PURIFYING AN UNKNOWN BURKHOLDERIA CONTAMINANS MS14 BACTERICIDAL

An Undergraduate Research Scholars Thesis

by

KEREN HERRERA DELGADO

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ABSTRACT

Purifying an Unknown *Burkholderia contaminans* MS14 Bactericidal

Keren Herrera Delgado
Department of Biology
Texas A&M University

Research Advisor: Dr. James Smith
Department of Biology
Texas A&M University

Recently, the rapid pace at which bacteria can successfully evade antibiotics has alarmed public health officials, since they could render even our most powerful antibiotics useless. With this in mind, it is evident to see the value and urgency in discovering novel antimicrobials. This project's focus is on the isolation and purification of an unknown bactericidal compound from *B. contaminans* MS14. While attempting to isolate and purify the unknown bactericidal agent, an interesting discovery was found. Ornibactin, a siderophore produced by MS14, has been shown to play a significant role in the bactericidal activity of the unknown antimicrobial. These two MS14 products seem to be working in tandem in some way, which reveals an interesting mode of action for our target antimicrobial.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

The world is in constant need for new drugs, especially with the rise of antibiotic resistance in Gram positive pathogens (1). In the US alone, there are 2 million cases of antibiotic. New antimicrobials have practical applications not just in medicine, but in the food industry and scientific community as well (2). Antimicrobials have a history of aiding in the expansion of our knowledge about microbial physiology and even our own.

Burkholderia contaminans MS14 is a Gram negative bacteria. It is a soil inhabitant, first isolated from a patch of soil that showed suppression to Brown Patch Disease of lawn grass (3). Originally, MS14 was observed to produce an antifungal, occidiofungin, when grown on minimal media (4). By culturing *B. contaminans* in complex media, antibacterial activity was seen. This unknown bactericidal product produced by MS14 is the focus of my project. Recent literature on *B. contaminans* focuses on its pathogenic effects. Our MS14 strain has been analyzed to be lacking the virulence genes responsible for pathogenesis in the capecia complex of Burkholderia. Although B. contaminans has shown potential as a plant growth promoted, its possible therapeutic applications have not been extensively studied. Therefore, any new bactericidal product isolated and purified from this bacterial strain will be novel, and an important contribution to the study of *B. contaminans*.

While trying to isolate the MS14 bactericidal, more information has been revealed about its siderophore product. Ornibactin is a small iron-chelating siderophore that exhibits bacteriostatic activity. Proximity assays have revealed that ornibactin is closely linked to the bactericidal product in MS14. Future directions in the project would include trying to unveil exactly in what way ornibactin interplays with the antibacterial to produce bactericidal activity.

CHAPTER II
METHODS

Culturing Methods & Media Preparation

Bacteria were kept fresh every week by streaking on Nutrient Broth Yeast Extract agar plates (487 mL dH_2O, 2.5 g peptone, 1.5 g Todd-Hewitt, 1.0 g yeast extract, and 7.5 g of agar). For antibacterial production, the following additional nutrients were added after autoclaving: 0.5 mL of 1 M $MgSo_4$, 10 mL of 20% glucose solution, and 5 mL of 1.0 g anhydrous K_2HPO_4 with 0.25 g KH_2PO_4. For antibacterial production bacteria were stabbed on glass pyrex baking trays with NBY soft agar (same ingredients as before, but this time only 3.5 g of agar). Bacteria were incubated at 37°C for three days and then frozen at -80 °C and heat killed for five hours at 65°C. The softened agar was then placed in 250 mL centrifuged bottles and centrifuged for 30 minutes at 23,000 X g. The supernatant was then collected for extraction. Chrome Azurol-S media for siderophore characterization was prepared according to manufacturer's instructions.

Characterizing Antibacterial Activity

Antibacterial activity was characterized by spotting samples on plates that had been overlaid with the indicator strain *Erwinia amylovora.* Overlay was prepared by diluting E. amylovora with dH2O to get an OD of 0.20, and then added to 5 mL of NBY soft agar. Plates were incubated at 37 °C and then analyzed the next day for inhibition zones showing clear or light halos indicating bactericidal activity or bacterial growth inhibition.

