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Microbial Analysis of Rhizosphere and Non-Rhizosphere Bacteria of *Lindera benzoin* & *Lithospermum canescens*

Kimberley Ndlovu

Missouri Valley College
Missouri Sigma Chapter

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Author contact information is available from tlindblom@alphachihonor.org or kvosevich@alphachihonor.org

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Abstract

Soils are a habitat to an incredible number of microorganisms and because no two soil samples are identical, there is great diversity of soil microbes, most of which we are still learning about today (Tecon & Or, 2017). Novel information about soil microbiology can be beneficial to human civilization, especially the medical field. For this study, analysis of select isolates of both the rhizosphere (R) and non-rhizosphere (NR) bacteria in soils surrounding the spicebush and hoary puccoon plants were performed. Isolates were identified and tested for antimicrobial activity against common human pathogens. A modified cross-streak method was used to evaluate the antimicrobial activity of the isolates. Of all the bacteria isolated from the soils, 12 were chosen for further analysis and seven of those 12 showed some antimicrobial activity against the test pathogens: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes* and *Klebsiella pneumoniae.* Molecular identification was carried out by 16S rRNA sequence. Genomic DNA was extracted using a silica protocol outlined by the Cold Spring Harbor Laboratory DNA Learning Center (2014). The samples were amplified by PCR and sent to GeneWiz® for identification. The sequences were submitted to NCBI MegaBlast. The most common genus amongst the isolates was *Pseudomonas* (58%) and the second most common was *Bacillus* (17%). Members of the genera *Duganella* and *Paenarthrobacter* were also isolated. The sequences were deposited into GenBank and given the accession numbers from MN861358-MN861369.

Keywords: antimicrobial activity, antibiotic resistance, rhizobacteria, 16S rRNA

Introduction

Soil Bacteria and Antimicrobial Activity

Bacteria are one of the most abundant living organisms on earth. They are found in nearly all habitats and soils contain the largest microbial diversity on earth (Mocali & Benedetti, 2010). It is estimated that a gram of soil contains about a billion bacteria, and that is not including other microorganisms like fungi and protozoans (Tecon & Or, 2017). Soil is one of the most challenging environments for microbiologists to study because it is a heterogeneous community whose biogeochemistry or the interaction of soil microbial life with soil and plant processes is not yet fully understood by microbiologists (Mocali & Benedetti, 2010). The reason behind this is because of the difficulty in culturing soil bacteria with available laboratory media. There is still a lack of information on specific growth requirements for soil bacteria (Qaisrani et al., 2019). Consequently, a large percentage of soil microbial life remains unexplored, uncategorized and rarely cultured even though the DNA of those microbes is widely studied without culturing them (Martin-Laurent et al., 2001). There is a great need for more research to be conducted on soil bacteria because they constitute a large unexplored reservoir of resources that would probably supply humans with useful innovative applications (Kapur & Jain, 2004). Humans have long been using microbes for food production and preservation, wastewater treatment, increasing soil fertility, tools for medical research, etc. Microbes are also major sources for anthelmintics, enzymes, antitumor agents, antimicrobial agents, and many other important medicinal compounds (Kapur & Jain, 2004). The discovery of these uses and agents are only a small fraction of what is likely present in nature; hence, there is a huge benefit to exploring the microbial diversity in soils.

Streptomycin, chloramphenicol, and tetracycline were among the first antibiotics used by humans and all were naturally produced by soil bacteria (Clardy, 2009). The antibiotic resistance pandemic is a threat to human health and is estimated to kill 2.4 million people over the next 30 years (Bengtsson-Palme et al., 2018; Nina, 2019). It is, therefore, important for more effective antibiotics to be found to treat infections, and soil bacteria are an unexplored area that could provide us with novel antibiotics. The aim of this study was to analyze the soil bacteria of the rhizosphere and non-rhizosphere of hoary puccoon (*Lithospermum canescens*) and spicebush (*Lindera benzoin)* plants for their antimicrobial activity against the common human pathogenic bacteria, *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Enterobacter aerogenes* and *Klebsiella pneumoniae.* Hoary puccoon was selected because it is a rare plant in Marshall, Missouri, and there is not a lot of research on its rhizobacteria. Spicebush was chosen for this experiment because it is an aromatic plant and the biochemistry of rhizobacteria in aromatic plants may be different from that of normal or non-aromatic plants. Plant growth promoting rhizobacteria (PGPR) in aromatic plants make compounds, such as ammonia and indole-3-acetic acid, that encourage the plant to grow (Malleswari & Bagyanarayana, 2013). It is, therefore, necessary to take on a search to find out the microbial activity rhizobacteria of an aromatic plant like spicebush.

