite having flushed her eyes and later seeking medical attention, it was not enough to save the worker's life. Just 42 days later, she died due to refractory respiratory failure from this seemingly minor exposure. A subsequent CDC/OSHA investigation found that the primate center believed the risk of CHV-1 exposure was thought to be low for the activity, that eye wash first aid was not conducted for at least 15 minutes, and that medical reporting and subsequent treatment was delayed. These factors contributed to a terrible outcome (1, 5).

Seemingly innocuous microorganisms can lead to serious LAIs as well. In 2009, an associate professor at the University of Chicago became the first person to die from an accidental exposure to an attenuated *Yersinia pestis* strain. The researcher worked with pigmentation-negative KIM D27, a strain considered avirulent because of its iron-acquiring limitations. However, host factors in the researcher appear to have made the laboratory strain virulent. Hemochromatosis is a hereditary medical condition in which the body absorbs too much iron and deposits excess amounts in organs. It is believed that after the researcher experienced a percutaneous or mucosal exposure in the laboratory, the KIM D27 strain was able to establish an infection due to elevated iron levels in

his body. An investigation revealed that the researcher did not wear appropriate personal protective equipment and did not have current biosafety training. This case highlights the importance of consistently practicing biosafety recommendations to mitigate unknown risk factors (2).

It is clear that improper adherence to biosafety practices and resulting LAIs could have a devastating impact on the individual. However, it is worth taking a moment to appreciate the bigger picture: the impact unsafe practices in the laboratory could have on co-workers, the general public, the environment, and the overall research enterprise. Recently, the Centers for Disease Control and Prevention (CDC) has been in the news media about several incidents where biosafety protocols and procedures have been breached. Most notably, the unintentional exposure of personnel to potentially viable anthrax and the cross-contamination of non-pathogenic avian influenza virus with the highly pathogenic avian influenza virus strain H5N1. In both cases, the lack of proper adherence to biosafety protocols led to potential exposures outside the laboratory. As a result, research activities of the laboratories involved in this incident were suspended. Significant time and funding were spent to investigate the incidents, re-inspect inventories, and retrain personnel. One could argue that the most important loss in the ensuing months has been the public trust and confidence in the scientific enterprise to conduct biomedical research safely with high-risk pathogens (3, 6). How can this trust be regained? How can future incidents be prevented? Diligent adherence to biosafety practices and procedures while working in the laboratory will ensure that workers are safe, the community and environment is protected and the progress of vital scientific research is not interrupted.

HOW CAN STUDENTS CONTRIBUTE TO BIOSAFETY?

Students can protect themselves and others by taking laboratory safety seriously. They should expect their departments to provide safe work spaces and comprehensive laboratory training. Before beginning research, students should make sure their project has undergone a formal risk assessment and that they feel comfortable performing it safely. Students should learn how to safely operate any equipment before using it and always follow standard operating procedures. Personal protective equipment must always be worn. Know what to do and whom to contact when an accident occurs, such as a spill, injury, or illness. If students have safety questions or concerns, they should make them known to their faculty or institutional safety office. Student researchers may even consider contributing to applied biosafety research, for example, by studying potential routes of contamination in a laboratory using a harmless surrogate or by evaluating the effectiveness of the institutional biosafety program.

SUMMARY

Biosafety is a multi-faceted discipline, a form of risk management designed to minimize the risk of biological exposures and prevent laboratory-associated infections. Biosafety professionals protect the research community's health and safety by assessing research and setting risk reduction strategies. For these strategies to be successful, student researchers and faculty advisors must commit to incorporating them into their daily research activities. Those who understand the value of biosafety will not only protect themselves and others but will also advance research by realizing that safe science is good science.