Antibacterial Extraction Methods

After centrifugation, 1 g of polyaromatic absorbance resin HP20 Diaion beads were added to the supernatant in a bottle and shaken for one hour. After the resin had settled to the bottom of the bottle, the supernatant was decanted, and the resin was r-esuspended in 10mL of 50% acetonitrile in water. This was mixed again, and after the resin had settled, the supernatant was collected and then lyophilized. The dried material was then re-suspended in 1 mL of 35% acetonitrile in water. HPLC was then performed on a C18 column with a hydrophobic resin (4.6 by 250 mm; Grace-Vydac catalog no. 201TP54) on a Bio-Rad Biologic DuoFlow F10 system with a QuadTec UV-visible detector. Sample was eluted off the column by using a 30 minute gradient of 90:10 water/acetonitrile with 0.1% trifluoroacetic acid to 20:80 water/acetonitrile with 0.1% trifluoroacetic acid.

Mutants for study

Mutants used in this study were courtesy of Dr. Shien Lu at the University of Mississippi. Mutations were performed using EZ-Tn5 Tnp transposome kit (Epicentre Biotechnologies). MT 577 has a mutation in a nonribosomal peptide synthetase, which disrupts the production of siderophore, while MT 357 has a mutation in a Lux-R like regulator.

CHAPTER III

RESULTS

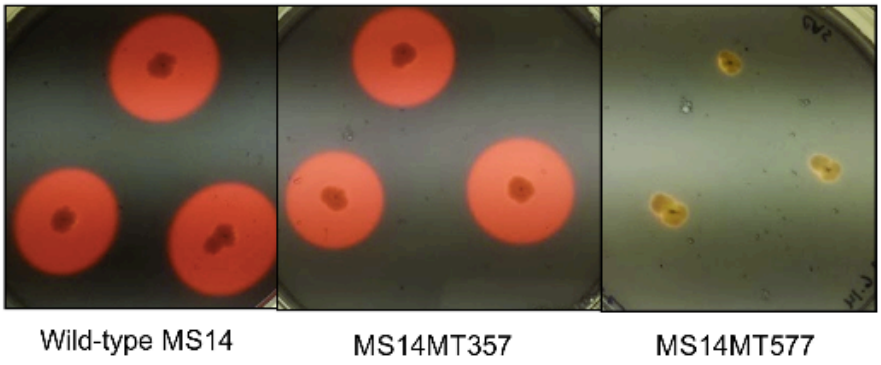

Figure 1. Siderophore production. Wild type and mutant colonies were stabbed on CAS (Chrome Azurol-S Plates) and incubated for 24 hrs. Production of siderophore is seen as bright orange halos around colonies as the siderophore quenches the iron bound to the blue dye in the plates. MT 577 colonies show no sign of siderophore production.

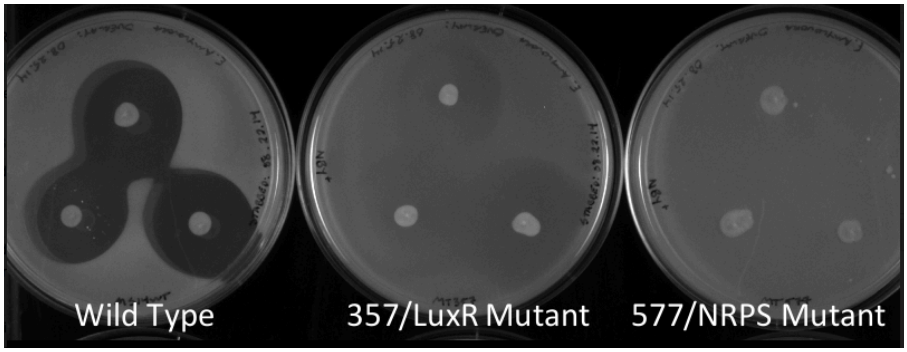

Figure 2. Loss of bactericidal activity in mutants. Wild type, and both mutant colonies were stabbed in modified NBY agar plates and then overlaid with indicator strains *E. amylovora*. Clear halos indicating bactericidal activity is seen in the wild type. Light halos are seen in the MT 357 mutant colonies indicating bacterial growth inhibition. Neither bacterial growth inhibition, nor bactericidal activity is seen in the MT 577 colonies.