Materials and Methods

Soil Sampling and Processing

Four soil samples of the spicebush rhizosphere, one non-rhizosphere sample, one sample for the hoary puccoon rhizosphere, and one non-rhizosphere sample (seven in total) were collected from different locations such as forests, prairies and backyards around Saline County and Boone County in Missouri. Rhizosphere soil samples were collected as follows: a clean shovel was used to excavate the roots at depths of 5-10cm and the roots were shaken to remove the soil from the roots. Non-rhizosphere soil samples were collected at a distance of at least 30cm from the plant but not more than 100cm. Care was taken to make sure that non-rhizosphere soil samples did not contain any plant roots. All soil samples were stored in clean polythene bags and stored at 4 °C until processed.

Isolation of Bacteria

For each soil sample, 1g of soil was mixed with 99ml of autoclaved deionised (DI) water and vortexed for 30-45 seconds. The homogenate was serially diluted to factors of 10^{-2} and 10^{-3}. The dilutions were spread in 20µl aliquots onto Reasoner's 2A (R2A) (Hardy Diagnostics, Santa Maria, CA) agar plates using a sterile hockey stick. R2A agar is a nutrient reduced medium developed for the enumeration of heterotrophic bacteria

in potable water. These bacteria tend to be slow-growing and would be suppressed in rich media by faster-growing bacterial species (Sandle, 2004). Ever since its development, R2A has been used to culture bacteria that will not readily grow in richer media (Kapetanovic et al., 2009). In this case, because there is a lack of specific growth requirements for soil bacteria, R2A was utilized to maximize the chances of culturing bacteria that would be otherwise suppressed in richer media. All agar plates were incubated at 20-22 °C for 48-72 hours (Kapetanovic et al., 2009). Colonies were picked from the isolation plates and re-streaked onto R2A agar plates. Colonies were selected based on their morphology and color. The aim was to pick as many different isolates as possible. Gram staining for cellular morphology was not used at this time. For long term storage, isolates were grown on R2A slants and stored at 4 °C. Slants were changed every ~two months.

Cross-Streak Method: Evaluation of Antimicrobial Activity

Antimicrobial activity was evaluated in the bacterial isolates using the cross-streak method. The bacterial isolate was seeded by a single streak in the centre of an R2A agar plate. The plates were incubated for 48 hours to allow any antibiotic substances to diffuse into the media. A modified cross-streak method (Velho-Pereira & Kamat, 2011) was used to seed the test pathogens and a template was also utilized for ease of inoculation as shown in Figure 1. The test pathogens used were common human pathogenic bacteria i.e. *Klebsiella pneumoniae*, *Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Streptomyces griseus* was used as a control and was also evaluated for its antimicrobial activity.

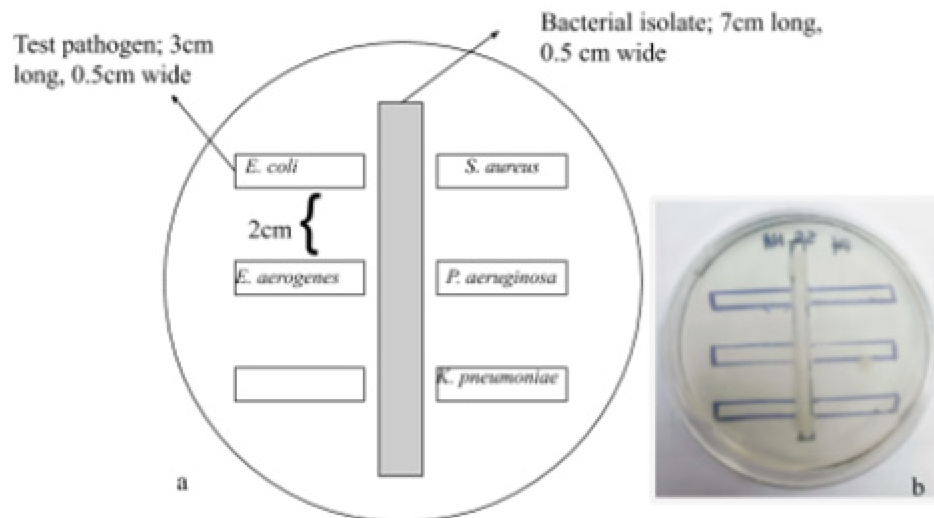

Figure 1: Modified cross-streak method (approx. life size)

Cross-streak method measurements used in (a) with a template for accuracy in inoculation (b).

