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

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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.

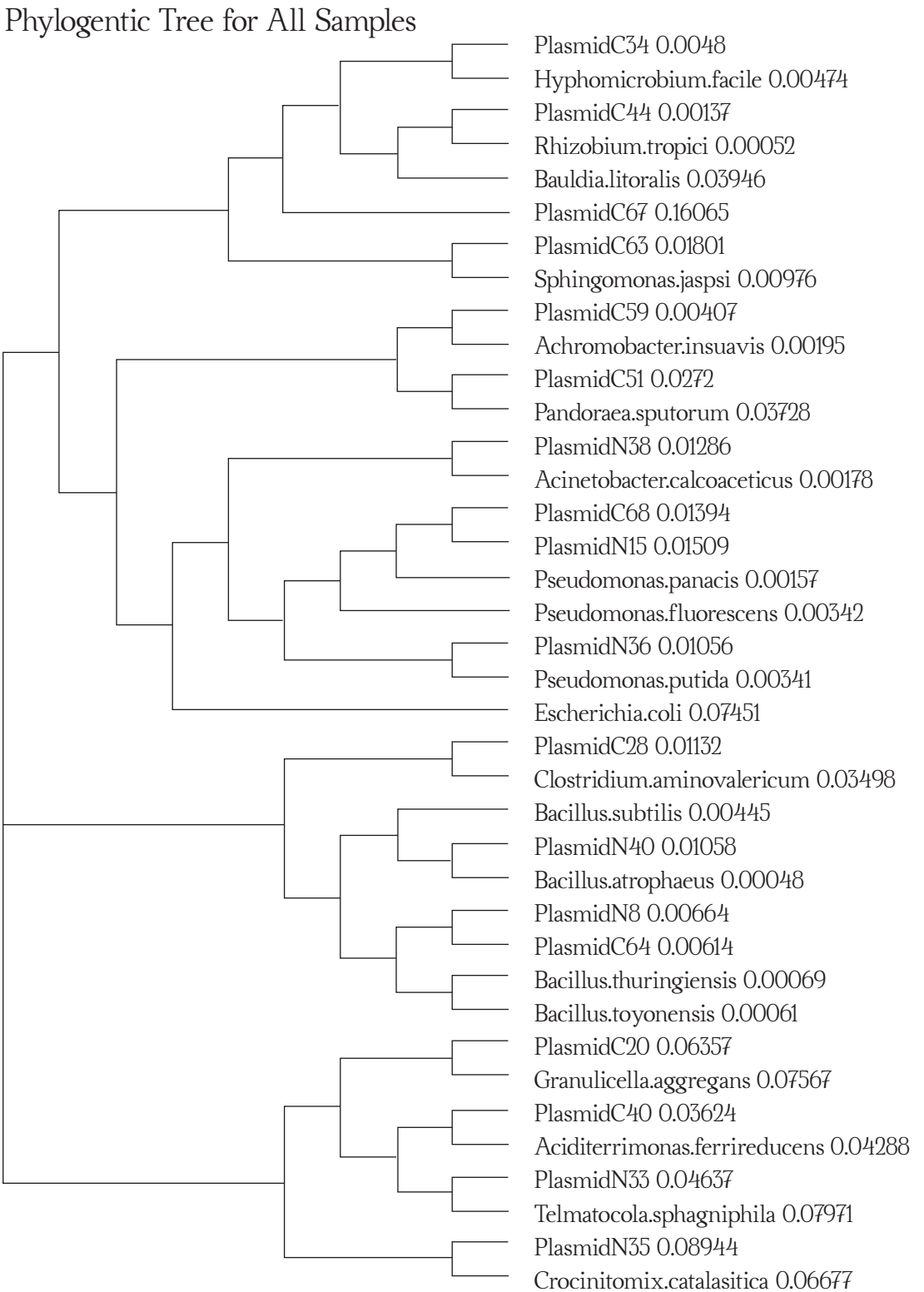

Figure 1. Phylogenetic Tree for all Non-vegetated (N) and Control (C) Samples.