As seen in Figure 2, both MT 357 and MT 577 have lost the bactericidal activity seen in the wild type strain. MT 357 does show to have some bacterial growth inhibition and this is seen as the light halos around the colonies in Figure 2. This bacterial growth inhibition activity is attributed to its own siderophore production, which can be clearly seen by the orange halos around MT 357 on Figure 1. Since MT 577 does not show any siderophore production on the CAS plates, it can be said that this is why no bacterial growth inhibition can be seen around its colonies on Figure 2.

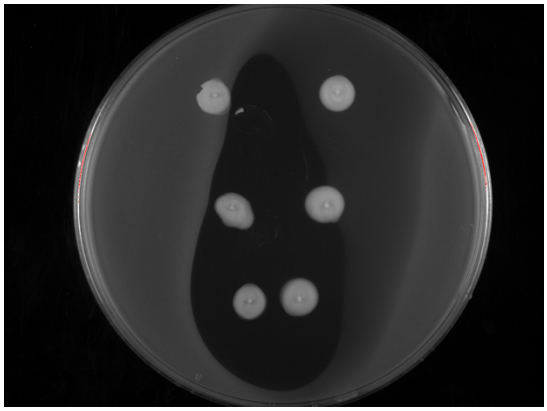

Figure 3. Mutant proximity assay. On a NBY plate overlaid with indication strain *E. amylovora,* MT 357 colonies were stabbed on the right-hand side and MT 577 colonies were stabbed on the left-hand side of the plate and incubated overnight. Bacterial growth inhibition is seen around the MT 357 colonies, which is attributed to its production of the siderophore ornibactin. Bactericidal activity is seen, as clear halos, to be restored the closer and closer the two mutants come with each other.

An interesting finding is seen on Figure 3. When both mutants were plated in close proximity to each other, bactericidal activity is restored in both of the mutants. The restoration of the bactericidal activity can also be seen when the two mutants are co-cultured together and even when ornibactin itself is diffused on top of MT 577 colonies.

Our unknown bactericidal also showed to be extremely sensitive to UV light, but resistant to heat up to 65 °C and low and high pH, as shown in Figure 4. The unknown bactericidal also proved to be effective against a panel of both Gram positive and Gram negative bacteria, although it seems to be more effective against the Gram negative strains, as shown on Figure 5.

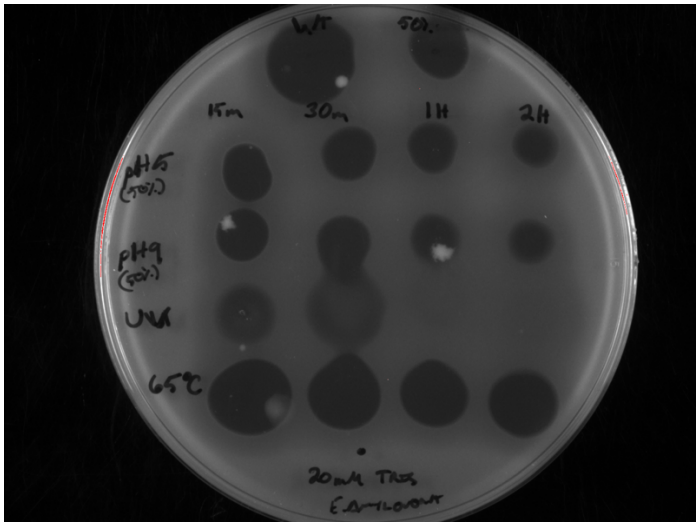

Figure 4. Stability Assays performed on the unknown bactericidal agent. The agent proved to be heat resistant, and can withstand pH levels of five and nine. This also shows that our unknown agent is extremely sensitive to UV light.

