Endophytic symbionts of *Acacia victoriae:*

diversity and potential for antibiotic production

By

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Abstract

The potential disease burden due to pathogenic microbes is increasing worldwide due to the evolution and spread of antibiotic resistance. Thus there is an urgent need to discover new antibiotics from diverse sources. One important source for new antibiotics is medicinal plants, some of which produce secondary compounds with antibiotic activity. Recently it has been shown that microbiomes of such plants may play a role in producing antimicrobials, some of which have been thought previously to be of plant origin. Here I examine the antibiotic potential of microbial endophytes of a focal medicinal plant, *Acacia victoriae*. In doing so I tested the hypothesis that endophytes from stems, a long-lived tissue, will have more potent antimicrobial activity than endophytes of shorter-lived leaves. I isolated over 70 endophytes from *A. victoriae,* including 32 from leaves and 39 from stem tissue. Isolates were organized to morphotypes, identified via DNA barcoding, and evaluated for antimicrobial activity against model strains of three pathogenic species (*Escherichia coli, Bacillus subtilis*, and *Candida albicans*). I did not find strong evidence of inhibition by focal endophytes under the culture conditions used here, but several endophytes overgrew the focal microbes, suggesting the potential for competitive interactions worthy of future exploration.

Introduction

As the human population continues to grow at a global scale, so too does the potential for new diseases and ailments (Jones et al. 2008). This potential reflects emerging infectious diseases (Morens and Fauci 2013) and disease due to stress and aspects of modern lifestyles (Salleh 2008). However, the growth in both the incidence and severity of disease also reflects a growing inability to treat common diseases of the past. Diseases caused by bacteria typically are treated with antibiotics (Opal 2016). However, antibiotics are becoming less useful in many cases due to the evolution of antibiotic resistance in diverse bacteria (CDC 2018). Recently drug-resistant strains of yeast have also become problematic in human healthcare (Nourani et al. 1997). There is a need to identify new sources of antibiotics, and medicinal compounds more generally, for the treatment of current and emergent human diseases (Livermore 2004).

Many medicines used today come from plants (Petrovska 2012). Medicinal plants have a long history of cultural use by diverse ethnic groups worldwide (Cragg and Newman 2013). It has come to light in recent years that microbes play a pivotal role in maintaining homeotic environments in both plants and animals alike (Berg et al. 2014). As with human microbiomes, plants host diverse combinations of microbes (i.e., plant microbiomes) that may influence the ability of plants to produce medicinal compounds (i.e., secondary metabolites) – or may actually be the producers of those secondary metabolites, instead of the plants themselves (Gouda et al. 2016). Harnessing the potential of these metabolites as antimicrobial agents is crucial as we enter the post-antibiotic era: widespread resistance, coupled with a limited chemical diversity of current antibiotics, is increasing the chances of deaths from bacterial infections that could be treated reasonably even a decade ago (CDC 2018). By identifying and applying antibacterial or

antipathogenic capacities of microbiomes associated with medicinal plants, the human population could be better equipped to combat diverse ailments (Koberl et al. 2013).

The goal of this study was to evaluate plant microbiomes for their production of medicinally important secondary metabolites. I focused on medicinal plants cultivated at the University of Arizona through the UA Campus Arboretum. I collected leaves and stems of two species and isolated endosymbionts (endophytic microbiomes) from one focal species. I characterized the microbes with molecular barcoding and screened them for antimicrobial activity *in vitro.* In doing so I tested the hypothesis that microbial endosymbionts in long-lived plant tissues (stems) will have a more potent antibiotic arsenal than those in short-lived, ephemeral tissues (leaves). I predicted this because those fungi that live in long-lived tissues have a greater likelihood of being important to maintaining the health of those tissues, whereas rapid turnover of tissues like leaves means that disease can be shed rapidly, and defense may be less important. Ultimately, I hope this work will contribute to a broader understanding of the potential medicinal properties of plant microbiomes.

Materials and Methods

In November 2018, I collected small branches from the north and south sides of one individual of each of two species: *Acacia victoriae* (Fabaceae) and *Taxodium mucronatum* (Cupressaceae). Both species are cultivated on the University of Arizona campus. *Acacia victoriae* is native to Australia and *T. micronatum* occurrs naturally in Mexico. The individuals were located at 32.230183 N, -110.954075 W and 32.230436 N, -110.954774 W respectively. I chose to study *Taxodium mucronatum* endophytes due to the tree's record of having antiarthritic properties

(Alonso-Castro et al. 2016). I chose *Acacia victoriae* due to its antitumoral properties and the role of its metabolites in influencing the cell cycle (Haridas et al. 2001).

