Antibiotic Prospecting From Plant Microbial Endophytes

Celeste Giannoulias[1], Lily Johnson[2], Olivia Patterson[1], Jeanmaire Molina (mentor)[3,4]

[1] The Brearley School, [2]Eleanor Roosevelt High School, [3]Pace University, [4]LIU-Brooklyn

ABSTRACT

Antibiotics are substances that kill bacteria and reduce the spread of bacterial infections. Many antibiotics are derived from natural metabolites produced by bacteria and fungi. However, antibiotic resistance is a growing problem resulting in a shortage of effective antibiotics. The Small World Initiative (SWI) hopes to address this crisis by crowdsourcing antibiotic discovery in soil samples. Another untapped but potential source of antibiotics may be found in endophytes. Endophytes are mutualistic microbes which live in plant tissues and may promote plant growth as well as enhance plant immune responses. Following SWI protocol, we isolated bacterial endophytes from plants known to have antibacterial properties and from the rare plant *Rafflesia*, whose microbiome is unexplored. We taxonomically identified 11 isolates by DNA barcoding. Unfortunately, none of the bacterial isolates were able to inhibit *Staphylococcus epidermidis* and *Escherichia coli* in our antagonistic assays. However, we found evidence in the literature that these bacteria we have isolated, or their close relatives, possess antibiotic activity. It is possible that the proper growth environment was missing for the antibiotic activity to be engaged. Regardless, our study has shed light on the diversity of plant bacterial endophytes, and how they may be explored as a resource for antibiotic prospecting.

INTRODUCTION

- Antibiotics are antibacterial substances used to treat infectious diseases, lysing the bacterial cell or interfering with the bacteria's metabolism (Ligon, 2004). Most pharmaceutical antibiotics are produced naturally by soil bacteria and fungi.
- Over the last few decades, increasing bacterial strains have evolved resistance against antibiotics (Andersson, 2003). To find new sources of antibiotics, the Small World Initiative (SWI) was spearheaded by Yale University in 2012 and seeks to crowd-source antibiotic discovery to college-level students by motivating students to collect soil and test antibiotic properties of soil bacteria against safe relatives of pathogenic bacteria.
- While SWI focuses on soil bacteria, bacteria are ubiquitous and also abound inside plant tissues. Termed endophytes, these microbes make up the plant microbiome which may have beneficial functions to the host plant such as producing plant-growth promoting hormones and secreting defensive metabolites (Turner et al., 2013; Babalola et al., 2020) which can potentially be antibiotic (Christina at el. 2013).
- As pathogens evolve to evade our current arsenal of antibiotics, antibiotic discovery must also keep pace, underscoring the importance of crowd-sourcing the process. Inspired by SWI, we propose to find novel antibiotic-producing bacteria from within plant tissues, instead of soil. We will focus on plant endophytes, which may be producing antibacterial compounds. To date, there are no pharmaceutical antibiotics developed from plants, and, importantly, there are no reports of bacteria evolving resistance against plant-based antibacterial compounds (Prasad et al. 2019). This is a strong case that plants—and their endophytes—could be one solution to the global antibiotic crisis.

MATERIALS & METHODS

- We followed the workflow of SWI with some modifications. Instead of sampling soil, representative plants from the families Lamiaceae (thyme leaves, *Thymus vulgare*), Lauraceae (bay leaves, *Laurus nobilis*), Myrtaceae (cloves, *Syzygium aromaticum*) and Zingiberaceae (ginger rhizome, *Zingiber officinale*; turmeric rhizome *Curcuma longa*), as well as seeds of *Rafflesia speciosa* were sampled. In addition, neem leaves (*Azadirachta indica*, Meliaceae) and guggul fruit (*Commiphora mukul*, Burseraceae), which have been reported to have antibacterial properties (Prasad et al. 2019) were sampled.
- Samples (0.1 g) were surface-sterilized with 2% NaOCl, rinsed with sterile distilled water, and then homogenized. An aliquot (100 ul) of the extract was swabbed on a nutrient agar plate (one sample/plate), which was incubated 48 hr at 37 C. Microbial isolates/colonies on the agar plate were picked-and-patched on new agar plates with the isolates labeled/numbered to make the master plates.
- For the antagonistic assay, *Staphylococcus epidermidis* (SE) and *Escherichia coli* (EC) were swabbed on two separate agar plates, onto which colonies from the master plates were picked and patched and incubated. Isolates were checked for zones of inhibition (ZOI) which indicated antibiotic potential.
- All isolates were DNA-barcoded using 16s bacterial primers (Klindworth et al. 2012). Those with PCR products after agarose gel visualization were submitted for sequencing. Geneious Prime (Biomatters Ltd) was used for sequence editing and BLAST analysis. Phylogenetic analysis was also performed for DNA barcodes of all bacterial isolates and their corresponding top BLAST hits. The identified microbe was then researched in the literature to find supporting evidence of its antibacterial properties.