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PERSPECTIVE

COME DINE WITH MICROBES: WHERE MICROBIOLOGY, FOOD, AND CULTURE MEET COMMUNITY OUTREACH AND STUDENT ENGAGEMENT

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INTRODUCTION

Thompson Rivers University (TRU) is located in the interior of the province of British Columbia in Canada. At TRU, we offer a cellular, molecular and microbial biology (CMMB) program alongside other biology programs. Students can select a three-credit elective upper level course, BIOL 4490, entitled "Selected Topics in Biology" which features an advanced seminar in a topic of interest. One of the BIOL 4490 courses was on Microbial Applications in Industry (Industrial Microbiology) for the 2014 winter semester and it highlighted the unlimited biochemical capabilities of microorganisms as well as a great variety of microbial fermented products. Principles of fermentation technology were introduced and various factors that have a great impact on the biochemical and physiological basis of industrial fermentation processes were discussed. Different fermented products and the microbes associated with their production were chosen and the students investigated them as case studies.

The general learning objectives of this course were to:

1. Recognize the role of microorganisms in industrial microbiology.
2. Understand the range of fermentation processes available for different products and the practical benefits and limitations of them.
3. Develop an awareness of the importance of microorganisms in industrial processes.
4. Learn the biochemical strategies used by microorganisms to produce metabolites that we can use to our benefit.
5. Explore the versatility of microorganisms, their diverse metabolic activities and their products.
6. Learn about industrial microorganisms and the technology required for large scale cultivation and isolation of fermentation products.
7. Learn to read and critically evaluate scientific literature.

With the intention of enhancing student engagement and community outreach with topics taught in class, the "Come Dine With Microbes" project was introduced. I was

Table 1. Student Evaluation for the BIOL 4490 Microbial Applications in Industry

Prior to 2014 Winter Semester	Winter 2014 Semester
In-class participation (10%)	In-class participation (10%)
Field trip report (10%)	Field trip report (10%)
Field trip participation (10%)	Field trip participation (10%)
Term paper (20%)	Individual presentation (10%)
Individual presentation (10%)	Group presentation (10%)
Group presentation (10%)	Come Dine With Microbes (50%)
Final exam (30%)	

inspired by a laboratory menu passed on to me years ago by a retired faculty member, Carolynne Fardy (Fig. 1). When I saw the menu again prior to the semester, I had a spark and I thought I should ask the class whether they were open to the unorthodox idea of putting on a dinner/fundraiser based on microbial fermented products instead of writing a final exam and a term paper for the 50% of the marks (Table 1). With this dinner, we, as microbiologists, could also help raise public awareness of the fact that not all bacteria are bad (fewer than one per cent of all bacteria cause human illness). The majority of microbes are good or neutral to us and in fact we cannot live without them. By introducing this initiative, I hoped that the experience would engage student learning and that at the completion of this event the students would be able to discuss the role of microorganisms in industrial microbiology and the range of fermentation processes available to produce different products with their practical benefits and limitations. I wanted the TRU students to communicate their knowledge at a layman's level by interacting with the NorKam Secondary School students and dinner guests. Furthermore, I hoped that they would communicate to this wider audience the importance of microorganisms in our daily life, the versatility of microorganisms, and their diverse metabolic activities resulting in products for our society. The dinner had the added benefit of intercultural learning through sampling different ethnic fermented foods. I would like to think that I am a part of creating the next generation of global minded scientists; one small class at a time!

“WITH THIS DINNER, WE, AS MICROBIOLOGISTS, COULD ALSO HELP RAISE PUBLIC AWARENESS OF THE FACT THAT NOT ALL BACTERIA ARE BAD (FEWER THAN ONE PER CENT OF ALL BACTERIA CAUSE HUMAN ILLNESS).”

In September 2014, we had discussed this idea with a local school, “NorKam Secondary School”, and their Culinary Arts, Foods and Home Economics Program. The instructors, Chefs Scott Roberts and Jo-Anna Allen agreed to help us put on the event on April 11, 2014. In collaboration, we were asked to dedicate two class-times prior to the dinner to go to the secondary school classes and demonstrate to the high school students the impact of microorganisms on food safety so the students can appreciate hand washing and no-touching-face/hair-during-cooking policy.