DNA Extraction

Genomic DNA was extracted from each isolate using the silica DNA isolation protocol method outlined by Cold Spring Harbor Laboratory DNA Learning Center (2014).

PCR Amplification of 16S rRNA Genes and Gel Electrophoresis

1µl of the extracted DNA was used for the PCR reaction. To amplify the 16S rRNA gene, the universal bacterial primers -- forward primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) -- were used, generating a PCR product of approximately 1500bp (Marchesi et al., 1998). The following PCR conditions were used: initial denaturation at 94 °C for 120s, followed by 30 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 90s, and a final elongation step of 72 °C for 90s. The reaction mixture (25µl) contained 12.5µl of 2X Sahara Hot Start PCR Master Mix (Chai, Santa Clara, CA), 0.625µl each of 63f and 1387r primers (IDT, Coralville, IA), 10.25µl of autoclaved deionised (DI) water and 1µl DNA. The PCR products were analyzed by agarose gel electrophoresis.

DNA Sequencing

PCR products were sent via mail to GeneWiz® (South Plainfield, NJ) for sequencing. The PCR products were not purified in our lab before being sent to GeneWiz®. Sequencing results from GeneWiz® were put into the NCBI MegaBlast classifier that converted the sequences to bacterial descriptions and percent identity with the closest related strain (Wang et al., 2007).

Results

Isolation of Bacteria

After bacteria were isolated, their colony morphology was photographed and described as shown in Figure 2. There were a total of 12 isolates chosen for further analysis from all the soils.

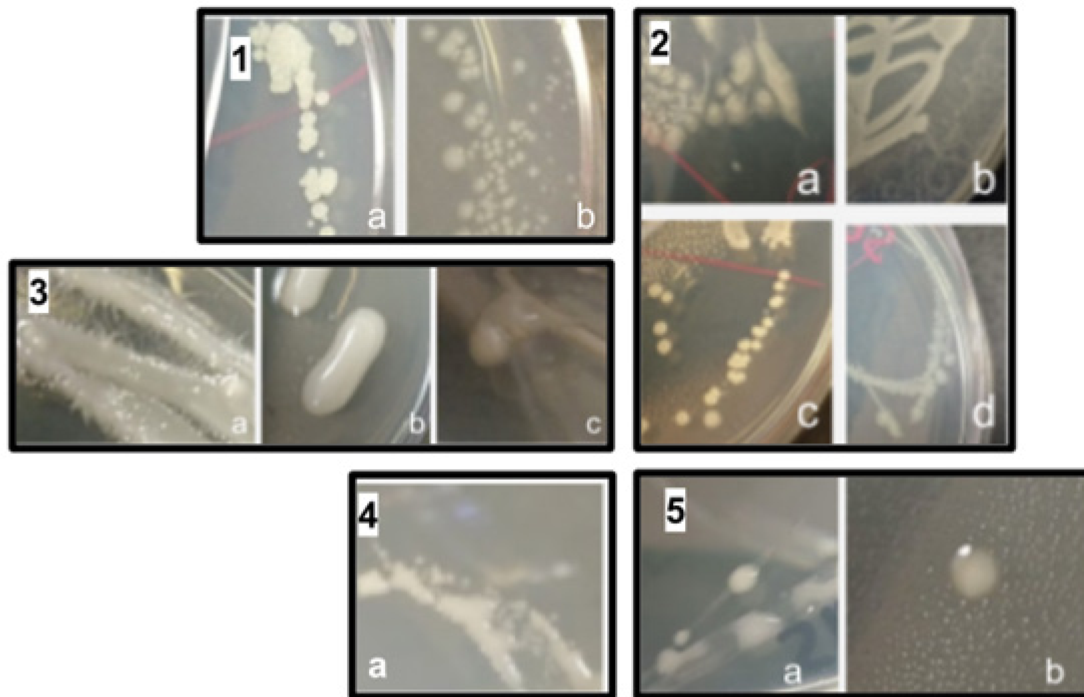

Figure 2: Morphology of bacterial isolates (approx. life-size)

2:1 Hoary puccoon bacterial isolates.
(a) The NR isolate; white and shine, circular, smooth edge (b) The R isolate; opaque white, yellowish, irregular, entire margins, slightly raised
2:2 Spicebush one (SB) bacterial isolates.
(a) The NR bacterial isolate; crateriform, white, irregular, filiform edges (b) The R1 bacterial isolate; filiform, irregular edge, circular, flat (c) The R3 bacterial isolate; crateriform, raised, off white, irregular edges (d) The R6 bacterial isolate; white, irregular edges, circular, flat.
2:3 Spicebush two (SB2) bacterial isolates.
All the isolates were from the rhizosphere. (a) R1; filamentous, flat and filiform margins (b) R2; circular, raised, entire smooth margins, shiny white (c) R3; circular, raised, entire smooth margins, off-white
2:4 Spicebush three (SB3) bacterial isolate.
Rhizosphere isolate, R2; irregular margins, slightly raised, circular.
2:5 Spicebush four (SB4) bacterial isolates.
All isolates were from the rhizosphere. (a) R1; circular, raised, entire smooth margins (b) circular, raised, entire smooth margins, off-white.

