ANTIMICROBIAL AGENTS OF THE *BURKHOLDERIA* GENUS AND THOSE PRODUCED BY *BURKHOLDERIA CONTAMINANS* MS14

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2020

Major Subject: Microbiology

ABSTRACT

The increasing threat of antibiotic resistant pathogens is a major problem in the management of infectious diseases, making it imperative that new antibiotics to combat them are discovered and sourced. However, limiting work to merely discovering these novel antibiotics is far too narrow of a scope. It is equally important to understand how the biochemistry and production of these secondary metabolites is regulated. Furthermore, a better understanding of the bioactivity and structure of novel variants of secondary metabolites may lead to the identification of products with enhanced properties, such as improved spectrum of activity or reduced minimum inhibitory concentrations.

Using a member of the *Burkholderia cepacia* complex, *Burkholderia contaminans* MS14, my dissertation studies have been aimed at identifying novel antimicrobial factors and to gain a better understanding of the antimicrobial secondary metabolites the bacterium is capable of producing. The *Burkholderia* genus is highly conserved; therefore many of the insights gleaned from its study could be applied to other members of its genus. This includes the more virulent strains such as *Burkholderia cepacia* and *Burkholderia pseudomallei*. My work led to the identification of a novel antibacterial produced by *B. contaminans* MS14. In these studies, it was discovered that MS14 also produces ornibactin, a powerful siderophore. Siderophores are commonly associated with iron acquisition. However, the most interesting aspect was not the production of ornibactin, but its role in regulating the production of a separate

antibacterial product. Thus we learned that ornibactin, rather than just being an additional product with minor antibacterial properties, has a direct effect on the antimicrobial capabilities of *B. contaminans* MS14. My studies clearly show an alternative function for this siderophore, as knockout strains that lacked the ability to make ornibactin completely lost bactericidal activity. While this antimicrobial product proved difficult to isolate, I was able to investigate and report several of its inherent properties, such as its estimated size, polarity, and stability. Interestingly, this product is highly resistant to common forms of damage, such as temperature and pH. However, direct UV exposure resulted in the destruction of this antibiotic, giving clues into its physical structure.

Occidiofungin is a novel lipopeptide that is a potent anticandidal fungicide and exhibits its mode of action by binding to actin, resulting in apoptosis. However, in addition several variants are also produced. Studies on the isolation of these different variants of occidiofungin have led to the discovery of a new product. Due to their potency against opportunistic pathogens of both humans and plants, these variants are possible candidates for medical or agricultural applications. In addition to the known variants, the discovery of a novel variant and the further structural elucidation of the compound using nuclear magnetic resonance, suggests that the non-ribosomal peptide synthetase (NRPS) module for diamino butyric acid has some promiscuity for other amino acids. Additional information pertaining to its spectrum of activity and anticandidal properties has been determined.

DEDICATION

This is for those who answer their wake-up call

and

To those who turn a stumble into a sprint.

ACKNOWLEDGEMENTS

I would not be where I am today without the guidance and support of my committee chair, Dr. James Smith. My future was indelibly altered for the better when he brought me in as an undergraduate researcher, which set the stage for my eventual ascension to graduate school, and for that I will forever owe him my endless thanks.

During my time in graduate school, Dr. Joseph Sorg has proven to be an invaluable source of knowledge, ideas and opinions. Beyond that, he has grown from being an irreplaceable member of my committee to also being one of my greatest friends, especially at times when I needed one the most. A bittersweet note when writing this dissertation was knowing that my time having morning coffee and discussing topics with him was coming to a close. I had some of my best ideas while standing in his office doorway, and some of them were even related to science.

I must also express my sincerest thanks to Dr. Samuel, Dr. Siegele, Dr. Lu, and Dr. Manson, for their past and present guidance as members of my committee during the course of my graduate studies. I would also like to extend my thanks to Dr. Dangott and the Protein Chemistry Lab here at Texas A&M for providing me access to his knowledge and equipment for mass spectrometry. Additional thanks are owed to the graduate student advisors during my tenure here, Dr. Arne Lekven and Dr. Rene Garcia as well as our graduate program coordinator, Jennifer Bradford.

I wish to say thank you to the members of the Smith Lab: Dr. Shawanda Wilson-Stanford, Dr. Akshaya Ravichandran, Dr. Jerome Escaño, Dr. Mengxin Geng, Dr.

Steven LaiHing, Dr. Min Ju, Dr. Evangel Kummari, McKinley Williams, Nopakorn Hansanant, Thushinari Joseph & Andrew Cothrell, for making my time in lab social and enjoyable. I was also fortunate to have a number of fantastic undergraduate researchers over the years, and I owe them my thanks as well.

Finally, thank you to my mother for being my biggest cheerleader, and to my dog Dorothy, who never knew what was frustrating me, but always knew how to make it better.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. James L. Smith (chair), Dr. Joseph A. Sorg, and Dr. Deborah Siegele of the Department of Biology, Dr. James Samuel of the Health Science Center, and Dr. Shien Lu of the Mississippi State Department of Biochemistry, Molecular Biology, Entomology & Plant Pathology.

The plasmids and random mutagenesis in Chapter 3 was provided by Dr. Shien Lu. All other work conducted for the dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by an assistanceship from the Texas A&M University Department of Biology and a research grant provided by the National Institute of Allergy and Infectious Diseases.

