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Isolation and Identification of Antimicrobial-Producing Microbes from Soil Associated with an Eastern Hemlock in the Great Smoky Mountains National Park

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Abstract

This research focused on the isolation and identification of antimicrobial-producing microbes from the Great Smoky Mountains National Park. Oligotrophic media was used to culture all microbial isolates. Antimicrobial screening was then performed by subjecting each soil isolate to potentially pathogenic microorganisms on nutrient agar test plates with growth of pathogens being inhibited if antimicrobial compounds were secreted into the culture medium. Select soil isolates that tested positive for antimicrobial activity were identified by analysis of sequences of small subunit rRNA genes following amplification by PCR. Antimicrobial production by *Nocardia grenadensis* strain X0885 isolated from soil obtained from the park has not been previously described.

1. Introduction

Soils contain enormous and diverse communities of microbes. While it is estimated that the number of microbes per gram of soil is 2×10^{9} [8], the number of microorganisms typically cultured from soil represents less than 1% of the total microbial community[9]. Since many natural products that are clinically or commercially useful are produced by the small proportion of soil microbes that have been described, it is likely that among the microbes not yet described, there will be many producers of useful natural products. Endophytic microbes that form an intimate relationship with their host plants are also relatively unexplored as potential sources of novel microbial species and novel antimicrobial compounds for commercial and medicinal use[12].

Microbes that have been found to produce antimicrobial compounds are mostly from the phylum *Actinobacteria*, and many are from the genus *Streptomyces*[4]. Endophytic *Actinobacteria* are also a very promising resource to investigate, as Qin et al.[12] show that many microbiologists overlook this potentially diverse source of uncharacterized microorganisms. In order to effectively grow these microbes, we employed oligotrophic isolation media containing carbon polymers that are representative of those found in natural soil[5,12].

Starch-casein and xylan-arginine media satisfy these requirements as both starch and xylan are polysaccharides which can only be utilized by certain microorganisms, and they resemble natural materials that *Actinobacteria* readily degrade in natural conditions[7,9]. This prevents substrate-accelerated death as seen in many instances where faster-growing bacteria effectively exhaust all nutrients on the plate, depriving the slower growing *Actinobacteria* of nutrients and preventing growth. Both of these polysaccharides prevent this, while also selecting for microorganisms we are particularly interested in since many common microbes cannot utilize these compounds as readily.

Thus, the goal of this work was to isolate and identify soil microbes from the Great Smoky Mountains National Park, as well as attempt to isolate a rare endophytic microbe as they are relatively unexplored and present a promising, rich source of novel microorganisms and antimicrobial compounds. Further work involved looking for

production of antimicrobial compounds and identifying the antimicrobial-producers by molecular and traditional techniques.

2. Methods

2.1. procurement of soil samples

Soil samples (1 cm x 5 cm) were obtained from an eastern hemlock (Figure 1) in the Great Smoky Mountains National Park (GPS coordinates N 35 34.897 W0S304.100). 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions of soil suspensions were plated on starch-casein and xylan-arginine media and subjected to a long term, room temperature, dark incubation. Replicas were prepared for each dilution to increase overall microbial diversity and inventory of potential antibiotic producers. Following incubation, soil isolates were selected from isolation plates and grown in pure culture on starch-casein and xylan-arginine media. Pure cultures were inoculated onto starch-casein and xylan-arginine slants for short-term storage for DNA isolation and PCR, and colony morphologies were recorded (Table 1).

Figure 1. Eastern Hemlock located at purchase knob at the great smoky mountains national park.

2.2. growth of soil microbes

Starch-casein and xylan-arginine media were prepared as described by Qin et al.[12] Plates were incubated for a suitable amount of time (~5 weeks) to allow optimum growth to occur. Endophytic microbes were selectively isolated using the procedures of Qin et al.[12]. Surface-sterilized root samples from Eastern Hemlock were then aseptically crumbled into small fragments and directly placed on starch-casein and xylan-arginine media and incubated for ~5 weeks.

