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Isolation and Characterization of Natural, Antibiotic-Producing Bacteria in Pure- and Co-Culture

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Abstract

Rapid emergence of bacterial antibiotic resistance has diminished the effectiveness of nearly all clinical antibiotics; thus, the search for novel compounds must continue in order to replenish the antibiotic drug pipeline. Natural products are a logical starting point for discovery of new molecules with antibiotic activity, and natural rhizosphere environments remain a practical source of new antibiotic producers. Increased success of culturing natural soil bacteria, combined with the knowledge that for every teaspoon of productive soil there are approximately 100 million to 1 billion bacteria, highlights the need to explore natural rhizosphere communities for undiscovered bioactive compounds. Furthermore, metabolite induction via microorganism co-culture, has been reported to activate cryptic biosynthetic gene clusters and enhance chemical diversity for novel drug discovery. Here, bacterial strains were isolated from various plant-, rhizosphere-, and aquatic- environments throughout Western North Carolina and screened for antibiotic production, both in pure culture and in pairwise co-cultures, using a high-throughput antagonism assay against a range of Gram-positive and Gram-negative bacterial targets including *Staphylococcus aureus* and *Escherichia coli*. Pure cultured-based screening reports 25 antibiotic-producing bacteria that were isolated from soil- and plant-associated environments. Each bacterium was phylogenetically identified via 16S rDNA amplification and sequencing. Antagonistic organisms include members of several common genera, including *Pseudomonas*, *Serratia* and *Streptomyces*, as well as less characterized groups including *Janthinobacteria* and *Rahnella* species. Antifungal activity against the pathogenic fungus *Fusarium* was also assessed, with 16% of the antibacterial isolates also exhibiting antifungal activity. Co-culture based screening of over 6,651 pairwise combinations of bacteria reports no activation of antibiotic production, despite recently published research results which suggest such interaction-induced production is common. Ongoing work seeks to characterize the bacterial producers and the natural products being produced, with the long-term goal being the discovery of novel compounds with clinical and agricultural applications.

Keywords: Antibacterial, Antifungal, Pure- and Co- Culture

1. Introduction

The impacts of infectious diseases were dramatically reduced following the introduction of clinical antibiotics, such as penicillin and streptomycin. However, widespread use of antibiotics in medicine and agriculture has led to the emergence of resistance to all known clinical antibiotics. Thus, there is a constant demand for the discovery and development of new antibiotics[1]. In 2013, the CDC reported more than 2,000,000 illnesses and 23,000 deaths due to antibiotic resistant bacteria in the United States alone; however, many large pharmaceutical companies have reduced or eliminated their antibiotic drug discovery programs. These facts highlight a growing need for the discovery, identification, and characterization of novel antibiotic compounds[2].

In addition to the role of antibiotics in a clinical setting, these compounds are also important in an agricultural context. A number of bacteria have been approved for biocontrol—the use of natural bacteria to inhibit plant

pathogenic bacteria, fungi, insect, and nematode pests[3]. The most notable historic use of antibiotics in agriculture was to control fire blight of apple and pear trees, which is caused by the bacteria *Erwinia amylovora*[4]. The emerging epidemics of this disease, as well as other plant bacterial pathogens, has caused an increasing dependency on antibiotics and chemical pesticides in agriculture. Concomitant with the use of antibiotics and pesticides to control bacterial diseases on plants, there has been a steady increase in the selection of resistance genes in the pathogenic plant bacteria rendering treatments useless[4]. Not only is this dependency negative due to treatments becoming ineffective, it is also allowing for these antibiotic resistant bacteria to be passed on to humans via many different agricultural outlets including livestock[5,6].

Antibiotic production by natural soil- and plant-associated bacteria represent an alternative to the use of chemical pesticides, through a process called biological control (biocontrol). Biocontrol strategies have proven effective in cases where conventional pesticides cannot be used, where no known control is available, or when the products must be certified organic[7]. Recent advances in bacterial cell culture have increased the number of organisms that can successfully be cultivated from soil, and have led to discovery of new antibiotics, including the promising new drug Teixobactin[8]. With approximately 100 million to 1 billion bacteria in every gram of soil, the discovery of Teixobactin may be merely the tip of the iceberg for a new generation of natural products drug discovery[2,9]. The rhizosphere, the plane of soil impacted by plant roots and exudates, is particularly high in nutrients and microbial activity[10], and thus can be exploited for the discovery of novel antibiotic and biocontrol compounds.