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1. INTRODUCTION

1.1 The antimicrobial activity of *Burkholderia* species

The world is continually facing the growing problem of antibiotic resistant bacteria and fungi. As of this writing, data from the Centers for Disease Control and Prevention (CDC) shows that each year over two million people are infected with an antibiotic resistant pathogen. Of these two million, these infections prove to be fatal in more than twenty-three thousand cases (1). More alarming still is the fact that these numbers are not a worldwide figure, but instead are only in cases within the United States. This continually rising threat has a broader scope than just medical concerns as they also increase the financial burden laid upon the individual, if they're fortunate enough to have healthcare. Recent estimates have found that patients infected with a bacterial or fungal pathogen that is antibiotic resistant can expect an increase of $1,383 to their treatment cost. This amounts to an additional nationwide cost of $2.2 billion each year on top of already existing expenditures (2).

When presented with information regarding antibiotic resistant pathogens, it is logical to assume that an average individual would only consider the threat they present as infectious agents in the human population. However, it is essential that when examining and addressing this concern efforts are also made to include the danger present to a nation's agriculture as well. This particular danger is multifaceted: not only is it possible for contaminated products to reach nationwide public consumption, but crops and livestock are also susceptible to pathogens as well, including those that exhibit

resistance to current treatments. These infections could lead to poor yields or outright crop loss, which in turn would have consequences that reverberate throughout the economy. Additionally, the strain placed upon maintaining a sustainable food supply should not be overlooked; there are numerous historical examples that demonstrate how devastating a rampant infection in agriculture could have on a nation, such as the Great Famine in Ireland during the 1840's (3), or more recent threats such as Race Ug99 (4). It is vital to remember that antibiotic resistant pathogens will not discriminate when it comes to infection.

Without new drugs to treat illnesses caused by these pathogens, we will not only face increasing treatment costs, but ultimately we will reach a point where our current stable of last resort drugs is driven to the point of ineffectiveness. Compounding this problem is the slow rate of discovery of new antibiotics: there is only so much a chemist in a lab can do when it comes to synthesizing new solutions. In the modern age of medicine we live in, it can be easy to forget that nature has been in the business of antimicrobial agents for a lot longer than we have (5) . In the search for novel antimicrobials, investigating for new medicinal candidates is not just limited to naturally occurring sources such as plants as one might expect but from microbes such as bacteria themselves, which are actually the source of most clinically available antibiotics.

Understandably, when most people think of natural selection and the idea of "survival of the fittest," there is a tendency to apply these concepts solely to creatures which have easily observable competitive traits. These traits, such as size, speed and aggression, are obvious when looking for the means creatures use to outcompete rivals

for resources. However, superior fitness extends from the largest creatures down to the smallest, such as microorganisms. There are numerous ways that bacteria in particular compete with neighboring microbes for nutrients and growth territory, such as having a higher numbers when compared to their competitors. Among these means there are numerous bacteria that produce secondary metabolites that halt the growth or kill outright the rival microorganisms in their local ecosystem. One such bacterium belongs to the genus *Burkholderia*.

Burkholderia is a Gram-negative, non-sporulating genus which, while ubiquitous and capable of growing within plants and groundwater, it is primarily a soil-dwelling bacterium. It derives its name from Walter H. Burkholder, who first encountered the organism in 1950 while investigating disease in young onion crops (6). At the time it was grouped with the genus *Pseudomonas*, but due to advances in genetic tools it was determined that it was more accurate to consider this a close relationship rather than a shared identity. This decision was made based on the 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics. As a result, in 1992 seven species were split from *Pseudomonas* Homology Group II and given its new designation of *Burkholderia* (7). As of this writing, this number now resides at 122 different species of *Burkholderia* that have been published or reported on (8). While this review will be focusing on the antimicrobial capabilities of the *Burkholderia* genus, some attention should be given to what are likely the three primary reasons why research into this topic is limited and approached with

trepidation: which are the potentially life-threatening illnesses caused by *Burkholderia mallei*, *Burkholderia pseudomallei*, and *Burkholderia cepacia*.

1.2 *Burkholderia mallei* and *Burkholderia pseudomallei*

While all three of the aforementioned species carry the genus name coined in 1992, knowledge of the organisms and their effects on humans, crops, and livestock are far from recent. This is especially true of *Burkholderia mallei*, which causes glanders. This infectious disease has been chronicled in ancient history, as the Greek physician Hippocrates first described its effects on equine species in 425 BCE (9). While primarily an infectious agent observed in livestock, human transmission is possible and its symptoms and severity is largely the same, regardless of the species unfortunate enough to contract the disease. In either case, the most common effects are observed in the upper respiratory tract, where chronic discharge, lesions and ulcers can appear. If untreated at this stage, *B. mallei* can increase its severity to an acute infection that causes septicemia. At this stage, glanders have a mortality rate up to 90% in as little as 7 to 10 days in untreated individuals (10). Due to the lack of recent cases in almost all well-developed countries, in addition to the initial symptoms being quite similar to pneumonia or the flu, it is conceivable that an infected individual would not seek proper treatment or may even be misdiagnosed, leading to an increased chance at the infection spreading to the bloodstream. Even in instances where adequate medical attention and the correct antibiotics are administered, the threat posed to those infected still remains alarmingly high, with the rate of mortality still reaching levels up to 50% in reported cases.