Once decided, we used some of our own class times to organize the event. Students designed and chose the menu (Fig. 2). We worked together to locate sponsors for the event. Once the tickets were ready (Fig. 3), we had them sold in a very short amount of time. We also met with Chefs Roberts and Allen a few times to go through aspects of getting the kitchen and materials ready for the dinner. Our TRU students were the main kitchen workforce with the NorKam students helping. Communications were maintained through email messages. Prior to the dinner, the TRU students spent two class-times talking to and introducing the NorKam students to the wonderful world of microbes with special emphasis on the relationships of microbes with food and food

Fig. 1. The menu passed on to the author that sparked the initiative (Source: Unknown)

Menu à la Laboratoire

Soup

Miso (soybeans arranged by *Aspergillus* and *Saccharomyces*)

Salad

Olives prepared by *Leuconostoc*
fleshy fungi (*Agaricus*) grown on thoroughbred manure
and seasoned by *Acetobacter* (vinegar)

Entrées

S. cerevisiae and (by request) *Lactobacillus* will prepare
rye, pumpernickel, and sourdough

Single-Cell Protein

A delightful casserole of sewage-fed cyanobacteria. Flavored with **poi**
(lactic-acid bacteria work their magic on taro root)

Fettucine Torula

Spicy noodles "proteinized" by
Torulopsis yeast

Beef Bonanza

Tender slices of *Methylophilus*-fed beef marinated in **soy sauce**
(produced by a symphony of microbes)

Carne Macha

An assortment of sausages from
Pediococcus and *Penicillium italicum*

Vegetables

Cabbage fermented to pH 3.5 by *L. plantarum*

Dessert

* *S. thermophilus* and *L. "Bul" garicus* will culture milk
Chef *Leu CoNostoc* will smother it in dextran, an α-1,6-glucose polymer

* Assorted cheeses
Streptococcus and *Lactobacillus*, assisted by *P. roquefortii* and *P. camemberti*

Drinks

Alcohol served by the sweet
fungus *Saccharomyces*
Beer
Wine

Nonalcoholic beverages
Lactobacillus' buttermilk
Saccharomyces' root beer
Erwinia's coffee

safety (Fig. 4). TRU students were marked on their presentations and interactions with the NorKam students. We spent a few hours of the day before the event purchasing ingredients and putting them in the NorKam kitchen (Fig. 5). The actual day of the event, all of us arrived at NorKam early to prepare the foods and the venue. We had a number of volunteers to help us as well.

At the dinner, we arranged a non-traditional buffet style by having food stations (for each chosen fermented food) and having TRU microbiology students and NorKam students man each station (Fig. 5). This facilitated communication with the guests when they came up to sample various foods. We successfully hosted 80 guests (approximately 100 people in total, including students and volunteers). Guests were very happy to taste delicious and healthy foods while learning about the production of microbially fermented foods through posters (Fig. 6) and communication with students. Some were pleased to win our raffle baskets filled with different fermented products donated and sponsored by local merchants (Fig. 5). A number of guests expressed how much fun it was and that they would like to see more events such as this. At the end, we raised $1,000 that was split between NorKam and TRU Microbiology teaching and research activity.

All in all, I think that we achieved what we set out to do given the success of the event. TRU students were engaged and showed their learning by teaching the high school students and dinner guests about the role of microbes in production of the various foods that were served. High school students and teachers responded enthusiastically to the microbiology exercises they performed and the food that they helped to prepare. Dinner guests were equally enthusiastic about the meal and the information provided by the students. The TRU students did very well explaining the role of microorganisms in industrial processes and food production in language that was easily understood by high school students and members of the public at the dinner. The students also demonstrated communication skills through posters (Fig. 6), brochures (Fig. 7), as well as informal verbal presentations at the dinner. They conveyed to a wide audience the importance of microorganisms in our daily life, the versatility of microorganisms, and their diverse metabolic activities resulting in products that can be used in our society.

On February 12, 2014 I was very happy to see an article entitled "Chefs and Microbiologists Break Bread Anew" published in the ASM-Microbes volume 9 (1) pp.13-17 (http://www.microbemagazine.org/index.php?option=com_content&view=article&id=6567:chefs-and-microbiologists-break-bread-anew&catid=1295&Itemid=1565). What perfect timing for our class project! My idea was not that far-fetched after all and I was glad that my students were on board and were much more enthusiastic than any class I ever seen before. Throughout the course, I found continuous enthusiasm and positive learning energy from my students even though they had to work harder than previous classes preparing for the dinner. This showed how engaged the students were through planning and executing the event. In my 13 years of teaching at TRU, I have never felt such a high level of ownership and pride demonstrated by students.

