Environmental *Pseudomonas* sp. Producing Antimicrobial 2,4-diacetylphloroglucinol

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Introduction

The overuse and misuse of antibiotics has selected for and increased antibiotic resistance to a crisis level (World Health Organization [WHO], 2012). There are fewer antibiotics to use for bacterial infections and new drug discoveries are not keeping up with the level of resistance. Soil bacteria naturally produce a wide range of secondary metabolites, which increase their survival by inhibiting the growth of nearby competitors. These natural products hold promise due to the recent discovery that soil dwelling *Streptomyces* spp. naturally produce sansazamycin, which displays significant activity against multi-drug resistant *Mycobacterium tuberculosis* (Tran et al., 2017). Previous research has shown that naturally occurring *Pseudomonas* spp. can produce bioactive metabolites that inhibit the growth of pathogens. Such bioactive compounds include heterocyclic phenazines, and the derivative 2-hydroxyphenazine, along with 2,4-diacetylphloroglucinol (2,4-DAPG).The objective of this project is to characterize a discovered secondary metabolite produced by a bacterium isolated from Las Vegas soil.

Materials and Methods

Sample Collection

- Soil samples were obtained from Mount Wilson, located in Las Vegas.
- Quantitative techniques were utilized to determine sample viability, followed by phenotypic analysis.
- Pure cultures were analyzed for antimicrobial production via patch plate method.
- Bioactive isolates were subjected to genotypic analysis

Polymerase Chain Reaction (PCR) Analysis

P. protegens Pf-5 *P. chlororaphis* 1.4

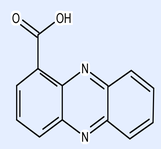
- Screened for the presence of antimicrobial biosynthesis genes commonly associated with *Pseudomonas.*
- Phenazine biosynthesis: *PhzF*
- 2,4-DAPG biosynthesis: *PhlD*

Bacteria	Template	Anneal (C°)	Product (bp)
P. protegens Pf-5	*PhlD*	58.0	629
P. chloroaphis	*PhzF*	62.0	427
1.4	Unknown	-	-

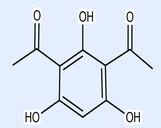
Antimicrobial Extraction & Identification

- Liquid-Liquid Extraction of antimicrobial
  - Solvent: Ethyl acetate
  - Crude extract subjected to TLC & HPLC
- Thin Layer Chromatography (TLC)
  - Silica gel aluminum sheet (5x20cm)
  - Mobile phase: chloroform/methanol (9:1)
- RP-High Performance Liquid Chromatography (RP-HPLC)
  - Kinetex 5 µm EVO C18 Column (150 x 4.6 mm)
  - Isocratic elution
  - PCA mobile phase: acetonitrile/water (30:70)
  - 2,4-DAPG mobile phase: acetonitrile/water (45:55)

Phenazine-1-carboxylic acid

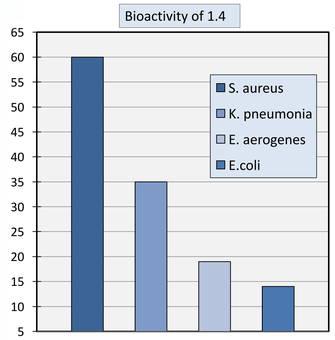


2,4-diacetylphloroglucinol

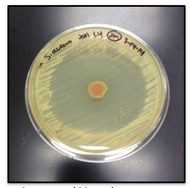


Results

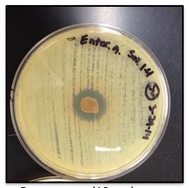
Antimicrobial Activity



- 1.4 isolate centrally inoculated on TSA containing one of the following lawn-inoculated test strains: *S. aureus, K. pneumonia, E. aerogenes* & *E. coli.*



S. aureus (60mm)



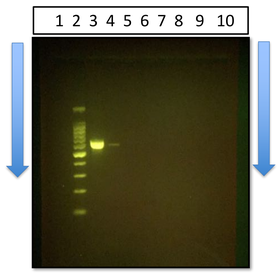
E. aerogenes (19mm)