Here, novel antibiotic producing bacteria were isolated from various natural environments, including samples from rhizosphere, aquatic, and plant environments. Cultured bacteria were screened for the ability to produce antibiotics when grown in pure culture; those that did not produce in pure culture were then screened in pairwise combinations, in an attempt to induce any silent antibiotic biosynthetic genes via bacterial interaction and competition. A number of antibiotic producers were identified, and subsequently genetically identified and characterized.

2. Methodology

2.1. Collection Of Environmental Samples

Soil samples were aseptically collected from two locations, one human-impacted area (the Rhodes Garden (RG), on the campus of UNC Asheville) and a second more pristine area (an adjacent forest, RF). Plant-associated samples were aseptically collected from the pitcher fluid of natural populations of *Sarracenia* pitcher plants. Pitcher plant samples were collected from undisclosed locations in Western North Carolina by Dr. Sarah Seaton and Dr. Rebecca Hale, and are referenced as CM and CP throughout this report. Additional environmental samples were collected from soil in an urban Asheville stream bed (STREAM), a backyard compost pile (COMPOST), and the rhizosphere of a bed of sphagnum moss in the Shining Rock Wilderness (MOSS).

2.2. Isolation And Purification Of Environmental Bacteria

Dilutions spanning from 1 and 1:1000 were made with six pitcher plant samples, each representing fluid collected in independent pitchers at two distinct geographic locations (CM and CP): CM 101, CM 131, CM 011, CP 141, CP 171, and CP 051. Each dilution was inoculated on 10% Tryptic Soy Agar (TSA) plates (3 g of Tryptic Soy Broth base/L solidified with 15 g/L of agar) and the 1 and 1:10 dilutions were inoculated on Actinomycete Isolation Agar (25 g/L of commercial Actinomycete base plus 5 g/L glycerol, solidified with 15 g/L agar). Plates were incubated at 25°C for several days and all morphologically distinct colonies were selected and streak purified.

For RG and RF samples, 0.35g of soil was suspended in 1mL Tryptic Soy Broth and vortexed. Particulate matter was allowed to settle to the bottom of the tube and the supernatant was decanted. Ten-fold dilutions of the supernatant were inoculated onto 10% TSA and AIA plates and incubated at 25°C for several days until morphologically distinct colonies could be selected and streak purified, as above.

Soil samples from other locations were processed similarly, allowing isolation of 88 morphologically distinct bacteria for subsequent antibiotic screening.

2.3. Screening Pure Cultures For Antibiotic Activity

Purified environmental bacteria were tested for antibiotic production using a standard zone of inhibition antagonism assay[11]. Briefly, target strains were grown overnight in dilute Tryptic Soy broth medium, and then spread plated onto TSA. Target strains used in this study included *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Micrococcus luteus*, and *Bacillus cereus*. To test the ability of isolated environmental bacteria to inhibit the growth of these target organisms, bacterial strains were grown overnight in dilute Tryptic Soy broth, standardized to an OD_{600} of 0.50 and 2 ul aliquots were spot-inoculated onto plates previously spread with the target organisms described above. Plates were incubated at 25°C and monitored for antibiotic production, as indicated by a zone of inhibition of the target strain. The diameter of the zones of inhibition were measured to determine the strength of the secondary metabolite being produced.

2.4. Antifungal Screening

All isolates that exhibited antibacterial activity were subsequently screened for their ability to inhibit growth of the plant-pathogenic fungus *Fusarium solani* ATCC ® 36031™ (Microbiologics). Briefly, a single colony of each environmental isolate was streak-inoculated onto dilute TSA plates. An agar plug, containing fungal hyphae was then added to the plate approximately 75 mm away from the bacterial inoculation point. Plates were monitored for antifungal production, as judged by an inhibition of *Fusarium* hyphae growth.

2.5. 16S rDNA Amplification And Phylogenic Identification Of Antibiotic Producing Bacteria

Genomic DNA was extracted using the Archive DNA Purification Kit (5 PRIME) following the procedure for Gram Positive Cultures.

Extracted DNA was subsequently used as the template in a PCR reaction to amplify a variable region of the bacterial 16S rRNA gene. Reactions were as follows: 20-50 ng of genomic DNA was used in a 50 µl reaction with OneTaq Hot Start 2X Master Mix with standard buffer (New England BioLabs) and 200 nM of universal bacterial 16S primers 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3')[12]. The conditions used to amplify the 16S rDNA region consisted of 30 cycles starting with the initial hot-start of 94°C for 30 sec, followed by denaturation at 94°C for 30 sec, annealing at 51°C 30 sec, and extending at 68°C 90 sec with a final extension of 68°C for 5 minutes. PCR products were examined by electrophoresis at approximately 107 V for 30 minutes in a 1% (w/v) agarose gel in 1 x TAE buffer. The products were compared to a 1Kb DNA ladder, for the expected product size of approximately 1470 bp. PCR products were then purified using the QIAquick Gel Extraction Kit (QIAGEN), and sequenced in both directions using the 27F and 1492R primers. DNA sequencing was performed by GeneWiz (Boston, Massachusetts).