Laboratory processing

Small branches from both trees were washed gently but thoroughly in running tap water to remove superficial debris. The leaves were stripped from the stems and cut into roughly 0.5cm^2 pieces for a total of 380 leaf segments. Stems were also cut into 0.5 cm segments for a total of 266 segments. The segments were surface-sterilized first for 10 seconds with 95% EtOH, then 0.5% NaOCl for 2 minutes, and finally with 75% EtOH for 2 minutes (Arnold and Lutzoni 2007). This process removes living microbes from the exterior surface of leaves and stems without killing endophytes within the segments (see Shaffer et al. 2017). The surface-sterilized segments were placed in a laminar flow hood to surface-dry under sterile conditions prior to plating, below.

Preparation of media

I prepared three growth media: 2% malt extract agar (MEA, 20.0 g agar, 20.0 g malt extract), Sabouraud dextrose agar (SDA, 40.0 g dextrose, 10.0 g peptone, 20.0 g agar), and lysogeny broth agar (LBA, 10.0 g tryptone, 10.0 g NaCl, 5.0 g yeast extract, 20.0 g agar). Each medium was prepared with 1 L of Milli-Q water. The media were sterilized in an autoclave by processing at 121 degrees Celsius for 60 minutes. MEA and SDA were each distributed evenly among 120 Petri plates (100 mm). These were allowed to cool and stored in the refrigerator until needed. LBA was pipetted into 155 Petri plates (60 mm), allowed to solidify, and cooled in the

refrigerator. MEA and SDA were used for the initial isolation of endophytic microbes. LBA was used for subsequent antimicrobial experiments.

Isolation of endophytes

Leaf and stem segments of *A. victoriae* and *T. mucronatum* from the north and south orientations were placed in groups of 10 on individual MEA and SDA plates, per above. Each plate was sealed with Parafilm and allowed to incubate at room temperature until growth was observed. No growth was observed from *T. mucronatum*, such that the remainder of this thesis focuses on endophytes of *A. victoriae.*

All plates were monitored until growth was observed. Once adequate growth was observed and before growth of any two cultures overlapped, individual isolates of bacteria and fungi were transferred to 60 mm MEA plates under sterile conditions in a biosafety cabinet via sterile, autoclaved toothpicks. All plates were sealed using Parafilm and allowed to grow individually. Isolates in pure culture were organized into morphotypes on the basis of cultural characteristics, including growth rate, surface color, colony shape and texture, coloration of the growth medium, the presence of aerial growth, and overall appearance.

DNA extraction

For isolates of fungi that were morphologically unique, DNA was extracted by first placing a small piece of mycelium from the culture plate into a sterile 1.5 ml microcentrifuge tube. The amount of agar included in the transfer was minimized in favor of as much mycelium as possible. The DNA extraction process followed Shaffer et al. (2017). Briefly, 100 µl of

extraction buffer was added and the tissue was ground using a sterile pestle. The tubes were heated in a heat block for ten minutes at 98 degrees Celsius and centrifuged. Finally, 100 µl of dilution buffer was added to each tube and the samples were vortexed briefly.

For unique bacterial isolates, a small quantity of cells were transferred to a 1.5 ml Eppendorf tube containing 20 µl of Y-PER buffer (Packeiser et al. 2013). Next, they were vortexed briefly and put in the heat block as above for 10 minutes. After heat lysis, the tubes were centrifuged at maximum speed for 30 seconds to separate the bacterial pellet from any debris. The supernatant was transferred to a new tube and diluted with 80 µl of molecular grade water.

Polymerase chain reaction (PCR)

I used PCR to amplify the barcode loci for fungi (nuclear internal transcribed spacer and 5.8S gene, plus ca. 300-500 basepairs of the adjacent nuclear ribosomal subunit) following Shaffer et al. (2017), and for bacteria (16S rRNA) following Hoffman et al. (2010). Table 1 shows the PCR recipe for fungi, which were based on Shaffer et al. (2017). The cycling reactions are described below.