DNA Sequencing

A total of 12 bacterial isolates from all the soils were chosen for further analysis. All the isolates were identified through the sequencing of PCR 16S rRNA and analyzing the sequences through NCBI MegaBlast. The sequences were analyzed and deposited into GenBank and were assigned the accession numbers shown in Table 1.

Isolate	Name of closely related strain	Maximum score	Identity (%)	Accession #
HP NR	*Duganella zoogloeoides* strain IAM 12670	1310	96.55	MN861363
HP R	*Pseudomonas koreensis* strain Ps 9-14	1295	96.50	MN861362
SB NR	*Bacillus wiedmannii* strain FSL W80169	1415	99.36	MN861361
SB R1	*Pseudomonas chlororaphis* strain NCBI 10068	1391	98.34	MN861358
SB R3	*Arthrobacter humicola* strain KV653	1378	99.08	MN861359
SB R6	*Bacillus wiedmannii* strain MCC 1A 00365	1437	99.28	MN861360
SB2 R1	*Pseudomonas putida* strain NBRC 14164	1323	99.18	MN861364
SB2 R2	*Pseudomonas putida* strain NBRC 14164	1362	99.20	MN861365
SB2 R3	*Pseudomonas entomophila* strain L48	1321	98.91	MN861366
SB3 R2	*Paenarthrobacter nicotinovorans* strain DSM 420	1327	98.65	MN861367
SB4 R1	*Pseudomonas putida* strain NBRC 14164	1339	99.06	MN861368
SB4 R2	*Pseudomonas putida* strain NBRC 14164	1308	98.77	MN861369

Table 1: Sequence analysis of the 16S rRNA genes of the isolates using NCBI MegaBlast.

Antimicrobial Activity

A total of 12 bacterial isolates were chosen for further analysis from all the soils. Of those 12, only seven showed any antimicrobial activity against the test pathogens. Table 2 shows the antimicrobial testing results. 33% of the isolates were active against *Klebsiella pneumoniae,* 25% against *Pseudomonas aeruginosa* and 17% of the isolates were active against *Escherichia coli, Enterobacter aerogenes,* and *Staphylococcus aureus.* Figure 4 shows the isolates that acted on some of the test pathogens and some that did not. The control *S. griseus* was only active against *K. pneumoniae.*

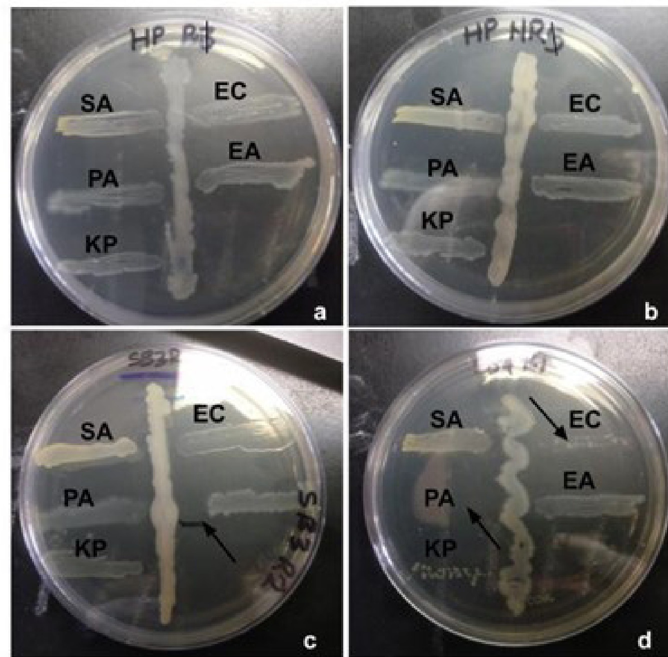

Figure 3: Antimicrobial activity of isolates (approx. life-size)

Isolates **(a)** HP R (*Pseudomonas koreensis*) and **(b)** HP NR (*Duganella zoogloeoides*) showing no inhibition zone on any of the test pathogens. Isolates **(c)** SB3R2 (*Paenarthrobacter nicotinovorans*) and **(d)** SB4R1 (*Pseudomonas putida*) show areas of inhibition.