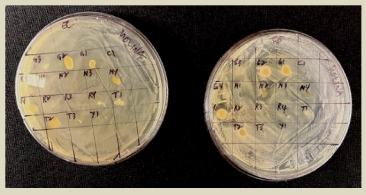

Fig. 1. Results of the antagonistic assay. Seventeen endophytic isolates were picked and patched on agar plates swabbed with EC (left) and SE (right). None of the 17 isolates showed antagonism/zone of inhibition against EC or SE.

Isolate name	Bacterial species ID	Size of PCR product	Pairwise identity with Genbank top hit	Family, Phylum	Brief description of ecology from literature
C1	*Staphylococcus epidermidis*	1388 bp	99.98%	Staphylococcaceae, Bacillota	*Staphylococcus epidermidis* is a common symbiont bacterium that can become infectious once inside the human host (Lee & Anjum 2022). No info was found on the antibiotic potential of *S. epidermidis* but antibiotic production was reported for its relative, *S. aureus* (Gardner 1949)
G3 and G4	*Priestia megaterium/ aryabhattai*	740-1391 bp	100.0%	Bacillaceae, Bacillota	*Priestia megaterium* is associated typically with plants and soil (Biedendieck et al. 2021) and was isolated as an endophyte of potatoes and orchid, with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Liu et al. 2023). The closely related *Priestia aryabhattai* is an endophyte of stress-tolerant wheat and was found to have antagonistic activity against fungal pathogens (Shahid et al. 2022)
N3	*Xanthomonas sacchari*	1391 bp	99.9%	Xanthomonadaceae, Pseudomonadota	*Xanthomonas sacchari* is a pathogen associated with banana plants. Its close relative *X. albilineans* has been found to produce the toxin albicidin, which is a DNA-gyrase inhibitor deleterious to most bacteria (Studholme et al. 2011).
R4	*Anthrobacter sp./Glutamicibacter*	1398 bp	99.6%	Micrococcaceae, Actinomycetota	*Anthrobacter* is often found in soil, on the surface of plants, and wastewater sediments (Gobbetti and Rizzello, 2014). Some spp. were reclassified as *Glutamicibacter* (Busse 2016) with one endophytic species from mangroves reported to produce antimicrobial peptides (Karthik et al. 2023)

Table 1. Representative endophytic bacterial isolates from 4 plant samples in this study (C: *Commiphora mukul,* G: *Zingiber officinale,* N: *Azadirachta indica,* R: *Rafflesia speciosa).* The DNA barcodes from the isolates were also phylogenetically analyzed to ascertain taxonomic identity (cf. Fig 2).

Fig. 2. Phylogeny of endophytic bacterial isolates (C1, G1-G4, N1-N4, R3, R4). Homologous sequences from corresponding top hits in BLAST were aligned with the 16s barcodes amplified from the isolates and phylogenetically analyzed.

RESULTS & DISCUSSION

- Our goal was to extract and isolate bacterial endophytes that may have antibiotic potential from plants with known antibacterial properties (Prasad et al. 2019)
- We were able to identify 17 unique colonies from four (C: *Commiphora mukul,* G: *Zingiber officinale,* N: *Azadirachta indica,* R: *Rafflesia speciosa)* of eight plants in this study.
- None of the colonies demonstrated antibiotic activity against EC or SE (Fig. 1), however this may have been due to not being able to provide the proper growth conditions for the bacteria to express antibiotic-production genes (e.g. missing a certain plant nutrient/metabolite). It is also possible that testing against other bacteria aside from EC/SE may produce antagonistic activity
- We were able to DNA barcode and taxonomically identify 11 of the 17 isolates (Fig. 2). Though none of the endophytic isolates were inhibitory to EC/SE, we were able to find evidence in the literature that the identified isolates in our study, or at least their close relatives, have antibiotic properties. Some representative isolates from each of the 4 plants are given in Table 1.
- Most pharmaceutical antibiotics such as streptomycin, erythromycin, and tetracycline have been derived from soil bacteria (Popowska et al. 2012). However, as pathogenic bacteria evolve resistance against these antibiotics, it is becoming increasingly important to search for new antibiotics. Plant tissues are a natural reservoir of microbes, of bacterial and fungal endophytes and offer an untapped resource for antibiotic prospecting.
- Fungal endophytes should also be investigated as studies have shown their antibiotic properties (dos Santos et al., 2015; Mao et al., 2021). After all, the first antibiotic, penicillin, was derived from a fungus (Ligon 2004). Our study has shed light on the diversity of plant endophytes, and how they may be explored as a resource for antibiotic prospecting.

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