COMMENTS AND IMPRESSIONS FROM STUDENTS AND DINNER GUESTS

Comment #1: "As for my experience, Come Dine With Microbes was the most rewarding project of my undergrad as it provided me with an opportunity to participate in a community outreach project and to network with the public, something our faculty does not emphasize. I not only valued the opportunity to be creative and learn the ropes of hosting a public event, but I also enjoyed that the theme we created which allowed us to educate the public on a scientific topic through the universal language of food! Having the opportunity to connect with the public provided me with an opportunity to learn how to communicate microbiology in the "real world" and in a way that left a lasting impression on the students we lectured at NorKam, as well as the gracious guests that attended our event!"

Comment #2: "I would say that overall the dinner was a success for the entire class as well as the community at large. Planning for this dinner allowed my classmates and I to discover just how many uses microbes actually have in the industrial world and in the culinary arts (I had no idea that chocolate is the result of fermentation). Furthermore, the presentations that we gave to the students at NorKam Secondary as well as the guests at the dinner allowed us to pass on what we had learned to the community. In the end, I think that the class as well as our audience members understood that most microbes are not bad; many are beneficial and necessary for the little luxuries we often take for granted. I had a great time in this course and would recommend that a similar approach is repeated for future classes."

Comment #3: "This class introduced me to the potential health benefits and industrial applications of microorganisms by giving me hands-on real life experience."

Comment #4: "This started out as a fun way to spread the word about the importance of microbial fermented food in a healthy diet, and ended up being an incredible learning experience. Not only did we discover fermented food products from all over the world and get to make several of them ourselves, we learned how to plan, organize, and carry out a fundraising event! Because this was our event and our food, everyone wanted to see it a success and I have never known an entire class to collectively put that much effort into an assignment before!"

Comment #5: "Come Dine With Microbes was an educational and fun event with lots of good food! The students did a fantastic job of presenting and serving the food. I thoroughly enjoyed meeting new people and old acquaintances as well."

Comment #6: "My spouse and I attended the "Come Dine With Microbes" event in April 2014. Dr. Cheepthams' passion and desire to promote microbiology concepts applicable to daily life events (i.e. food) was most evident in the enthusiasm and energy her students exhibited throughout the evening. Each menu item was delicious and accompanied by a narrative with regard to the microbes utilized in the dish. The event was well organized and educational. In my value judgment, a meaningful dining and learning experience was had by all participants."

Comment #7: "I found 'Dinner with the Microbes' to be such a unique and creative learning experience for me and my children (age 4 and 7), in addition to the students who worked so hard to bring it to fruition. I was not sure what to expect with respect to a dining experience, but was intrigued by the concept and believed in the educational merit. To my surprise the food was amazing and my children and I learned a great deal