Sequences were compared to public databases of bacterial 16S sequences, using the Basic Local Alignment Search Tool (BLAST)[13] provided by the National Center for Biotechnology Information and also using the Alignment Tool provided by the Ribosomal Database Project. Phylogenetic identification at Genus-level discrimination was possible for most isolates.

2.6. Co-Culture Antibiotic Screening

All bacterial isolates that did not exhibit antibiotic activity were subsequently screened in pairwise co-cultures. Isolates from CM/CP and RG/RF were combined with other non-producers from the same sample location, after being cultured in 10% TSB in 96-welled sterile plates. The 96-welled plate was set up in a manner that allowed for all possible combinations among the isolates to be made, with appropriate controls. Using a 96-pronged pin replicator, the pairwise combinations were spot inoculated onto 10% TSA plates streaked with either *S. aureus* or *E. coli* target strains. These plates were monitored for antibacterial production.

All bacterial isolates from locations STREAM, COMPOST and MOSS were co-cultured and screened similarly.

3. Data

3.1. Pure-Culture Screening

In total, 88 bacteria were cultivated and purified. Of these, 25 were found to exhibit antibacterial activity against at least one target organism (Table 1, Table 2, Table 3). 20/88 of the isolates showing antibacterial activity were against Gram-positive organisms, while 11/88 showed activity against Gram-negative organisms. From these, one-quarter of the antibiotic producers exhibited broad spectrum antibacterial activity against both Gram-negative and Gram-positive targets. Of the antibiotic-producing bacteria, 52% were identified by 16S rRNA gene sequencing to be members of the genus *Pseudomonas*, while 8% were *Janthinobacterium* and *Chromobacterium.* Other genera identified as producers included: *Xanthomonas*, *Streptomyces*, *Pedobacter*, and *Bacillus*.

Table 1. Plant-associated bacteria (PA)

			Gram-Negative Targets		**Gram-Positive Targets**	
Isolate Number	**Isolate Name**	**Genus**	***E. coli* Inhibition**	***P. fluorescens* Inhibition**	***S. aureus* Inhibition**	***M. luteus* Inhibition**
1	**Uri**	***Chromobacterium***	Yes	Yes	Yes	Yes
2	**Agar**	***Xanthomonas***			Yes	Yes
3	**Strep**	***Streptomyces***			Yes	Yes
4	**CP2SS IV**	***Chromobacterium***			Yes	Yes
5	**JB**	***Pseudomonas***			Yes	
6	**Leidtke**	***Pseudomonas***	Yes		Yes	
7	**RL021 Col 6**	***Pseudomonas***			Yes	
8	**Long**	***Rahnella***			Yes	
9	**CM/ CP B2**	***Pseudomonas***			Yes	Yes
10	**CM/CP D5**	***Pseudomonas***	Yes		Yes	Yes
11	**CM/CP H5**	***Janthinobacterium***	Yes			
12	**CM/CP E3**	***Janthinobacterium***	Yes			Yes
13	**CM/CP G1**	***Pseudomonas***	Yes			
14	**CM/CP B4**	***Pedobacter***				Yes
15	**CM/CP C5**	***Pseudomonas***				Yes
16	**CM/CP F3**	***Pseudomonas***	Yes			

Table 2. Bacteria from agricultural soil (Rhodes Garden (RG))

			Gram-Negative Targets		Gram-Positive Targets		
Isolate Number	**Isolate Name**	**Genus**	***E. coli* Inhibition**	***P. fluorescens* Inhibition**	***S. aureus* Inhibition**	***M. luteus* Inhibition**	***B. cereus* Inhibition**
1	**B7**	***Bacillus***	Yes				
2	**B8**	***Pseudomonas***			Yes	Yes	Yes
3	**B9**	***Pseudomonas***			Yes		Yes
4	**B10**	***Pseudomonas***	Yes		Yes	Yes	
5	**C6**	**Not Determined**			Yes	Yes	
6	**C7**	***Pseudomonas***					Yes
7	**C10**	**Not Determined**				Yes	Yes
8	**E6**	***Pseudomonas***		Yes	Yes	Yes	Yes