Key: EA =*Enterobacter aerogenes* KP = *Klebsiella pneumoniae*
PA =*Pseudomonas aeruginosa* SA =*Staphylococcus aureus*
EC =*Escherichia coli*

Identified Isolate	*S. aureus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*	*K. pneumoniae*
S. griseus (control)	-	-	-	-	+
HP NR	-	-	-	-	-
HP R	-	-	-	-	-
SB NR	-	-	-	-	-
SB R1	+	+	+	-	-
SB R3	+	+	-	-	+
SB R6	-	-	-	-	+
SB2 R1	-	-	-	+	-
SB2 R2	-	-	-	-	-
SB2 R3	-	-	-	-	-
SB3 R2	-	-	-	+	-
SB4 R1	-	+	+	-	+
SB4 R2	-	-	-	-	+

Table 2: Antagonistic activity of isolated soil bacteria (identified in Table 1) against test human pathogens by modified cross streak method. *- indicates no zone of inhibition* + *indicates zone of inhibition*

Discussion

DNA Sequencing and Antimicrobial Activity of Isolates

The aim of this study was to explore culturable soil bacteria, identify them and test them for their antimicrobial activity on common human pathogenic bacteria. The soil environment was chosen as an area for study because the microbial community in soils exceeds that of any other environment (Gislin et al., 2018; Mocali & Benedetti, 2010). In terms of sequencing results, the genera *Bacillus, Arthobacter* and *Pseudomonas* are commonly found in soils (Bodelier & Laanbroek, 1997; Eschbach et al., 2003; Vilain et al., 2006). *Pseudomonas* was the most common isolated genus composing of the 58% of the isolated and identified strains. *Bacillus* was the second most common with 17% of isolates. The genus *Duganella* is a relatively new genus with only six species of bacteria. The species of this genus have previously been isolated from agricultural soil or forest soil (Li et al., 2004). *Paenarthrobacter* is also a new genus whose members are primarily found in soil (Busse, 2016). Six species (*A. aurescens, A. histidinolovorans, A. ilicis, A. nicotinovorans, A. nitroguajacolicus and A. ureafaciens*) that were previously of the genus *Arthrobacter* were reclassified as *Paenarthrobacter* (Busse, 2016).

There were a total of 12 bacteria isolated from all the soils and seven of those isolates showed any antimicrobial activity against the test pathogens. Most of the isolates showed antimicrobial activity against *K. pneumoniae,* a gram-negative bacterium (Table 1). The reason for the low percentage in antimicrobial activity might be two-fold. The first reason might be because the isolates did not grow on the plates long enough for any secreted antimicrobial compounds to diffuse into the media. Some experiments showed positive inhibition with two days incubation of the isolates (Gislin et al., 2018; Lertcanawanichakul & Sawangnop, 2008). However, some experiments have let the bacterial isolate cultured on growth agar for seven days (Wadetwar & Patil, 2013). Another simple reason is that the bacterial strains of the isolates did not produce any antimicrobial substances that could cause inhibition on the test pathogens. Of the test pathogens, 80% were gram-negative bacteria, which are much more resistant to antimicrobial activity due to their outer membrane (OM), which has phospholipids, liposaccharides and outer membrane proteins like porins. Gram-negative bacteria may have some changes in the OM like mutations in the OM proteins or modifications in their hydrophobic proteins that can create resistance to antibiotics (Breijyeh et al., 2020). Gram-positive bacteria lack this layer and have an outer peptidoglycan layer instead, which is ineffective, therefore making them more susceptible to antimicrobial activity (Scherrer & Gerhardt, 1971).

Conclusion

This study provided the basis for future research on antimicrobial-producing bacteria found in soils. It can be concluded that some soil microbes produce antimicrobial compounds that can be exploited to find newer and more effective antibiotics. Of the 12 isolates chosen for further analysis, seven showed antimicrobial activity against the test pathogens. This means that there is a great potential for more research into discovering and exploring novel species of soil bacteria and their antimicrobial activity against common human pathogens. This information can be exploited to help the medical community as there is a great need for antibiotics now. More research needs to be conducted into understanding the heterogeneous community of soils and the relationships between microorganisms that reside in those soils. By finding effective methods of culturing soil bacteria, we can then begin to study and comprehend those interactions.

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