Furthermore, the devastation and disruption that *B. mallei* could cause in warfare has not gone unnoticed as well, with German troops using it as a weapon to disrupt horse and mule-driven supply lines in World War I, and Japanese forces purposely infecting prisoners, civilians and livestock in the Pacific Theater of World War II (11).

Despite the severity of infection and the possibility of transmission to humans, actual infections in the human populace are extremely rare, with transmission rates also being comparably low. Furthermore, human-to-human transmission has never been reported in the United States (12, 13). Both of these facts are likely due to an atypical characteristic of *B. mallei*: it is an obligate mammalian pathogen. In essence this means that outside of laboratory conditions it cannot be found in the soil, water, or plants one would expect in other Burkholderia species, and instead it is only found in infected hosts. However, because in the rare event of a human infection manifesting in dangerous symptoms and a high mortality in untreated cases, as well as its capability of being transmitted through inhalation, *Burkholderia mallei* has been assigned a classification as a biosafety level 3 agent, with 4 being the highest level an organism can be assigned. In addition to this, and its history of having been used in warfare, the Centers for Disease Control and Prevention considers *B. mallei* a candidate for being developed into a bioterrorism weapon and have thus classified it as a Category B disease-causing organism. This is the second highest classification the CDC has (14); for comparison, threats in this category includes cholera and ricin.

Contrary to *B. mallei*, *Burkholderia pseudomallei* is found in its typical environment of soil, thriving in subtropical and tropical climates of Southeast Asia and

Northern Australia (15). This particular species of *Burkholderia* is most well-known for causing melioidosis (also sometimes referred to as Whitmore's disease). Melioidosis infections most frequently occur when an individual or livestock comes into contact with soil or standing water contaminated with *B. pseudomallei,* often through cuts or abrasions on the skin, however infections in the lungs can occur through the inhalation if these sources are aerosolized (13). The largest danger with regards to a melioidosis infection is two-fold: it is capable of multiple infection sites (localized, pulmonary, and septic) and, similar to an infection with *B. mallei,* can be difficult to initially diagnose. Further complications arise due to the initial symptoms appearing flu like, with common pneumonia or possibly a tuberculosis infection being the most typical diseases melioidosis is mistaken for. Completely untreated, melioidosis has a mortality rate as high as 90%; however a basic course of antibiotic treatment drastically lowers this number. Even though *B. pseudomallei* is naturally resistant to antibiotics due to the presence of efflux pumps, the treatments that do exist for individuals who contract melioidosis cause the mortality rate to drop to less than 40%, and this number falls even further to less than 20% for those that seek more intensive treatment options. This, along with the fact that human-to-human transmission of melioidosis is extremely rare could lend credence to an argument that the threat of *B. pseudomallei* is not very high, however this is not the case (16). Both *Burkholderia pseudomallei* and glanders share the common traits of possible infection through inhalation, nor having a vaccine to prevent initial infection (17). This, as well as the fact that closely related *B. mallei* has indeed been used on the field of battle, was likely taken into consideration when

classifying its possible danger. As such, *B. pseudomallei* shares the same level of classification: it is a biosafety level 3 organism that is also considered a Category B threat by the CDC.

While *Burkholderia mallei* and *pseudomallei* are the only members of the genus that are direct causative agents of disease in non-immunocompromised individuals, the severity of their safety classifications has led to most of the remaining members of the genus being classified as biosafety level 2 organisms. This classification could be considered inaccurate for many species of *Burkholderia*, as numerous examples exist that are completely avirulent, such as *Burkholderia thailandensis.* This species is closely related to *Burkholderia pseudomallei*, but requires a 1000-fold increase in dosage to reach lethal levels (18). Still, there exists one remaining group of *Burkholderia* that poses a threat as human-infectious agents, and that is *Burkholderia cepacia* and the species closely related to it.

1.2.1 The Burkholderia cepacia *complex (BCC)*

Despite the two previously described species having a much longer history in regards to disease and warfare, the most infamous member of the *Burkholderia* genus is likely *Burkholderia cepacia*, due to its occurrence and complications in modern medicine. Although not particularly virulent on its own *B. cepacia* has gained notoriety as a colonizer in the respiratory airways of immunocompromised patients, particularly those with cystic fibrosis, with the first reported case occurring in 1977 (19). Due to the excess, highly viscous fluid typical of the disease, the airways and lungs become an ideal

growth environment for bacteria. The infections quickly become chronic and are eventually cleared through antibiotic treatment; however each subsequent infection leaves behind increasing amounts of scar tissue. The accumulation off scar tissue is what eventually contributes to respiratory failure and death in CF patients.

The *Burkholderia cepacia* complex itself was designated in 1997, when samples of the namesake bacterium species were isolated from cystic fibrosis patients and recognized as having five distinct genomic species (20). As of today, there are at least nine recognized genomovars containing at least 20 closely-related species (21). Of all the species included within the BCC, *Burkholderia cenocepacia* has risen as the most deadly of the species. Although there are some other members of the BCC that can cause severe infections the reported mortality rate is still lower than that of *B. cenocepacia* (22). Despite *Burkholderia cepacia* being the first species of the genus found in an infected cystic fibrosis lung, the species most prevalent in CF patients are *B. cenocepacia* and *B. multivorans*, with *B. cepacia* now being found most commonly among patients with a non-CF associated lung infection (23).