Fig. 2. The menu that students selected to represent at our dinner

Come Dine With Microbes Menu

Starter
Kimchi Crostini

Soup
Miso Soup

Salad
Pickled Root Vegetable Salad

Entrees
Tempeh Sliders

Sausage and Sauerkraut

Dessert
Chocolate Dipped Fruits

Nonalcoholic Beverages
Ginger Beer

Tea

Coffee

Fig. 3. The ticket for the event

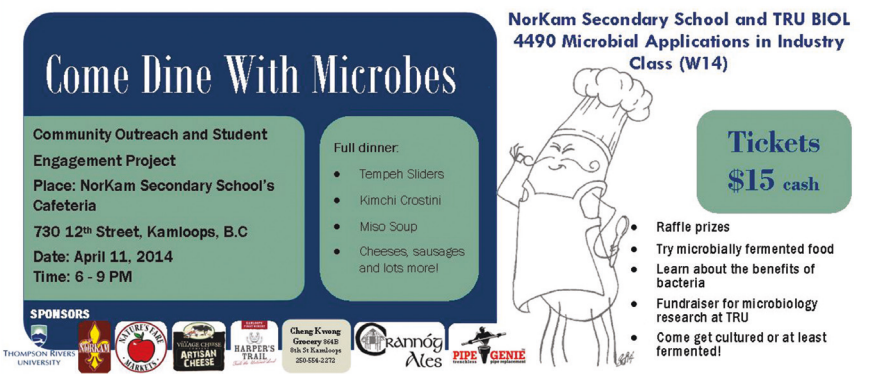

Fig. 4. TRU student presentation on the wonderful world of microbes to NorKam's Culinary Arts, Foods and Home Economics Program students prior to the dinner event: a) Class presentation; b) Demonstration of bacteria cultured on the plates from swabs that NorKam students did; c) Chef Scott Roberts and the NorKam students interacted with the TRU students; d) Glow Germ hand washing exercise.

about microorganisms in a way that was engaging and memorable. It was evident that the students who participated in this event were inspired by the innovative teaching techniques offered through this event. I would not hesitate to attend such a function again in the future and could see an event like this growing with time as it was received so well by those who attended."

Comment #8: "My wife, 7 year old daughter, and I all attended the "Come Dine with Microbes" dinner at NorKam secondary. This event was really enjoyable. First, as we walked through the food buffet, we enjoyed talking with both the TRU microbiology students and the NorKam science students about how the food served were representative of different microbial and fermentation processes. It was clear the students were enthusiastic about sharing their scientific knowledge in this practical way. Moreover, it was a very interesting experience for my family to gain a greater appreciation for the different production processes of the food at the different buffet stations! Also, while sitting and eating my family and I also enjoyed talking with other guests and students. Everyone at the event agreed how we had gained a greater

“THIS DINNER WAS A GREAT ‘AWARENESS TOOL’ THAT SHOWED THE PUBLIC THAT NOT ALL BACTERIA ARE HARMFUL AND HAVING A REGULAR INFLOW OF NEW MICROBES INTO OUR SYSTEMS CAN ACTUALLY BE BENEFICIAL AND HEALTHY.”

appreciation, and understanding of our food through this event. I really believe events like this contribute to increasing our awareness and knowledge of scientific areas such as microbiology.”

Comment #9: “I was intrigued when I heard there were tickets being sold for “Come Dine With Microbes” so decided to attend. I found the whole experience very delicious and interesting. The students did a wonderful job of presenting the dishes and were ready to answer our questions on the food and the microbes that created them.”

Comment #10: “Come Dine with Microbes was a wonderful, unique and engaging way to bring a more positive perspective to the community about the often hidden value of microbes in our lives. My husband and I thoroughly enjoyed the microbial based foods prepared by the enthusiastic students and came away with a better understanding of the health benefits of fermented foods.”

Comment #11: “The Dining with Microbes Dinner was an amazing opportunity to show just how essential microbes are to life. As students we were able to present a delicious array of food that was possible due to the actions of microorganisms. I think everyone at the dinner had a great time eating the food, winning various prizes all while being educated about the many jobs of microorganisms. The dinner was a great success and I believe our class and the participants had a blast!”

Comment #12: “I was thrilled with the dinner with microbes’ event last year. It was really beyond my expectations for everything. The students had done a great job providing interesting information about their particular food and they presented it well. The food was delicious, well-prepared and the tempeh sliders were my favorite! I will not hesitate to attend another event such as this.”

Comment #13: “The event was one of the most interesting things that I have done at TRU. It really encouraged me to work well with my fellow students in a way that a regular structured class does not provide. I helped out mostly with decorating the hall, design of posters, media things such as music and the tending to the miso soup station. Throughout the entire process I feel like I got to know my instructor and fellow classmates on a more intimate level. Many times classes have you sitting and staring at a projector screen trying to absorb somewhat boring words or material. This class and the dinner event focused on the community aspects more, which is something that I really enjoy. The dinner also engaged the public into a world of microbes that maybe they were never aware of existed in these foods. Some of the dishes also provided the public and students with a greater idea of how to make/prepare and enjoy these foods in our own home. This dinner was a great ‘awareness tool’ that showed the public that not all bacteria are harmful and having a regular inflow of new microbes into our systems can actually