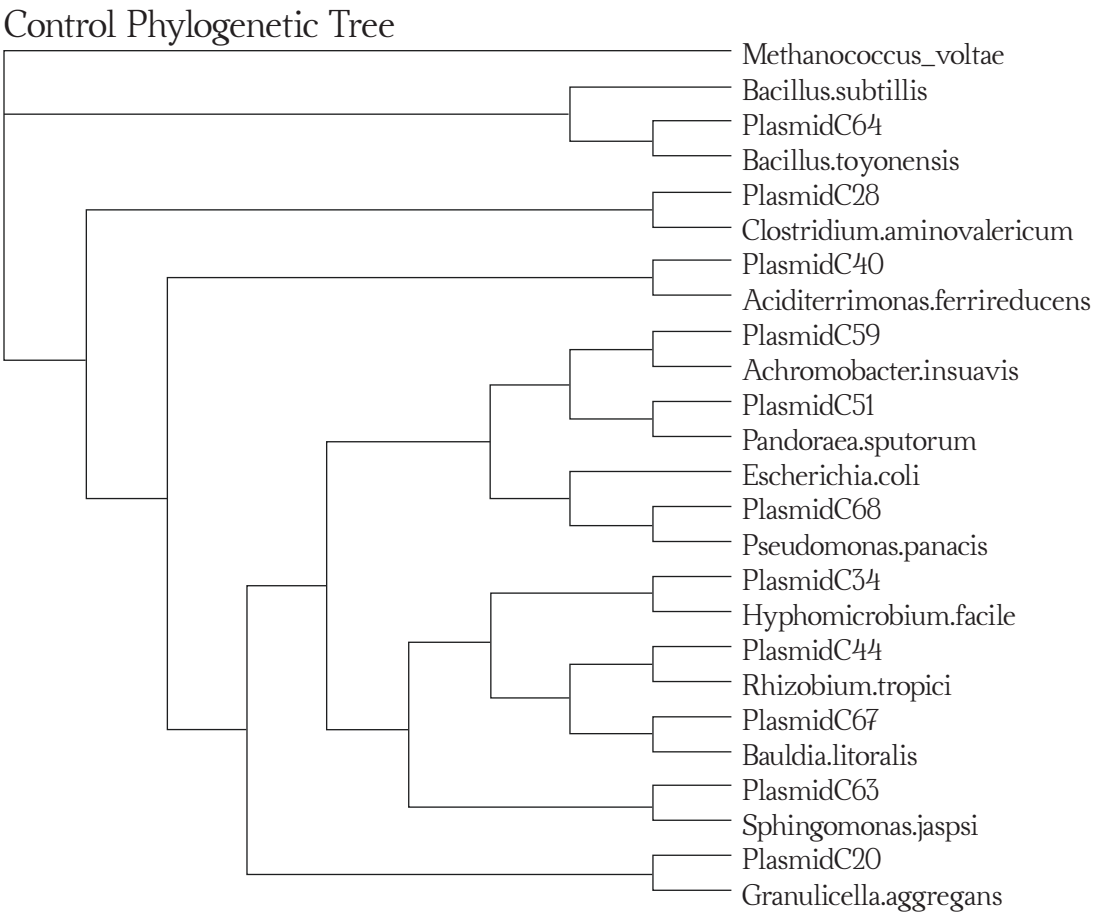

Figure 2. Outgroup Phylogenetic Tree for Control Samples.