Given the serious nature of the disease and infections caused by the aforementioned *Burkholderia* species one can easily understand why it would not be a researcher's first choice when learning about its non-virulent properties. However, despite being as severe as a biosafety level 3 organism it is important to acknowledge that not all species of *Burkholderia* are threatening to the human population, with some species being completely avirulent. Not only this, but some members, such as *Burkholderia ambifaria* ,are already well known for their use in agriculture, as it has

antagonistic activity against several plant pathogens, fixes nitrogen, and can aid in bioremediation (24).

While the antimicrobial properties of the *Burkholderia* genus have been known for quite some time, including when it was still classified as *Pseudomonas*, the positive identification of the compounds uniquely produced by the various species is still relatively new and unexplored. An example of this is *Burkholderia ambifaria* 2.2N, a species that was undescribed until the year 2000 (25). While the antibiotic capabilities of the then-unknown bacterium were first documented then, it wasn't until 2010 that the unique antifungal compounds were characterized (26).

Although the process of identifying antimicrobial products is no easy task, there have been major strides in four different types produced by *Burkholderia*:

1.2.2 Xylocandins, cepacidines, occidiofungins and burkholdines

Xylocandins, cepacidines, occidiofungins and burkholdines are among a class of novel lipopeptide and glycolipopeptide antifungal antibiotics produced by different *Burkholderia* species. Many of these have subvariants that are produced in smaller quantities, distinguished by slight differences in their peptide components or smaller modifications to the standard peptides present in the majority product. Structurally they are very similar in many respects, and their powerful antifungal activity is not new (27, 28) (Figure 1.1). However, it was not until occidiofungin was discovered in 2009 that researchers began to explore in detail its structural properties as well as its mechanism of action for fungicidal activity. Although not elucidated in full, xylocandins and

cepacidines (along with the burkholdines which were first described in 2012) share such a high degree of similarity that it would not be implausible to hypothesize that they share not only a similar means of production when compared to occidiofungin, but also a similar, if not identical, method of killing its fungal target (29).

Although peptidal in nature, occidiofungin does not involve the use of ribosomes or messenger RNA in its genesis. Instead, it relies on a hybrid system of a polyketide synthetase (PKS) coupled with a non-ribosomal peptide synthetase (NRPS), both of which rely on multi-domain enzymes to modify or elongate the growing peptide in some fashion. This nonstandard means of production often means that their products can result in modified amino acids different from the standard 20 amino acids most often encountered and are typically referred to as novel amino acids (NAA). In the case of occidiofungin (of which there are two currently known variants, Occidiofungin A and Occidiofungin B), its production begins in with its nonribosomal peptide synthetase, however the hybrid system involves the polyketide synthetase's products during key points of its production (30).

The end result is a cyclized lipopeptide (or glycolipopeptide in the case of Occidiofungin B) with bactericidal activity against a broad spectrum of plant and animal pathogens, in particular yeast of the *Candida* genus (31, 32). Occidiofungin induces apoptosis by targeting actin, a family of proteins responsible for the formation of microfilaments in eukaryotic cells.

1.2.3 Siderophores

Iron acquisition is vital in *Burkholderia* species, whether in nutrient poor soil or in the iron-limited environment of the lungs. Because of the highly conserved genome, up to four common siderophores can be observed being produced by the various species of the genus: ornibactin, pyochelin, cepabactin and cepaciachelin (33) (Figure 1.2). While the primary function of these compounds is the chelation and uptake of iron for use by the producing organism they have also been shown to have antimicrobial effects, as well as a play a role in the regulation of additional antibiotic capabilities. Likely due to the fact that their primary role is in iron-acquisition, the direct antimicrobial effect of *Burkholderian* siderophores is not explored in great detail. However, evidence has been shown that these play a role in growth inhibition of surrounding organisms, with bacteriostatic growth inhibition being attributed to iron-starvation of competing species. *Burkholderia paludis* MSh1T was shown to have antimicrobial activity when spotting dichloromethane extracts of culture supernatant but it is also a known producer of pyochelin, known primarily as a siderophore, which has bacteriostatic activity against four *S. aureus* strains and three *E. faecalis* strains. While not identified, the siderophore produced by *Burkholderia cepacia* XXVI was shown to inhibit the growth of *Colletotrichum gloeosporioides,* the fungal plant pathogen responsible for bitter rot in many tropical perennials (34, 35). Finally ornibactin, the most commonly produced siderophore amongst the different Burkholderia species, has shown to have bacteriostatic activity against the Gram-negative plant pathogen *Erwinia amylovora*. More interestingly, there appears to be a secondary function to ornibactin, as its production has

been directly linked to bactericidal activity against the pathogenic species (36). Siderophore production and iron chelation has previously been shown to have a relation to the virulence of more pathogenic species of *Burkholderia*. This research suggests that attenuation of ornibactin production could open a new avenue to novel therapeutic approaches to these particular species.

Of all the members of the *Burkholderia* genus, members of the *Burkholderia cepacia* complex contain the most published data examining their antimicrobial properties and products. As mentioned previously, these properties are not a new discovery. As a soil bacterium, *Burkholderia* are able to colonize the rhizosphere, providing growth promotion in plants and commercial crops (37). In addition to fixing nitrogen, use of BCC members and their antimicrobial products have possible applications in biocontrol and bioremediation, but need further research. An example being *B. cepacia* SCAUK0330, which showed strong bioactivity against numerous plant pathogens, but was also found to assist in solubilizing phosphate (38).