Table 1. PCR reactions for fungi.

Reagent	1x, µl	15x, µl
Red Taq	10	150
ITS1F, 10µl	0.8	12
LR3, 10µl	0.8	12
Mol H2O	7.9	118.5
Total	19.5 / rxn	292.5
DNA / well	0.65	0.65

Cycle
94°C *3 min*
94°C *30 s*
54°C *30 s*
72°C *1 min*
Repeat 36 times
72°C *10 min*
15°C *until retrieval*

For bacteria I used the reaction mixtures detailed in Table 2 and 3. Bacterial DNA prepared for PCR amplification was run on the DreamTaq program on an MJ Research Thermal Cycler. All bacterial isolates were rerun through PCR amplification in attempt to yield better results than the first amplification, as the first amplification did not yield strong bands.

Table 2. Bacteria PCR reactions, first attempts.

Reagents	1x, µl	13x, µl
Green Taq	12.5	162.5
27F	1	13
1492R	1	13
Mol H2O	9.5	123.5
Total	24 / rxn	312
DNA / well	0.5	0.5

Table 3. Bacteria PCR reactions, second attempts.

Reagents	1x, µl	13x, µl
Green Taq	10	120
27F	0.8	96
1492R	0.8	9.6
Mol H2O	7.9	94.8
Total	19.5 / rxn	234
DNA / well	0.5	0.5

Cycle		
94°C		*3min*
	94°C	*30s*
	55°C	*30s*
	72°C	*30s*
	Repeat for 40 cycles	
72°C		*10 mins*
10°C		*until retrieval*

Gel electrophoresis

Amplified PCR products were evaluated via gel electrophoresis with 5 µl of a one kilobase ladder run as a standard. For each product, 3 µl of PCR product was added to individual wells with 2 µl of SYBR. SYBR was used as a dye that binds to DNA so that we could visualize it in a 1% agarose gel under UV light (Hoffman and Arnold 2010). The presence of bands and their approximate length, based on distance traversed on the gel relative to the ladder, were evaluated after 20-30 min at standard voltage.

PCR cleanup

Successful PCR amplification products were purified prior to sequencing. A total of 1 µl of ExoSAP™ product was added to each PCR tube. This was then run through the ExoSAP™ program in the thermal cycler as follows:

Cycle
37°C for 1 h
85°C for 15 min
4°C until retrieval

DNA sequencing

Validated and cleaned PCR products were sent to the University of Arizona Genetics Core. The products were sequenced bidirectionally on the Sanger sequencing platform (Heather et al.

2016). Bidirectional sequencing overlaps similar sequence fragments and allows for more accurate representation of nucleotides at the ends of strands (Valenzuela 1989). These contigs were edited manually, and consensus sequences were compared against GenBank with the Basic Local Alignment Search Tool (BLAST) program (Neumann et al. 2014) to identify the strains tentatively.

Antimicrobial assays

Ten endophytes were chosen to test against three focal microbes. The focal microbes were *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans*. These strains represent a diversity of pathogenic speices. *Escherichia coli* is a Gram-negative bacterium. Most *E. coli* are harmless and some are important components of human microbiomes, but some are virulent against humans (Lim et al. 2010). *Bacillus subtilis* is a Gram-positive bacterium. Members of its genus include anthrax and other pathogenic bacteria, as well as diverse soil- and plant-endophytic strains. *Candida albicans* is a single-celled fungus. Members of its genus are highly virulent pathogens in humans, and this species is known for causing thrush and candidiasis.

In preparation for antimicrobial assays, the three focal microbes were incubated individually in a sterile 10 ml Falcon tube with 5 ml of sterile lysogeny broth (prepared as for LBA without agar). A small (1 ml) amount of cells for each focal microbe was extracted from lab stocks and placed in the tubes via a sterile spatula. The tubes were then placed horizontally in a rotatory incubator at 27 degrees Celsius for 24 hours. At that point visible growth could be seen for all microbes when compared to the controls (lysogeny broth with no inoculum).

Next, 100 µl of each microbial suspension was pipetted onto LBA in individual 60 mm plates and spread using a sterile spreader to ensure uniform growth. The plates were sealed using Parafilm and allowed to grow for 24 hours. After 24 hours of room temperature incubation, lawns were visible on the growth medium.