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

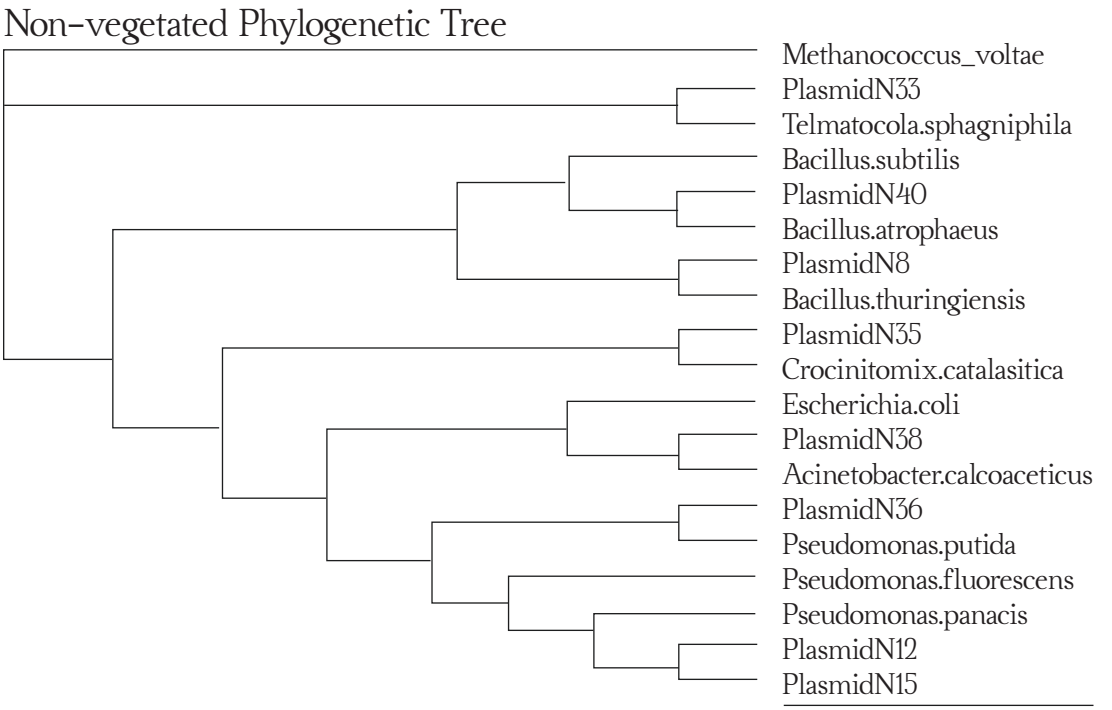

Figure 3. Outgroup Phylogenetic Tree for Non-vegetated Samples.

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

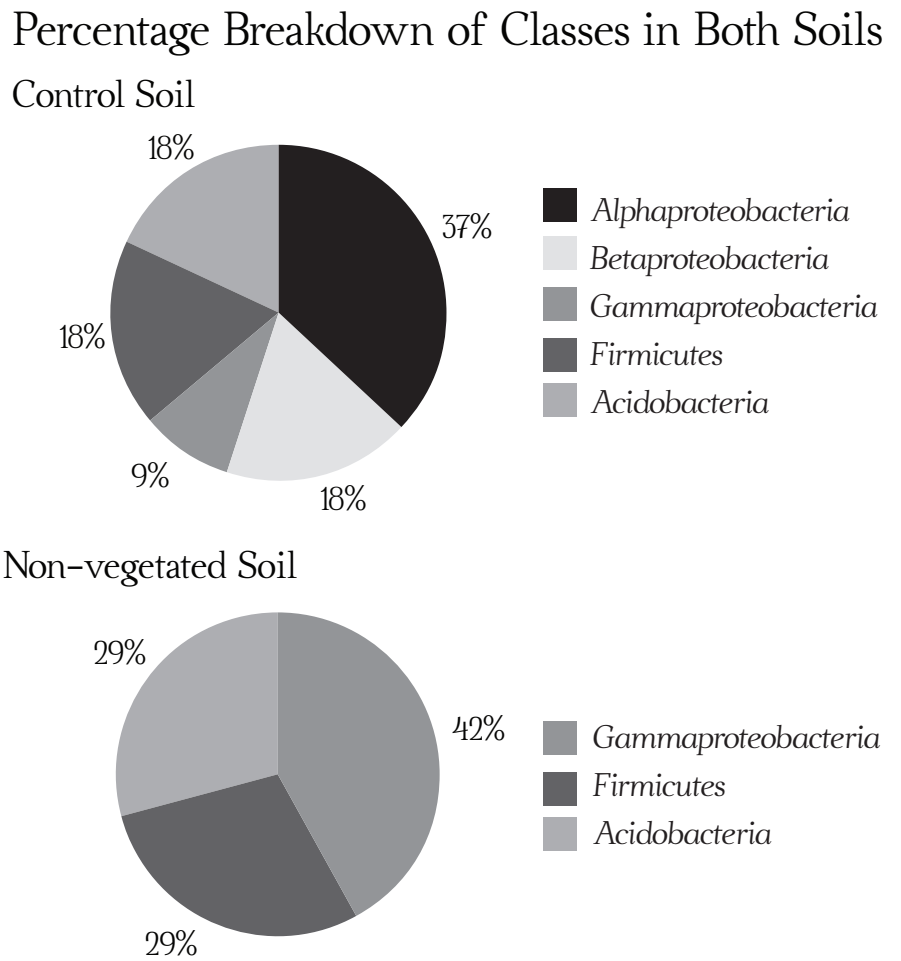

Figure 4. Pie charts representing the class diversity in the control soil and non-vegetated soil.