1.3 *Burkholderia cenocepacia* products

Despite being one of the major colonizers of cystic fibrosis patients, *Burkholderia cenocepacia* has also been shown to produce compounds of interest. Among the strains tested, *Burkholderia cenocepacia* TAtl-371 has been examined the furthest. Siderophore production is evident, and antimicrobial activity of this particular strain has shown it to be active against Gram-negative species, including against other *Burkholderia* species. In addition, *B. cenocepacia* TAtl-371 also has strong activity against yeast and fungal species. Although success was had in isolating this activity, only one compound was successfully purified ("Compound one"), and it only had activity against one tested strain of *Tatumella terrea* (39).

Another promising product comes from *B. cenocepacia* strain BC0425. This strain produces a tailocin, which is a phage tail-like bacteriocin. This tailocin was designated BceTMilo and proved to be quite effective against other members of the *Burkholderia cepacia* complex as well as 90% of the non-BCC *Burkholderia* species tested, opening up a possible avenue for new therapeutics against more pathogenic species that infect humans or crops (40).

Finally, although not fully explored, *Burkholderia cenocepacia* P525 has also been studied for its antimicrobial capabilities, where a bacteriostatic oxidizer was found that was effective against species of *Enterobacter*, however it was not fully characterized.

1.4 *Burkholderia cepacia* products

The pathogenic *Burkholderia cepacia* also produces antimicrobial products. For example *B. cepacia* NB-1 has a broad spectrum activity. However, the organisms it is active against vary, with different pathogens appearing as sensitive in agar diffusion tests vs microtiter plates. Several strains have been recorded as producing the siderophore pyrrolnitrin including *B. cepacia* NB-1 (41). Some strains have also been shown to produce analogs of pyrrolnitrin as well. *B. cepacia* J82rif and J51rif, still identified by the antiquated Pseudomonas designation at the time of its publication, was reported to produce aminopyrrolnitrin and monochloroaminopyrrolnitrin (42). In addition to standard pyrrolnitrin, *B. cepacia* K87 also produces two pyrrolnitrin analogs: 3-chloro-4-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one (2) and 4-chloro-3-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one, however it was found that the antimicrobial activity of K87 is unstable when exposed to sunlight, and was readily metabolized in vivo (43). Finally, *Burkholderia cepacia* XXVI has also been reported to produce a siderophore with activity against the fungal pathogen *Colletotrichum gloeosporioides*; however the specific siderophore found in the study was not identified.

The antifungal produced by *B. cepacia* CF-66 is the most characterized antimicrobial produced by the different strains of this particular species. The compound, designated as CF66I, was first reported in 2005 with its activity against *Rhizoctonia solani* (44, 45). Further studies expanding on this knowledge, as it exhibited activity against a wide range of plant pathogens, such as *Fusarium* and C*olletotrichum*. In addition to this, CF66I displayed remarkable stability in varying pH levels and

temperature, even maintaining a measure of bioactivity when briefly exposed to temperatures as high as 160°C (46). It was later shown to be active against *Candida albicans* as well; however a definitive mechanism of action was not determined. It was hypothesized that its activity was due to its effect on the assembly and integration of cell wall components, possibly by interrupting the interactions of hydrogen and hydrophobic bonds. When exploring its effects on *Fusarium*, at fungicidal concentrations CF66I was found to act on the cell membrane, which resulted in the cytoplasm leaking and ultimately leading to cell death. While it's activity was notable against fungi and yeasts, it has no antibacterial activity (47).

Alkyl-quinolones have also been reported as products of *Burkholderia*, with the entirety of genomovar I of *B. cepacia* producing two analogous 2-alkylquinolones with activity against *Aspergillus niger*. These two compounds were given the designation of Bc-255 and Bc-257, with Bc-255 having twice as much activity as Bc-257 (48) (Figure 1.4). While this is a broadly produced antifungal, more detail was provided when *B. cepacia* Cs5's antifungal activity was examined. Originally found in the rhizosphere of almond trees, three antifungals isolated from this strain were named Cs5-255, Cs5-257 and Cs5-446, with Cs5-255 and Cs5-257 being found to be analogous (Figure 1.4). These antifungals were found to have activity against *Aspergillus niger*. Additional activity was found against *Alternaria alternata*, *Fusarium culmorum*, *F. graminearum*, *F. oxysporum* and *Rhizoctonia solani* however this was found while testing activity with supernatant extracts of Cs5, so the product directly responsible for this activity is yet to be named (49).

The remaining products from *B. cepacia* have not been explored further since the 1990's. Although some were published while still designated under the genus *Pseudomonas*, they have been changed to *Burkholderia* to reflect their correct nomenclature here. In 1994, *B. cepacia* AF 2001 was found to produce two closely related compounds, cepacidine A1 and cepacidine A2. Once combined, the singular mixture was called cepacidine A, which has strong antifungal activity against several plant and human pathogens, which was diminished in the presence of serum. Furthermore, this particular compound exhibited no antibacterial activity (28, 50). The similarly named cepalycin I and II was isolated from *B. cepacia* JN106. Of the two, cepalycin I was the most active, having stronger antibiotic activity against *S. cerevisiae* and *C. neoformans* with a smaller measure of activity against *C. albicans*. Cepalycin II had reduced activity against *S. cerevisiae* and *C. neoformans*, with no activity against *Candida* (51). Finally, an antifungal designated as AFC-BC11 was produced by *Burkholderia pyrrocinia* BC11 (formerly *B. cepacia* BC11), and was hypothesized to be a lipopeptide. This antifungal was active against *R. solani, Pythium ultimum, Colletotrichum sp., Helmthosporium maydis, Botrytis cinerea, Fusarium sp., Rhizopus stolonifer, Rhodotorula glutinis, Sclerotium rolfsii, Scopulariopsis brevicaulis*. Interestingly, although previously described cepadines are active against *Candida*, *Saccharomyces*, and *Aspergillus*, this particular antibiotic was not (52).