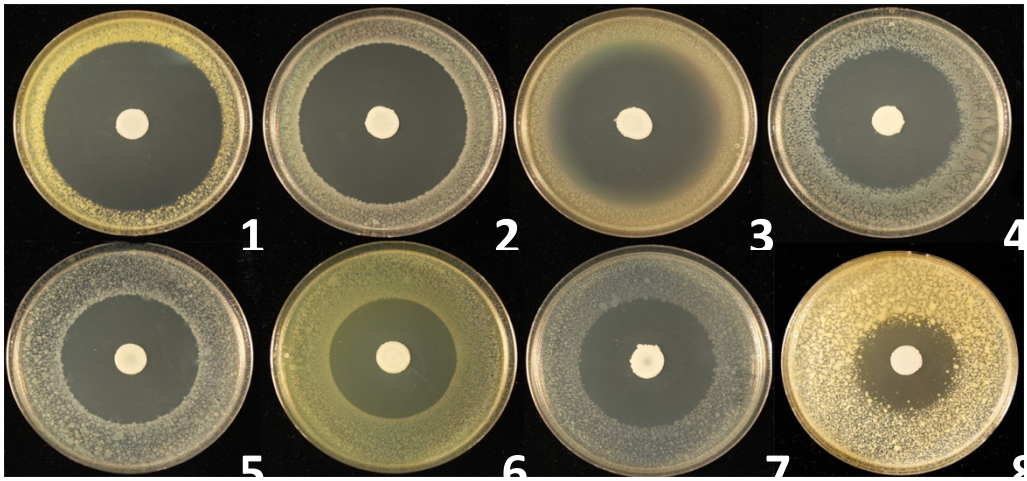

Figure 5. Antibacterial activity of the wild type strain of *Burkholderia contaminans*. Wild type colonies were stabbed on NBY plates and then overlaid with either a Gram positive or Gram negative strain as follows: **(**1) *Xanthomonas citri* pv. *malvacearum;* (2) *Pectobacterium carotovorum* WSCH1; (3) *Ralstonia solanacearum;* (4) *Pseudomonas syringae* B301; (5) *Erwinia amylovora;* (6) *Burkholderia glumae* 291(7) *Escherichia coli;* (8) *Clavibacter michiganensis* subsp. *Michiganensis*

Further Kupchan extractions with hydrophobic solvents indicated that our unknown antibacterial might actually be hydrophilic. As shown in Figure 6, none of the solvents were able to successfully extract the bactericidal component.

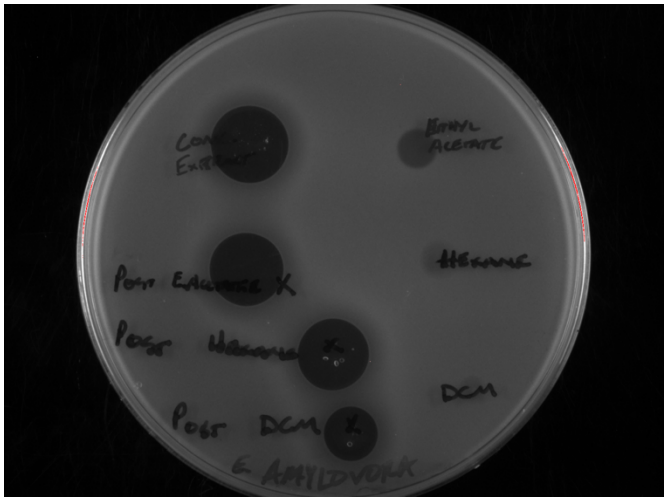

Figure 6. Kupchan extraction fractions tested for bactericidal activity. No activity is seen to be taken out by neither one of the solvents indicating that our unknown bactericidal might not be hydrophobic at all.

CHAPTER IV

CONCLUSION

We found that our antibacterial was effective against both Gram positive and Gram negative bacteria, but more so towards the Gram negative bacteria. We also conclude that the antibacterial is small, due it being able to pass through a 1kD filter. Another important conclusion is that the unknown bactericidal is sensitive to UV light.

From the results, there is clearly an interaction going on with the siderophore, ornibactin, and the unknown bactericidal agent. This might be in two possibilities, either ornibactin and the antibacterial agent are working in tandem in some way to confer bactericidal activity, or maybe ornibactin is behaving in a regulatory manner and activates a set of genes responsible for the production of the antibacterial product.

There is still much to be explored with this unknown bactericidal and also the interplay it has with ornibactin. Some future directions of this project would include trying different extraction methods, such as using an Amelite resin which separates compounds based off of charge instead of hydrophobicity which is what our current resin was doing. Mutagenesis would be continued to try to identify a biosynthetic gene cluster for the bactericidal agent, and more bioassays would be performed to try to unveil whether the interplay between ornibactin and the bactericidal agent is a regulatory one or not.

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