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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REFERENCES

1. Akhtar, M. S., Chali, B., & Azam, T. 2013. Bioremediation of Arsenic and Lead by Plants and Microbes from Contaminated Soil. *Res. Plant Sci.* 1:68–73.

2. Camargo, F. A., Okeke, B. C., Bento, F. M. & Frankenberger, W.T. 2003. *In-vitro* reduction of hexavalent chromium by a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}. *Appl. Microbiol. Biotechnol.* 62:569–573.

3. Celleto, V. R., Oliveira A. J. B., Gonçalves, J. E., Watanabe, C. S. F., Matioli, G., & Gonçalves, R. A. C. 2012. Biosynthesis of Indole-3-Acetic Acid by New *Klebsiella oxytoca* free and immobilized cells on inorganic matrices. *Scien. World J.*

4. Dipu, S., Kumar, A. A., & Thanga, S. G. 2012. Effect of chelating agents in phytoremediation of heavy metals. *Remed. J.* 22:133–146.

5. El-Shanshoury, A. E. R., Elsilk, S. E., & Ateya, P. S. 2013. Uptake of some heavy metals by metal resistant *Enterobacter* sp. isolate from Egypt. *Afric. J. Microbiol. Res.* 7:2875–2884.

6. Francis, A. J. 1998. Bioremediation of uranium contaminated soils and wastes. International conference and workshop: Uranium-mining and hydrogeology. Freiberg in Sachsen (Germany) 340–346.

7. Fulekar, M. H. 2010. *Environmental Biotechnology.* Boca Raton, FL: Taylor & Francis Group.

8. Gao, W. & Francis, A.J. 2013. Fermentation and Hydrogen Metabolism Affect Uranium Reduction by Clostridia. *ISRN Biotechnol.* 2013:1–11.

9. Gibbons, H. S., Broomall, S. M., McNew, L. A., Daligault, H., Chapman, C., Bruce, D., Karavis, M., Krepps, M., McGregor, P. A., Hong, C., Park, K. H., Akmal, A., Feldman, A., Lin, J. S., Chang, W. E., Higgs, B. W., Demirev, P., Lindquist, J., Liem, A., Fochler, E., Read, T. D., Tapia, R., Johnson, S., Bishop-Lilly, K. A., Detter, C., Han, C., Sozhamannan, S., Rosenzweig, C. N., & Skowronski, E. W. 2011. Genomic signatures of strain selection and enhancement in *Bacillus atrophaeus* var. *globigii*, a historical biowarfare simulant. *PLoS One* 6:e17836.

10. Gleba, D., Borisjuk, N. V., Borisjuk, L. G., Kneer, R., Poulev, A., Skarzhinskaya, M., Dushenkov, S., Logendra, S., Gleba, Y. Y., Raskin, I. 1999. Use of plant roots for phytoremediation and molecular farming. *Proc. Nat'l Acad. Sci.* 96:5973–5977.

11. Gomes, N. C., Kosheleva, I. A., Abraham, W. R., & Smalla, K. 2005. Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. *FEMS Microbiol. Ecol.* 54:21–33.

12. Goris, J., De Vos, P., Coenye, T., Hoste, B., Janssens, D., Brim, H., Diels, L., Mergeay, M., Kersters, K., & Vandamme, P. 2001. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend. *Int. J. Sys. Evol. Microbiol.* 51:1773–82.

13. Gupta, D. K., Srivastava, A., & Singh, V. P. 2008. EDTA enhances lead uptake and facilitates phytoremediation by vetiver grass. *J. Environ. Biol.* 29:903–6.

14. Han-Jen, R. E., Wai-Fong, Y., & Kok-Gan, C. 2013. *Pandoraea* sp. RB-44, A Novel Quorum Sensing Soil Bacterium. *Sensors (Basel)* 13:14121–14132.

15. Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60:579–598.