1.5 *Burkholderia ambifaria* products

Although not many antimicrobials have been reported from *Burkholderia ambifaria* strains, burkholdines, which are structurally similar to occidiofungins, xylocandins, and cepacidines, have been isolated from one strain. From *Burkholderia ambifaria* 2.2N, Bk-1119, Bk-1213, Bk-1215, as well as Bk-1097 and Bk-1229 were all isolated (29). Similar to other antifungals produced by other *Burkholderia* species, these compounds exhibited strong antibiotic activity against *S. cerevisiae*, *A. niger*, and *C. albicans* (26). Among the more unique products of *B. ambifaria* is enacyloxin IIa and its stereoisomer iso-enacyloxin IIa, produced by strain AMMD. While this particular antibiotic is effective against multidrug resistant pathogens such as *Acinetobacter baumannii*, importantly it also has activity against its own genus, with examples including *B. multivorans* and *B. dolosa.*

1.6 *Burkholderia pseudomallei* group products

Burkholderia thailandensis was mentioned previously as being closely related to, yet lacking the virulence of *B. pseudomallei*. Although it is not the only member of the *B. pseudomallei* group that produces antimicrobials, it is likely the most studied. Currently it is known to produce bactobolin, a member of the polyketide-peptide family consisting of a C6-polyketide fused to a chlorinated hydroxy-valine residue. This particular compound was first found as a secondary metabolome in *B. thailandensis* strain E264, as it was noted a few years prior that production of this activity was under quorum-sensing control (53, 54). This is not the only secondary metabolite of interest as

well, as *B. thailandensis* has been shown to also produce both bacteriocins and bacteriophages that were active against more virulent species of *Burkholderia* (55).

1.7 *Burkholderia xenovorans* group products

While the positive effects of *Burkholderia* has in agricultural applications is not new, often the source of the benefits remain unexplored. Within the *Burkholderia xenovorans* group there are four species which warrant further study: *Burkholderia graminis*, *Burkholderia bryophila*, *Burkholderia megapolitana*, and *Burkholderia phytofirmans PsJ* have all been reported as having plant growth promotion, antifungal activity, or both yet have not been studied further (56-59).

1.8 Other *Burkholderia* products

Several different strains of *Burkholderia gladioli* have been shown to possess promising antimicrobial activity. In particular *Burkholderia gladioli* BCC0238, a strain which was originally isolated from a cystic fibrosis patient, produces gladiolin (Figure 1.5). This novel macrolide has been shown to inhibit RNA polymerase effectively in several *Mycobacterium tuberculosis* clinical isolates, while maintaining a low mammalian cytotoxicity (60). This is not the only antibiotic produced by BCC0238, as it has been shown to produce at an two-tailed lipopeptidiolide antibiotic known as icosalide A1 (Figure 1.3). Remarkably, this particular compound was originally isolated from a fungal culture before this bacterial origin was uncovered (61, 62). Among other *B. gladioli* strains there exist several other promising, albeit unexplored candidates. The

most promising clinical products come from *B. gladioli* OR1, which produces at least five different products with bioactivity against *Staphylococcus* and *Candida*.

Burkholderia gladioli pv. Cocovenenans has been shown to produce antifungal and antibacterial polyketides, but this activity was only when co-cultured with the fungus *Rhizopus microspores* (63). Finally, there appears to be agricultural applications for this particular species as well, as *B. gladioli* pv. Agaricicola and *B. gladioli* NGJ1 exhibit broad-spectrum antifungal activity against the agricultural pathogens *Rhizoctonia solani*, *Fusarium oxysporum*, *Magnaporthe oryzae*, and *Venturia inaequalis* (64, 65). As of this writing these products have not been characterized.

Burkholderia glumae also has similar possible applications in agriculture, as numerous strains exhibited activity against *Rhizoctonia solani*. Strain 411gr-6 produced a pigmented compound that also had strong antifungal activity against *Colletotrichum orbiculare*, however, none of the bioactive compounds in this particular study were further characterized, with a small exception (66). When investigating the antimicrobial pigmented compound of *B. glumae* 411gr-6 in a later study, two derivatives of the antibacterial phencomycin were uncovered as well (4-hydroxyphencomycin and 5,10-dihydro-4,9-dihydroxyphencomycin methyl ester) (Figure 1.5) (67). Among other notable antibacterials produced by *B. glumae* strains designated as #3729 and #8657 from the International Collection of Microorganisms from Plants were found to produce two products. Although one of these products was inactive once purified the second, an oxygenated pyrazole, displayed strong inhibitory activity against *Erwinia amylovora*, the causative agent of fire blight disease. Structure elucidation of this second compound

determined it to be 3-[L-alanyl-L-homoserinyl-L-aspartyl-beta-carboxy]-4-hydroxy-5-oxopyrazole, which had activity against *Erwinia* as well as *Psuedomonas* and *Xanthmonas* species.