Table 3. Bacteria from forest soil (Rhodes Forest (RF))

			Gram-Negative Targets		Gram-Positive Targets		
Isolate Number	**Isolate Name**	**Genus**	***E. coli* Inhibition**	***P. fluorescens* Inhibition**	***S. aureus* Inhibition**	***M. luteus* Inhibition**	***B. cereus* Inhibition**
1	**H9**	**Not Determined**	Yes				

The strongest secondary metabolite activity against *Staphylococcus aureus* was exhibited by the following isolates: Rhodes Garden (RG) Isolates B9 (10.2 mm), B10 (11.4 mm), and C6 (9.3 mm); Plant-Associated (PA) Isolate CP2SS IV (14.8 mm) and Isolate Leidtke (10.2 mm) (Figure 1A). Antibacterial activity in isolates against *Escherichia coli* was less notably strong with the largest inhibition diameter associated with PA Isolate CM/CP G1 (8 mm) (Figure 1B). Several isolates showed broad-spectrum antagonism against both the Gram-positive and Gram-negative target including RG Isolate B10 and PA Isolate Leidtke and CM/CP D5 (Figure 1A, Figure 1B).

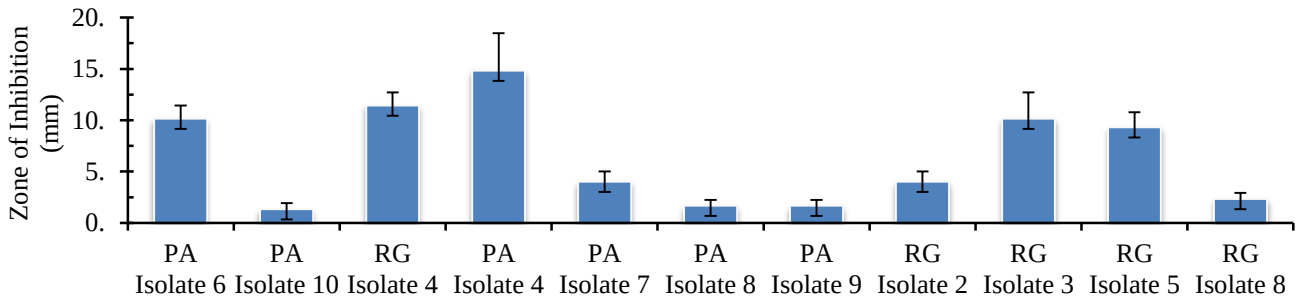

Figure 1a: activity against *staphylococcus aureus*

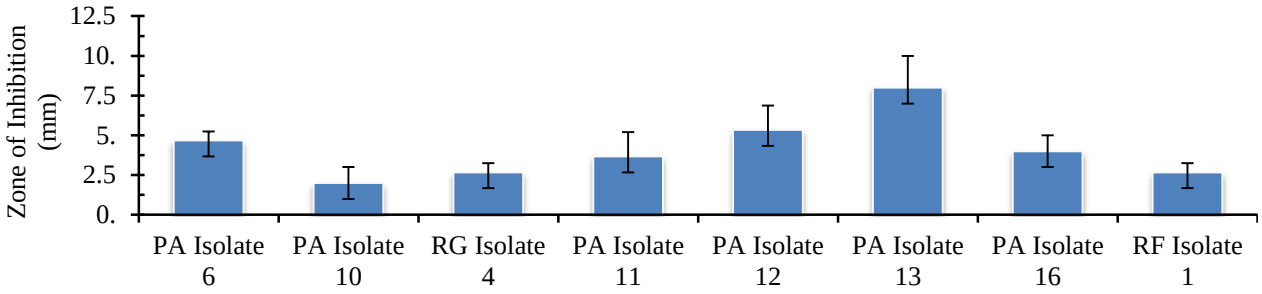

Figure 1B: Activity against *Escherichia coli*

Antifungal assays showed 4/25 antibiotic producing isolates were additionally able to inhibit the growth of the plant pathogenic fungus, *Fusarium solani.* These included isolates Uri, Strep, Leidtke, and CM/CP E3 (data not shown). Three of these four, showed antibacterial effects against both Gram-positive and Gram-negative targets (Table 1), indicating production of a broad spectrum compound with antibacterial and antifungal activity.

3.2. Co-Culture Screening

Out of the 6,651 pairwise combinations tested, no significant antibacterial activity was observed from any of the co-cultures.