16S Sequence Distance Matrix

	1.4 Isolate	*Donghuensis*	*Vranovensis*	*Alkylphenolica*	*Putida*	*Chlororaphis*	*Protegens*
1.4 Isolate		99.013%	97.619%	97.479%	96.779%	96.148%	93.978%
Donghuensis	98.599%		99.013%	99.449	97.487%	97.498%	96.265%
Vranovensis	97.619%	99.013%		99.174%	97.28%	96.95%	96.055%
Alkylphenolica	97.479%	99.449%	99.174%		99.346%	97.065%	96.355%
Putida	96.779%	97.487%	97.28%	99.346%		97.087%	95.342%
Chlororaphis	96.148%	97.498%	96.95%	97.065%	97.087%		97.978%
Protegens	93.978%	96.265%	96.055%	96.355%	95.342%	97.978%	

- The obtained 16S rRNA sequence from isolate 1.4 was compared to known sequences in the NCBI database.
- ClustalW used to calculate the percentage of nucleotide sequence identity between 1.4 and the top NCBI database matches.

2,4-DAPG Biosynthesis Gene Fragment (629 bp)



2% Agarose Gel

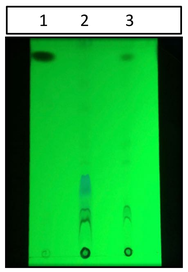
2) Ladder (100 bp)

3) *P. protegens* Pf-5
- Positive *PhlD* (629 bp)

4) 1.4 Isolate
- Positive *PhlD* (620 bp)

5) Negative (No template)

Thin Layer Chromatography



1) PCA standard
Rf: 0.9

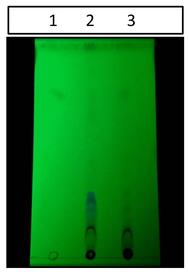
2) 1.4 Isolate

3) *P. chlororaphis*
Rf: 0.9

1) DAPG standard
Rf: 0.7

2) 1.4 Isolate
Rf: 0.7

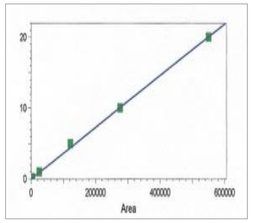
3) *P. protegens* Pf-5
Rf: 0.7



Results Cont'd

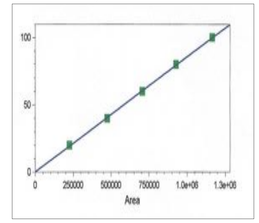
HPLC Calibration

PCA



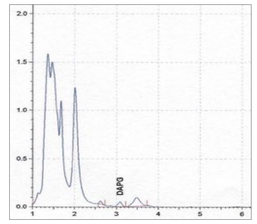
- PCA absorption maxima 250nm
- Five point calibration
- (0.2, 1.0, 5.0, 10, 20 µg)
- (r^2): 0.998787

2,4-DAPG

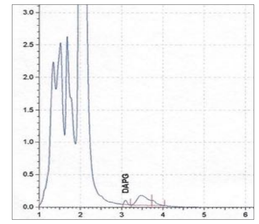


- DAPG absorption maxima 270nm
- Five point calibration
- (20, 40, 60, 80, 100 µg)
- (r^2): 0.999594

HPLC Identification of 2,4-DAPG



- *P. protegens* Pf-5 extract
- Positive for 2,4-DAPG production
- 2,4-DAPG peak at 3.1 min



- 1.4 extract
- Positive for 2,4-DAPG production
- 2,4-DAPG peak at 3.1 min

Conclusion

The results of the preceding study show an environmental bacteria species capable of producing broad-spectrum antimicrobial activity. Phylogenetic analysis based on the obtained 16S rRNA sequence indicates the identification of a *Pseudomonas* species. With a 99% sequence similarity to *P. donghuensis.* Our study identified the presence of the biosynthetic loci involved in the production of the antimicrobial 2,4-DAPG. This bioactive compound was successfully extracted from a liquid matrix and identified via TLC and RP-HPLC. The future work will consist of further verification of the presence of 2,4-DAPG by means of gas chromatography-mass spectrometry. Gene knockout methodologies will be utilized to determine that 2,4-DAPG is solely responsible for the antimicrobial activity

Acknowledgements

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References

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