2.3. antimicrobial testing of soil isolates

Cross-streak agar assay testing was performed on selected soil isolates with *Bacillus subtilis* IA30, *Escherichia coli*, and *Staphylococcus aureus* used as test indicator organisms grown in 1% tryptone 1% yeast extract broths. Colonies were selected from each of 6 soil isolate pure culture plates, and suspended in 1 ml of deionized water in a microfuge tube. Tubes were then thoroughly mixed, and sterile swabs were used to inoculate fresh nutrient-agar plates with a single streak of the suspended soil isolate down the middle of the plate. Plates were then incubated for ~7 days, and test indicator strains were streaked at right angles to the soil isolate with sterile swabs from broth cultures (Figure 3). Plates were then allowed to incubate for 1 additional day, and results were read the next day for determination of antimicrobial production (Figure 4,5). Lack or absence of growth of the test indicator microorganisms from the selected soil isolate indicated that an antimicrobial compound was being secreted into the agar medium, and positive isolates were kept on agar slants to await DNA extraction and amplification by PCR.

Table 1. Colony morphologies and cross-streak agar assay results for soil isolates.

Soil Isolate Code	Isolation Plate (dilution)	Size of Colony	Color	Consistency	Colony Appearance	Edge	Elevation	Results of Cross-Streak Agar Assay
BI	10^{-4} Xylan-arginine rep. 4	1-2 mm	Clear/light yellow	Translucent	Irregular	Lobated	Raised	Pos. against *B. subtilis* IA30
4	10^{-2} Starch-casein rep. 2	3-4 mm	White/brown	Opaque	Circular	Filamentous	Raised	Pos. against *B. subtilis* IA30
UV	10^{-3} Xylan-arginine rep. 2	1-2 mm	White	Dull	Circular	Filamentous	Raised	Pos. against *B. subtilis* IA30
Act.	10^{-4} Xylan-arginine rep. 3	1-2 mm	Orange	Shiny	Circular	Filamentous	Raised	Pos. against *B. subtilis* IA30
WA (endophyte)	10^{-3} Starch-Casein rep. 2	1-2 mm	White/clear	Dull	Circular	Filamentous	Raised	Pos. against *B. subtilis* IA30
Q	10^{-3} Xylan-arginine rep. 1	1-2 mm	White/creamy	Shiny	Circular	Circular	Raised	Pos. against *B. subtilis* IA30

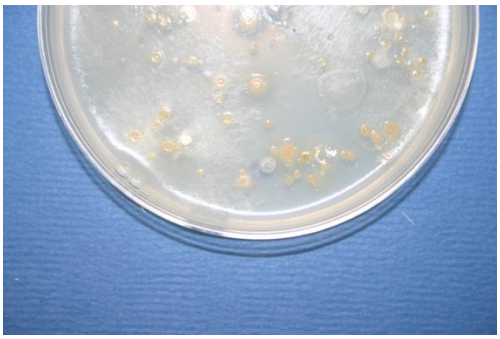

Figure 2. Primary (10^{-3}) isolation plate with various types of bacterial colonies present. Colonies were chosen at random and tested for antimicrobial-activity.

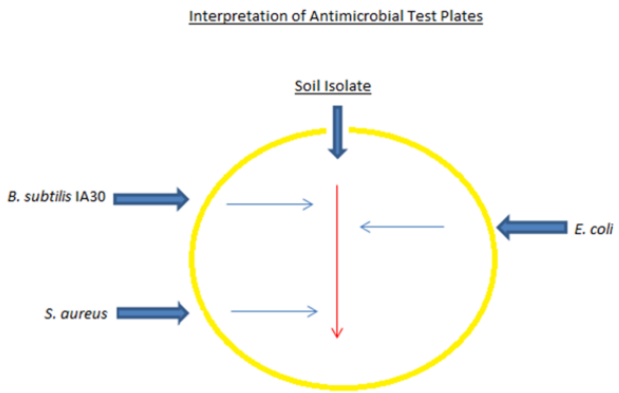

Figure 3. Interpretation of antimicrobial test plates.