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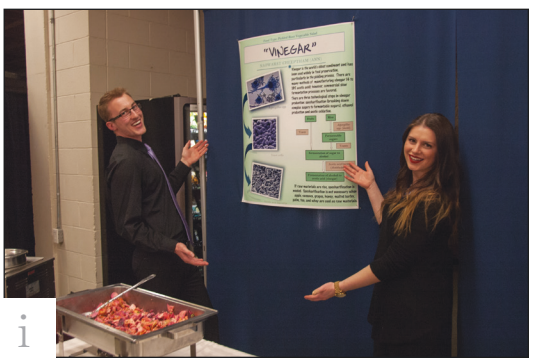

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Fig. 5. April 11, 2014 the event: a) Both TRU and NorKam students were briefed by Chef Scott Roberts; b to g) Students were having fun on food preparation and setting up the venue; j) Raffle ticket drawing and announcement; k) The team; l to o) Our happy dinner guests with their raffle baskets; p) Cleaning crew!

be beneficial and healthy. Overall, it was an awesome experience and it taught me a lot about microbes and how they interact with our foods."

Comment #14: "I think there is a lot of unfortunate misinformation about microbes spread by the media. At the very least, the focus is kept on the negative aspects of pathogenic bacteria, which obviously represents only a small portion of the known microorganisms and are actually exceptionally rare considering how many we encounter on a daily (or even hourly)

basis. I understand why these negative aspects are considered more "newsworthy" –– a story about contaminated food killing two people in Toronto is more shocking than a species of bacteria living in our digestive tracts helping us to break down our food and preventing illness. However, I think if the mainstream media isn't going to promote these ideas then somebody should. I know far too may people who constantly disinfect everything they may touch and can't go an hour without using hand sanitizer. These actions represent someone terrified of "bugs" and "germs" because they see them as purely negative, able to infect you at any moment and cause life-threatening illnesses! Of course, we know that this isn't the case and we understand that having communities of microbes inside your body is a requirement of a healthy individual, but I can understand why the average person gets frightened. Caution is completely fine (nobody wants food poisoning!) but I think people take it to a new level. That's why I think projects like ours are important. Everyone knows yogurt is made using microbes but many don't realize the other food products we gain or the importance of microbes in the process. After the dinner was held, my aunt (an attendee) made one of the dishes we served for Easter dinner and spread the word about the fermentation process to the rest of my family because she thought it was interesting. I think the dinner is a good format to spread the word because (if it's successful) it's something that people remember easily because they had fun and learned some new information! The same can be said for my own personal engagement. I will always remember it because I had fun and learned new information. I had no idea most of the foods we served were fermented. It was a very memorable learning opportunity! As for how I think we could make it better in the future... (if you do decide to do this with your classes in the future). I think everything worked out in the end which is awesome, but the process of organizing the dinner could also probably be streamlined. I think it would be more beneficial if, instead of people taking on certain individual jobs, if we had people (or small groups of people, 1-3) taking on general roles instead: Decorations (table cloths, table placement, centerpieces, screens/ screen placement, music); Signage (posters, pamphlets, advertising); Tickets (designing them, coordinating the selling of them, ideas for selling outside the university); Food (getting donations, figuring out amounts for the final diner (scaling up recipes), being in charge of menus); Raffle/Donations (getting donations, putting together the raffle baskets, coordinating sponsor logos for the tickets/ pamphlet); Secretary (going to all the NorKam meetings & taking notes, taking notes at in-class discussions, sharing the notes with the class, making a game plan for how everything will go during dinner

"I THINK THE DINNER IS A GOOD FORMAT TO SPREAD THE WORD BECAUSE (IF IT'S SUCCESSFUL) IT'S SOMETHING THAT PEOPLE REMEMBER EASILY BECAUSE THEY HAD FUN AND LEARNED SOME NEW INFORMATION!"

Fig. 6. Examples of posters describing microorganisms and fermentation processes involved in the food served.