1.9 Conclusion

The purpose of this chapter is to provide an overview of the antimicrobial products that are currently known to be produced by the *Burkholderia* genus. While this Gram-negative soil bacterium does have a small number of pathogenic species, the majority of the genus is avirulent, with almost all member species capable of producing useful antimicrobial products that could potentially be a new means of therapeutics. This chapter also highlights the need for new treatment options, as the increasing prevalence of antibiotic-resistant pathogens, coupled with the declining rate of discovery for new antibiotic treatment options, could lead to a global crisis is not addressed.

Table 1.1: Antimicrobials produced by *Burkholderia* species

Strains	Antibiotic Isolated
B. ambifaria 2.2N	Burkholdines (Bk 1119, 1213 & 1215)†
B. contaminans MS14	Occidiofungin†‡
B. ambifaria 2.2N	Burkholdines (1097 & 1229)†
B. paludis MSh1T	Unknown
B. paludis MSh1T	Pyochelin
B. cepacia XXVI	Unknown siderophore‡
B. cepacia SCAUK0330	Unknown‡
B. cenocepacia TAtl-371	Compound 1‡
B. cenocepacia TAtl-371	Likely numerous unknowns‡
B. cenocepacia TAtl-371	Lectin-like bacteriocin LlpA88‡
B. cenocepacia strain BC0425	Talocin (BceTMilo)
B. cepacia NB-1	Pyrrolnitrin
B. cepacia NB-1	Pyrrolnitrin
B. cepacia J82rif and JSlrif	Pyrrolnitrin, aminopyrrolnitrin, and monochloroaminopyrrolnitrin‡
B. cepacia K87	Pyrrolnitrin‡
B. cepacia germnovar I	Bc-255 and Bc-257 (2-alkylquinolones)
B. cepacia Cs5	Cs5-255 and Cs5-257 (alkylquinolones)
B. cepacia Cs5	Cs5-446 (didecyl-phthalate)
B. cepacia Cs5	Unknown
B. pyrrocinia BC11	AFC-BC11
B. bryophila	Unknown
B. megapolitana	Unknown
B. phytofirmans PsJN	Unknown‡
B. gladioli BCC0238	Gladiolin
B. gladioli pv. *cocovenenans* HKI 10521 (DSM 11318; ATCC 33664)	Bongkrekic acid and Toxoflavin
B. gladioli pv. *agaricicola*	Unknown
B. gladioli NGJ1	Unknown‡
B. cepacia ATCC 39277	Xylocandin (A1 & A2)
B. cepacia AF 2001	Cepacidine (A1 & A2)
B. cepacia CF-66	CF66I‡
B. thailandensis E264	Bactobolin
B. gladioli Lv-StA (HKI0739)	Icosalide A1
B. glumae 411gr-6	Phencomycin
B. cepacia ATCC 39277	Catacandin
B. cepacia JN106	Cepalysin
†Indicates possible medical applications	‡Indicates possible agricultural applications
*See Figures 1.1-1.5 for structure	