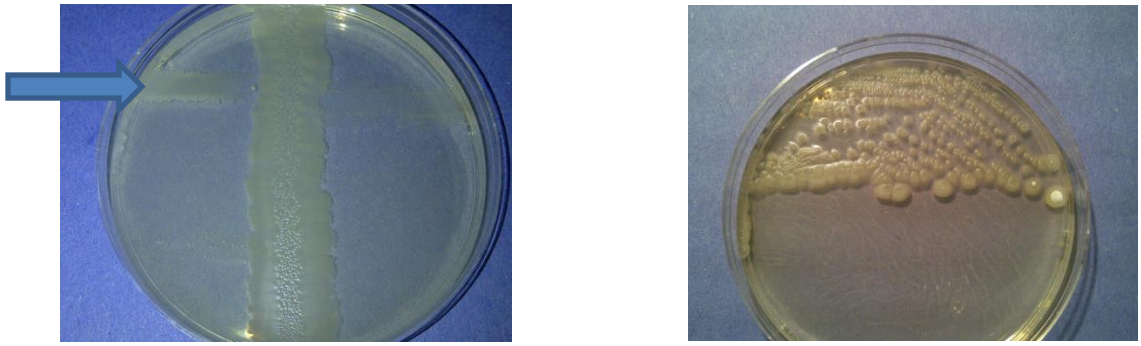

Figure 4. Soil isolate 4 positive cross-streak agar assay inhibiting *B. subtilis* IA30 (left) from a soil isolate 4 pure culture (right).

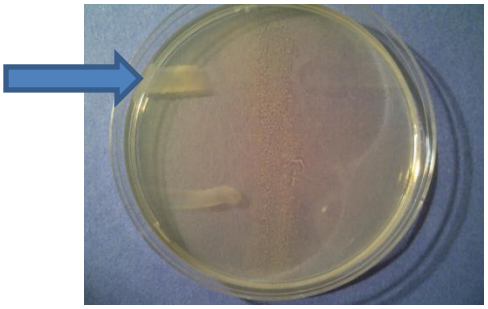

Figure 5. Soil isolate BI positive cross-streak agar assay inhibiting *B. subtilis* IA30 (left).

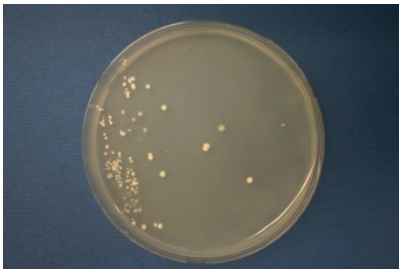

Figure 6. *Nocardia grenadensis* strain XO885 (soil isolate UV) that was recently discovered by Kämpfer et al.[10] in 2012.

2.4. DNA extraction and amplification by PCR

A single bacterial colony was selected from each of the 6 soil isolate plates, and DNA was isolated from the selected soil microbes using a FASTPREP™ DNA Spin Kit from MP Biomedicals®. The manufacturer's protocol was followed for all isolations. Small subunit rRNA genes of purified DNA from each isolate were amplified by PCR following the protocol of Frederickson et al[7]. PCR products were then subjected to electrophoresis in 1.5% agarose gels and visualized with UV transillumination.

3. Results

Soil isolates were selected from isolation plates and grown in pure culture on starch-casein and xylan-arginine media. Pure cultures were inoculated onto starch-casein and xylan-arginine slants for short-term storage for DNA isolation and PCR, and colony morphologies were recorded (Table 1). Results of antimicrobial tests are also shown

in Table 1. DNA was successfully extracted from chosen antimicrobial-producing microbes, and small-subunit rRNA genes were amplified by PCR. PCR products resulting from amplification were then sent to GENEWIZ® for sequencing, and DNA sequences were inserted into the BLAST computer algorithm tool to ascertain identities of microbial isolates being analyzed as shown in Table 2.