Endophytes AVE064, AVE042, AVE041, AVE039, AVE031, AVE070, AVE063, AVE037, AVE055B, and AVE022 were evaluated for their interaction with the focal microbes. A plug of endophyte mycelium was transferred from a source culture under sterile conditions and placed with a sterile toothpick on the surface of each lawn. Each endophyte was tested in triplicate against each microbe (i.e., 9 plates were analyzed per endophyte).

Results

Overall, 71 endophytes were isolated from leaves and stems of *A. victoriae* at the University of Arizona campus. Of these, 39 were derived from leaf tissue and the remaining 32 were derived from stem tissue. The number of isolates from each medium, tissue type, and aspect (north or south side) of the tree are shown in Table 4.

Table 4. Endophyte isolates and morphotypes as a function of isolation medium, branch orientation, and tissue type. MEA = malt extract agar, SDA = Sabouraud's agar. In general we obtained more isolates on SDA than MEA, and more morphotypes on SDNA than MEA. In most cases more endophytes were found in north-facing branches than in south-facing branches, except the isolation of endophytes from south-facing branches on SDA.

	Leaves				Stems			
	MEA		SDA		MEA		SDA	
	North	South	North	South	North	South	North	South
Total Number of isolates	11	1	10	17	9	4	13	6
Total Number of morphotypes	4	1	4	3	4	2	8	4
Fungal Morphotypes	1	0	1	2	1	1	3	2
Bacterial Morphotypes	3	1	3	1	3	1	5	2

The strains isolated here represent 31 distinct morphotypes. From those, 8 fungal endophytes and 8 bacterial endophytes were sequenced for identification. The focal bacteria included *Bacillus licheniformis, Cutibacterium acnes, Bacillus megaterium, Bacillus amyloliquefaciens / velezensis, Bacillus subtilis, Williamsia muralis,* and *Enterobacter aerogenes.* The fungi included *Paradendryphiella salina, Aureobasidium pullulans, Alternaria tenuissima,* Pezizomycetes sp., Dothideomycetes sp., *Preussia* sp., and *Smardaea australis*. Isolate AVE031 was not identified. *Bacillus licheniformis, Cutibacterium acnes, Preussia* sp., and AVE031 were derived from leaf tissue; all other focal isolates were derived from stem tissue. These strains were used in the antimicrobial assays. Table 5 shows each sequenced endophyte and BLAST results.

Table 5: BLAST sequencing results, original medium, derived tissue type, branch orientation, and inhibition assay results. MEA = malt extract agar, **SDA =** Sabouraud's agar. Numbers indicate the number of plates (of three total) in which a response different from the control plates was observed. X/3 = the number of plates with a distinctive response; distinctive responses are inhibition unless marked with *, which indicates that overgrowth was observed.

Isolate	BLAST results	Medium	Tissue Type	Branch Orientation	B. subtilis Inhibition	C. albicans Inhibition	E. coli Inhibition
AVE022	*Bacillus licheniformis*	MEA	Leaf	N	2/3	0/3	2/3*
AVE037	*Cutibacterium acnes*	SDA	Leaf	N	0/3	0/3	0/3
AVE044	*Bacillus megaterium*	SDA	Stem	S	Not used for Inhibition Assays		
AVE057	*Bacillus amyloliquefaciens / velezensis*	SDA	Stem	N	Not used for Inhibition Assays		
AVE058	*Bacillus subtilis*	SDA	Stem	N	Not used for Inhibition Assays		
AVE063	*Williamsia muralis*	MEA	Stem	N	0/3	0/3	0/3
AVE070	*Enterobacter aerogenes*	MEA	Stem	N	3/3*	0/3	2/3*
AVE055B	*Cutibacterium acnes*	SDA	Stem	N	2/3	0/3	0/3
AVE039	*Preussia* sp.	MEA	Leaf	N	1/3	0/3	1/3*
AVE041	*Smardaea australis*	SDA	Stem	S	1/3	0/3	0/3
AVE042	Pezizomycetes	SDA	Stem	S	0/3	0/3	0/3
AVE047	*Paradendryphiella salina*	MEA	Stem	S	Not used for Inhibition Assays		
AVE053	*Aureobasidium pullulans*	SDA	Stem	N	Not used for Inhibition Assays		
AVE060	*Alternaria tenuissima*	SDA	Stem	N	Not used for Inhibition Assays		
AVE061	Pezizomycetes	SDA	Stem	N	Not used for Inhibition Assays		
AVE064	Dothideomycetes	MEA	Stem	N	0/3	0/3	0/3
AVE031	no significant match	SDA	Leaf	S	1/3*	0/3	0/3