16. Itoh, T., Yamanoi, K., Kudo, T., Ohkuma, M., & Takashina, T. 2011. *Aciditerrimonas ferrireducens* gen. nov., sp. nov., an iron-reducing thermoacidophilic actinobacterium isolated from a solfataric field. *Int. J. Sys. Evol. Microbiol.* 61:1281–1285.

17. Järup, L. 2003. Hazards of heavy metal contamination. *Brit. Med. Bull.* 68:167–182.

18. Leeds, M. & Yang, M. 2010. Rhizofiltration using sunflower (*Helianthus annuus* L.) and bean (*Phaseolus vulgaris* L. var. *vulgaris*) to remediate uranium contaminated groundwater. *J. Hazard. Mat.* 173:589–596.

19. Lone, M. I., He, Z., Stoffella, P. J., & Yang, X. 2008. Phytoremediation of heavy metal polluted soils and water: Progresses and perspectives. *J. Zhejiang University SCIENCE B.* 9:210–220.

20. Lopez, M. L., Peralta-Videa, J. R., Parsons, J. G., Benitez, T., & Gardea-Torresdey, J. L. 2007. Gibberellic Acid, Kinetin, and the Mixture Indole–3-Acetic Acid–Kinetin Assisted with EDTA-Induced Lead Hyperaccumulation in Alfalfa Plants. *Environ. Sci. Technol.* 41:8165–8170.

21. Nwachukwu, S. C., James, P., & Gurney, T. R. 2001. Inorganic nutrient utilization by "adapted" *Pseudomonas putida* strain used in the bioremediation of agricultural soil polluted with crude petroleum. *J. Environ. Biol.* 22:153-62.

22. Ormeño-Orrillo, E., Menna, P., Almeida, L. G. P., Ollero, F. J., Nicolás, M.F., Rodrigues, E. P., Nakatani, A. S., Batista, J. S. S., Chueire, L. M. O., Souza, R. C., Vasconcelos, A. T. R., Megías, M., Hungria, M., & Martínez-Romero, E. 2012. Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). *BMC Genomics* 13:735.

23. Park, Y. D., Lee, H. B., Yi, H., Kim, Y., Bae, K. S., Choi, J. E., Jung, H. S., & Chun, J. 2005. *Pseudomonas panacis* sp. nov., isolated from the surface of rusty roots of Korean ginseng. *Int. J. Sys. Evol. Microbiol.* 55:1721-1724.

24. Rahman, M. M., Azirun, S. M., & Boyce, A. N. 2013. Enhanced Accumulation of Copper and Lead in Amaranth (*Amaranthus paniculatus*), Indian Mustard (*Brassica juncea*) and Sunflower (*Helianthus annuus*). *PLoS ONE* 8:e62941.

25. Roane, T. M. 1999. *Bioaugmentation with Metal-Resistant Microorganisms in the Remediation of Metal and Organic Contaminated Soil* (Doctoral dissertation). University of Arizona, Tucson.

26. Salt, D. E., Blaylock M., Kumar, N. P. B. A., Dushenkov, V., Ensley, B. D., Chet, I. & Raskin, I. 1995. Phytoremediation: A novel Strategy for the Removal of Toxic Metals from the Environment Using Plants. *Biotechnology* 13:468–474.

27. Shi, S., Richardson, A. E., O'Callaghan, M., DeAngelis, K. M., Jones, E. E., Stewart, A., Firestone, M. K., & Condron, L. M. 2011. Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiol. Ecol.* 77:600-610.

28. Silver, S., & Phung, L. T. 1996. Bacterial heavy metal resistance: new surprises. *Ann. Rev. Microbiol.* 50:753-789.

29. Tsai, Y. L. & Olson, B. H. 1991. Rapid method for direct extraction of DNA from soil and sediment. *Appl. Environ. Microbiol.* 57:1070–1074.

30. United States Environmental Protection Agency. 2014. *Learn about Lead*. Retrieved From the Environmental Protection Agency website: http://www2.epa.gov/lead/learn-about-lead

31. Varivarn, K., Champa, L. A., Silby, M. W., & Robleto, E. A. 2013. Colonization strategies of *Pseudomonas fluorescens* Pf0-1: activation of soil-specific genes important for diverse and specific environments. *BMC Microbiology* 13:92.