Table 2. Results of BLAST analysis using DNA sequences obtained from soil isolates

Soil Isolate Code	Sequence analyzed	Accession	Description	Max score	Total score	Query coverage	E Value	Max. Ident
UV	Partial sequence of 16S ribosomal RNA gene	JX857481.1	Nocardia grenadensis strain X0885	1282	1282	92%	0.0	99%
BI	Partial sequence of 16S ribosomal RNA gene	JN791035.1	Pseudomonas sp. PTAS6	1007	1007	91%	0.0	89%
4	Partial sequence of 16S ribosomal RNA gene	KC462544.1	Streptomyces sp. TCA20038	1343	1343	91%	0.0	99%
WA	Partial sequence of 16S ribosomal RNA gene	KC441749.1	Bacillus licheniformis strain B51	1280	1280	93%	0.0	98%
Q	Partial sequence of 16S ribosomal RNA gene	KC492058.1	Staphylococcus saprophyticus strain CD250Y	1325	1325	93%	0.0	100%
Act.	Partial sequence of 16S ribosomal RNA gene	KC355257.1	Brevibacterium iodinum strain KUDC1716	990	990	81%	0.0	93%

4. Discussion

We have used techniques designed to cultivate microbes that are likely to produce antimicrobial compounds from hemlock and hemlock-associated soil. We have tested whether the isolated cultures produce antimicrobial compounds that are effective against antibiotic-sensitive bacteria. We have amplified the small subunit rRNA genes of the antimicrobial-producing isolated soil microorganisms to determine the identity of the isolates. As a result of obtaining amplified PCR products, sequences were analyzed in BLAST® to obtain the identities of the microbial isolates in question.

Soil isolate UV was determined to be a *Nocardia grenadensis* strain, an *Actinobacterium*, with a 99% similarity between the query and subject sequences over the length of the 16S rRNA gene. An interesting note is that this strain was recently discovered in sand from the Carribean Sea in 2012[10]. With regards to antimicrobial compounds secreted by *Nocardia grenadensis*, further work will need to be done to characterize the secreted compound as this strain has just recently been recognized.

Soil isolate BI was identified as a *Pseudomonas* species, yet the actual species could not be identified. An 89% similarity was determined between the query and subject sequences over the length of the 16S rRNA gene. However, *Pseudomonas* species often produce one or more antibiotics including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), and phenazine compounds[15].

Soil isolate 4 was determined to be a *Streptomyces* species, which are ubiquitous in soil. A 99% similarity was determined between the query and subject sequences over the length of the 16S rRNA gene. Different *Streptomyces* species produce about 75% of commercially and medically useful antibiotics[3]. They have provided more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds[11].

Soil isolate WA was identified as *Bacillus licheniformis*, which produces the antibiotic bacitracin[1]. A 98% similarity was determined between the query and subject sequences over the length of the 16S rRNA gene. This

bacterium was an endophyte isolated from the root system of Eastern Hemlock. Sgroy et al.[14] isolated a *B. licheniformis* strain from the root system of *Prosopis strombulifera*. Therefore, this bacterium seems to be common microorganism forming endophytic relationships with their plant hosts.

Soil isolate Q was identified as *Staphylococcus saprophyticus*, with a 100% similarity between the query and subject sequences over the length of the 16S rRNA gene. This isolate produces an extracellular enzyme complex that inhibits growth of gram-positive and gram-negative bacteria[5]. Therefore, this isolate was likely a false positive.

Lastly, soil isolate Act. was identified as *Brevibacterium iodinum*. A 93% similarity between the query and subject sequence was determined over the length of the 16S rRNA gene. *B. iodinum* is recognized for producing iodinin, which is a chemical intermediate in the synthesis of the antibiotic, myxin[2]. In conclusion, 5 out of the 6 microbes isolated are likely producers of antimicrobial compounds. *S. saprophyticus* is not recognized as producing any antibiotics, inhibits growth of gram-positive and gram-negative bacteria, leading to a false-positive result. Future work could involve identifying and amplifying polyketide gene clusters (PKS) that are known to code for the synthesis of antimicrobial compounds through primer-specific PCR.

5. Acknowledgements

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