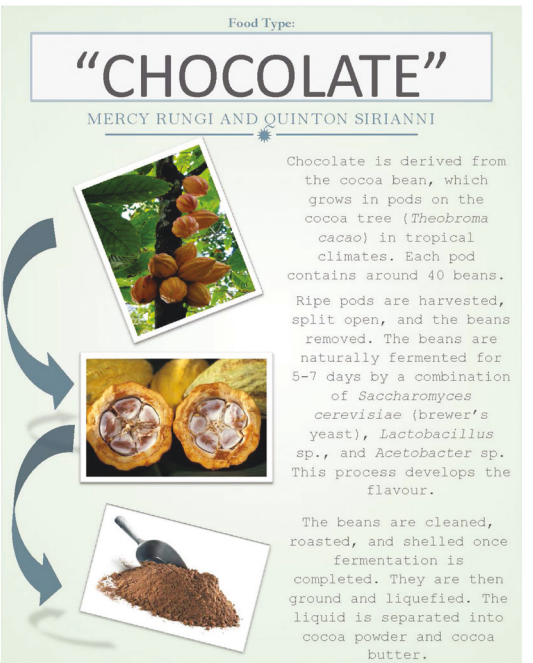
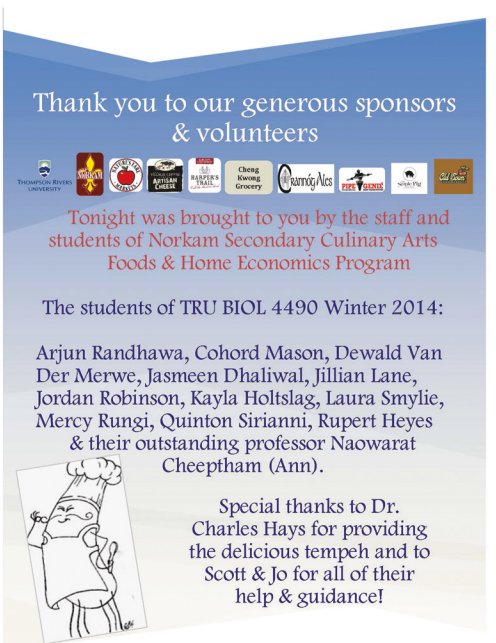
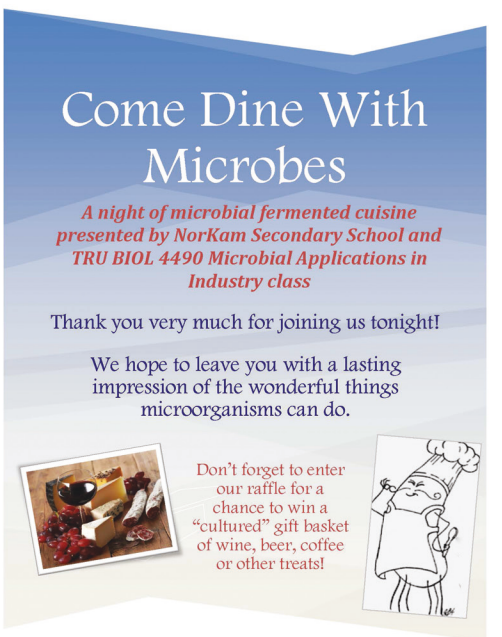


Fig. 7. Examples of student-generated brochures





preparation, etc.). Those are six roles and as I assume you generally have approximately 12 people in your class during a semester maybe two people could be assigned to each role? It worked out for us that everyone got all the work done but only because everyone tried really hard and we managed to have a lot of invested people! It may be beneficial to assign roles to make sure everyone does their part in the future. Also, this would reduce the amount of classes you would have to hold to discuss things -- two people would be responsible for one aspect and could then share ideas with the class and move on. On that subject, I think you could also reduce the amount of class time spent discussing the project by having a plan for each class -- what we want to discuss and have a set amount of time for each topic so we don't run overboard. This would be far easier now that we have a general timeline and some general ideas. I also think we should attempt to sell tickets to people in the community beyond those we know personally. Perhaps setting up a table at the mall? Or putting posters/flyers out around town? Either way I think it would really help the community outreach aspect of it! As a final suggestion, it may be beneficial to try and hold the dinner in the culinary arts building in the future (aka make plans around their schedule) as I think it would reduce a lot of the work and it's a nicer building!

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