4. Conclusion

Our study identified a number of common plant- and rhizosphere-associated bacterial genera, including members of *Pseudomonas, Xanthomonas,* and *Chromobacterium*. Species within these genera have previously been associated with antibacterial and antifungal characteristics[14,15,16,17,18]. These genera mostly dominated the antibiotic producing bacterial and antifungal isolates, as well as other genera typically found in natural environments including *Streptomyces*, *Pedobacter*, and *Bacillus*. Overall, *Pseudomonas* species exhibited more broad spectrum activity, including plants-associated isolates Leidtke and CM/CP D5 (Table 1) and soil isolates B10 and E6 (Table 2). Of these, the isolate designated Leidtke offers more promise for producing stronger secondary metabolites, due to the large zones of inhibition created on *S. aureus* and *E. coli* (Figure 1A, Figure 1B) as well as exhibiting antifungal activity (data not shown). The other broad spectrum *Pseudomonas* isolates also show promise for producing secondary metabolites that could eventually produce broad spectrum antibiotics, due to their ability to kill off many gram-positive and gram-negative organisms. Another isolate that showed broad spectrum activity was the *Chromobacterium* PA Isolate Uri, which exhibits great promise as it was able to kill and inhibit the growth of all gram-positive and gram-negative strains as well as the fungi, *Fusarium solani*. However, due to the current use of *Pseudomonas* and some *Chromobacterium* species, some of the compounds being produced by the isolates may not be novel.

Of the identified genera, the *Janthinobacterium* and the *Rahnella* were the least expected to exhibit antibacterial activity due to their rarity and human pathogenicity. Of the isolates with the rarer genera, PA Isolate CM/CP E3, a *Janthinobacterium*, exhibited broad spectrum antibacterial activity as well as antifungal activity. This isolate produced a zone of inhibition on *E. coli* with a diameter of 5.33 mm (Figure 1B). The other *Janthinobacterium* and the *Rahnella* isolate, PA Isolates Long and CM/CP H5, did not show broad spectrum antibacterial activity, large zones of inhibition, or antifungal activity. However, due to their rarity and lack of explored antibacterial capabilities, these isolates, as well as the broader acting PA isolate CM/CP E3, will be further characterized in hopes of future clinical and agricultural applications due to their potential novelty.

Furthermore, the co-culture antibacterial screening, which produced no significant results, despite the large number screened, is inconsistent with recent reports in the scientific literature. Due to there being a large amount of secondary metabolic gene clusters found in bacterial genomes, it was expected that bacterial isolate could be able to produce a wider variety of novel antibacterial compounds if different signal pathways are triggered[19]. Co-culture mixtures have been shown to trigger these signal pathways, allowing bioactive antibiotic compounds to be produced by organisms in mixed culture that were not produced by the same organisms grown in pure culture. Thus supporting the hypothesis that cell-cell interactions and interspecies competition are a driving force for antibiotic production[20,21,22,23]. In order to confirm procedural techniques, multiple different retesting of combinations happened using a variety of overlay and streaking techniques. However, no significant, consistent zones of inhibition were observed in co-culture screens.

Overall, both Gram-positive and Gran-negative pathogens are a cause for concern when developing new antibiotics. For example, methicillin-resistant *Staphylococcus aureus (*MRSA), a gram-positive pathogen, kills more citizens in the US alone than the combined deaths from emphysema, homicide, Parkinson's disease and HIV/AIDS[24]. While, on the other spectrum, increasingly prevalent multi-drug resistant Gram-negative bacilli pathogens are becoming increasingly pan-resistant, causing a crisis in the medical field[24]. However, despite both Gram-positive and Gram-negative organisms being a concern, broad-spectrum antibiotics are not necessarily the cure for knocking both out effectively. Broad-spectrum antibiotics are useful in a clinical emergency, however, overuse and abuse of them will kill off patients' natural, normal flora and create even more bacterial resistance. The isolates that exhibited antimicrobial activity with either Gram-positive or Gram-negative organisms provide the best outlet to developing bacterial-specific antibiotics. Isolates that exhibited broad-spectrum antimicrobial activity would work in an emergency clinical setting where fast bacterial death is essential. The isolates that exhibited antifungal activity in general would be most beneficial in the agricultural setting to treat fungi and bacterial infections while maintaining a crop's organic certification

Future work seeks to isolate and characterize the antibiotic compounds being produced by the novel bacterial strains isolated during this work. Particular focus will be on characterization of strains that showed antifungal and broad spectrum antibacterial activity, as well as species belonging to rare genera, that have not previously been characterized as antibiotic producers. Identification of any novel compounds may have future relevance for clinical and biocontrol uses.

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