We observed the antimicrobial assay plates for evidence of inhibition or lysis. Classical evidence for such interactions, such as a small zone of clearing around the endophyte, indicating lysis or

inhibition of the growth of the focal microbe, was not observed. Six plates (represeting four pairings of endophytes with *B. subtilis)* were observed with no visible lawn at the end of the observation period; in each case, a lawn had been observed at the start. In these cases, it is posssible that volatiles from the endophytes led to death of the focal microbial cells; however, not all replicate plates for each endophyte/microbe pairing showed significant effect of the endophytes (Table 5). In those cases, the endophytes were *Smardaea australis* (AVE041 and *Cutibacterium acnes* (AVE055B) from stem tissue (together, 3 of 6 plates), and *Bacillus licheniformis* (AVE022) and *Preussia* sp. (AVE039) from leaf tissue (together, 3 of 6 plates) (Table 5). In another case, the endophytes overgrew the focal microbe, suggesting competition or other interactions (but not antibiosis per se). In those cases the focal microbe was *B. subtilis,* and the endophytes were *Enterobacter aerogenes* (AVE070) from stem tissue and *Bacillus licheniformis* (AVE022), *Preussia sp.* (AVE039), and AVE031 from leaf tissue (Table 5). We never observed lysis, inhibition, or overgrowth in any pairings with *C. albicans*, and most of the interactions we observed were most striking in *B. subtilis.*

Discussion

Given the spread of robust antibiotic resistance, an important future direction for medicine depends on identifying new antibiotics and antimicrobials, ideally from novel sources (Moloney 2016). This study produced a culture library of diverse endophytic microbes, including bacteria and fungi, from leaves and stems of a medicinal plant. This work shows that *A. victoriae,* a medicinal plant, harbors many microbial associates within its healthy tissues. We did not observe classical inhibition or lysis of the focal microbes over the short timeframe of our antimicrobial assays, and the results were ambiguous regarding the potential for volatile products to have

eradicated growth of some plates of *B. subtilis*. Moreover, we never observed any potential chemical interaction (volatile effects, inhibition, lysis) for *C. albicans* or *E. coli.* The timeframe used here perhaps was not sufficient for us to see inhibition if it were occurring – it is possible that more time would be needed to see the interactions (Laget and Smith 1979). Thus, we are continuing to monitor the inhibition assays. For this reason, I do not have a conclusive result for addressing my hypothesis, except that I did not see a difference in inhibition between stem and leaf endophytes in the present study. My work incorporated only a few focal microbes and was done only under one growth environment. It is possible that working with different media and different pathogens could bring out distinctive antimicrobials not witnessed here (Lazazzara 2017). The observed overgrowth of *B. subtilis* and *E. coli* by some endophytes was striking, suggesting potential ecological interactions relevant to competition and worthy of future exploration.

Perspectives on endophyte isolation

This study yielded a slightly higher number of isolates from long-lived stem tissue than shorter-lived leaf tissue. This result is consistent with previous work (e.g., Massimo et al. 2015). A higher isolation frequency can be expected in long-lived tissues because endophytes accumulate through tissue lifetimes via horizontal transmission (Saikkonen 2004).

These results suggest that in future work, more efficient capturing of endophytes will be possible with stem tissues instead of leaf tissues, at least in the case of desert plants. I also recommend considering the medicinal plant species in its native habitat, where the microbes may be different and may represent a longer history of association with the host plant. Such

associations could filter for more beneficial microbes, which in turn could have potential applications in human health.

Conclusions

My work shoes that endophytic microbes are common and diverse in leaves of a non-native medicinal plant cultivated on the University of Arizona campus. None showed classical inhibition against a panel of focal microbes under one set of culture conditions. No distinctive interactions were observed between endophytes and the single-celled fungus *C. albicans.* Overgrowth of some plates of *B. subtilis* and *E. coli* by several endophytes suggests an interesting interaction for future work.

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