Table 1.1 Continued

Known Spectrum of Activity	Covalent Structure Available*	Molecular Weight Data Available	Sources
S. cerevisiae, C. albicans, A. niger	Yes	1119, 1213, 1215 Da	16
C. albicans LL, *C. albicans* TE, *C. albicans* 66027, *C. glabrata* TE, *C. glabrata* 66032, *C. parapsilosis* 90018, *C. tropicalis* 66029, *G. candidum* , *S. cerevisiae* BY4741**	Yes	1200, 1216 Da	17
S. cerevisiae, C. albicans, A. niger	Yes	1097, 1229 Da	21
E. faecalis ATCC 29212, *E. faecalis* ATCC 700802, *S. aureus* ATCC 29213, *S. aureus* ATCC 700699, *E. coli ATCC* 25922	No	N/A	23
E. faecalis ATCC 29212, *E. faecalis* ATCC 700802, *E. faecalis* JH-22, *S. aureus* ATCC 29213, *S. aureus* ATCC 700699, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P	Yes	325 Da	23
Colletotrichum gloeosporioides	No	N/A	24
Exserohilum turcicum SCAU3564, *Helminthosporium maydis* SCAU3321, *Mycogone perniciosa* Magn SCAU3216, *Rhizoctonia solani* Ktihn SCAU3111, *Alternaria alternata* (Fries) Keissler SCAU3471, *Colletotrichum gloeosporioides* SCAU3725, *Selerotium rolfsii* Sacc SCAU3025, *Fusarium graminearum* Sehw. SCAU3741, *Fusarium oxysporum* SCAU3221	No	N/A	27
Tatumella terrea SHS2008	No	391 Da	28
Acinetobacter baumannii ATCC BAA-747, *P. aeruginosa* PAO1, *S. aureus* 55C1	No	N/A	28
Only Bcc strains	No	N/A	28
Bcc strains, *P. aeruginosa*	No	N/A	29
Streptomyces antibioticus, S. violaceoruber, Paecilomyces variotii, Penicillium puberulum	Yes	257 Da	30
Ustilago maydis, C. albicans, Hansenula anomala, Arthrobacter oxidans, Bacillus coagulans, B. lichenifernis, B. subtilis, B. thuringiesis, B. megaterium, B. polymyxa, B. pumilus, Corynebacterium glutamicum, Micrococcus luteus, S. aureus, S.	Yes	257 Da	30
Sclerotinia sclerotiorum	Yes	257, 227, 223 Da	31
Rhizoctonia solani	Yes	N/A	32
A. niger	Yes	255. 257 Da	37
A. niger	Yes	255, 257 Da	38
A. niger	Yes	446 Da	38
F. oxysporum, F. culmorum, F. graminearum, R. solani, A. niger, A. alternata	No	N/A	38
R. solani, Pythium ultimum, Colletotrichum sp., *Helmthosporium maydis, Botrytis cinerea, Fusarium* sp., *Rhizopus stolonifer, Rhodotorula glutinis, Sclerotium rolfsii, Scopulariopsis brevicaulis*	Yes	733 Da	41, 58
V. dahliae, R. solani, C. albicans, X. campestris, P. ultimum	No	N/A	46
Verticillium dahliae, R. solani, Erwinia carotovora, S. aureus, Xanthomonas campestris, P. ultimum	No	N/A	46
Botrytis cinerea	No	N/A	47
Mycobacterium tuberculosis, Enterococcus faecium DSM25390, *S. aureus* DSM21979, *C. albicans* SC 5314	Yes	779 Da	49
R. microsporus	Yes	486.6, 193.1 Da	52
B. cinerea, A. flavus, A. niger, P. digitatum, P. expansum, S. sclerotiorum, P. cactorum	No	N/A	53
R. solani, Magnaporthe oryzae, Ventura inaequalis, Fusarium ocysporum	No	N/A	54
C. albicans, C. tropicalis, C. krusei, C. parakrusei, C. pseudotropicalis, C. guilliermondii, C. stellatoidea, C. glabrata, Trichophyton mentagrophytes,	No	1215, 1199 Da	14a
C. albicans, C. glabrata, Cryptococcus neoformans, S. cerevisiae, A. niger, Microsporium gypseum, M. canis, Epidermophyton floccosum, Trichophyton mentagrophyte, T. rubrum, F. oxysporum, Rhizopus stolonifer	Yes	1215, 1199 Da	15a, 39
C. albicans, R. solani, F. oxysporium, F. sambucinum, Rosellinia necatrix, A. flavus, A. niger, Cochliobolus carbonum, Botrytis cinerea, Mucor heimolis, Penicillium chrysogenum, Rhizopus oryzae, Cryptococcus neoformens, Pichia membranae, S. cerevisiae	No	N/A	34, 35, 36
S. aureus, Streptococcus pyogenes	Yes	383.2 Da	42, 43, 58
Bacillus thuringensis, Paenibacillus larvae, S. pyogenes	Yes	712 Da	50, 51
Collectotrichum orbiculare	Yes	299.06, 329 Da	55, 56
C. albicans, C. tropicalis, C. krusei, C. parakrusei, C. pseudotropicalis, C. guilliermondii, C. stellatoidea, C. glabrata	Yes	344.4 Da	14a
S. cerevisiae, C. neoformans		240 Da	40

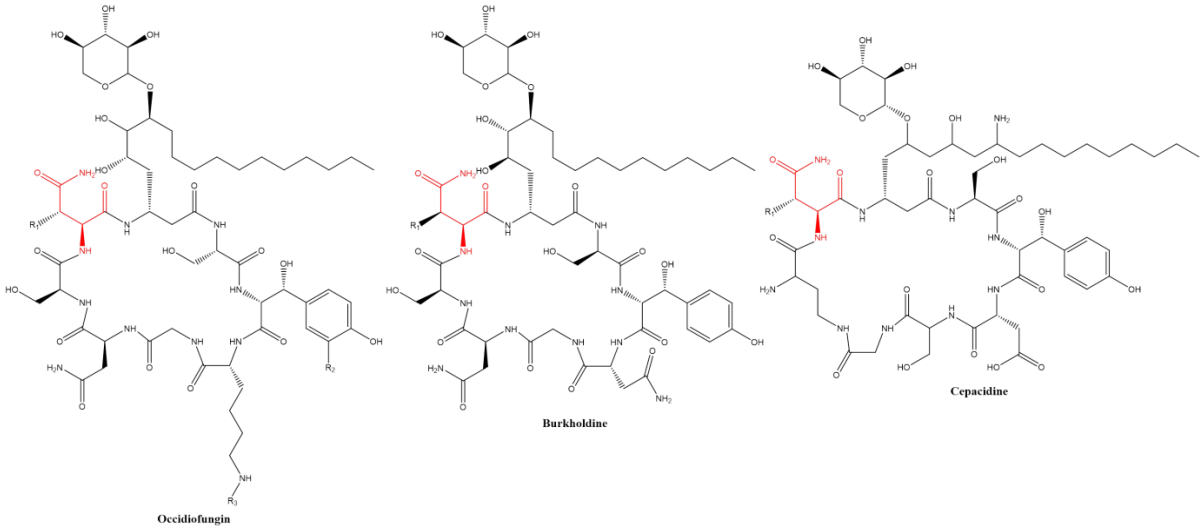

Figure 1.1: Structures of hybrid PKS-NRPS antifungals occidiofungin, burkholdine and cepecidine. The structures of these cyclic peptides are highly similar, and the serine located in their first position has been highlighted.

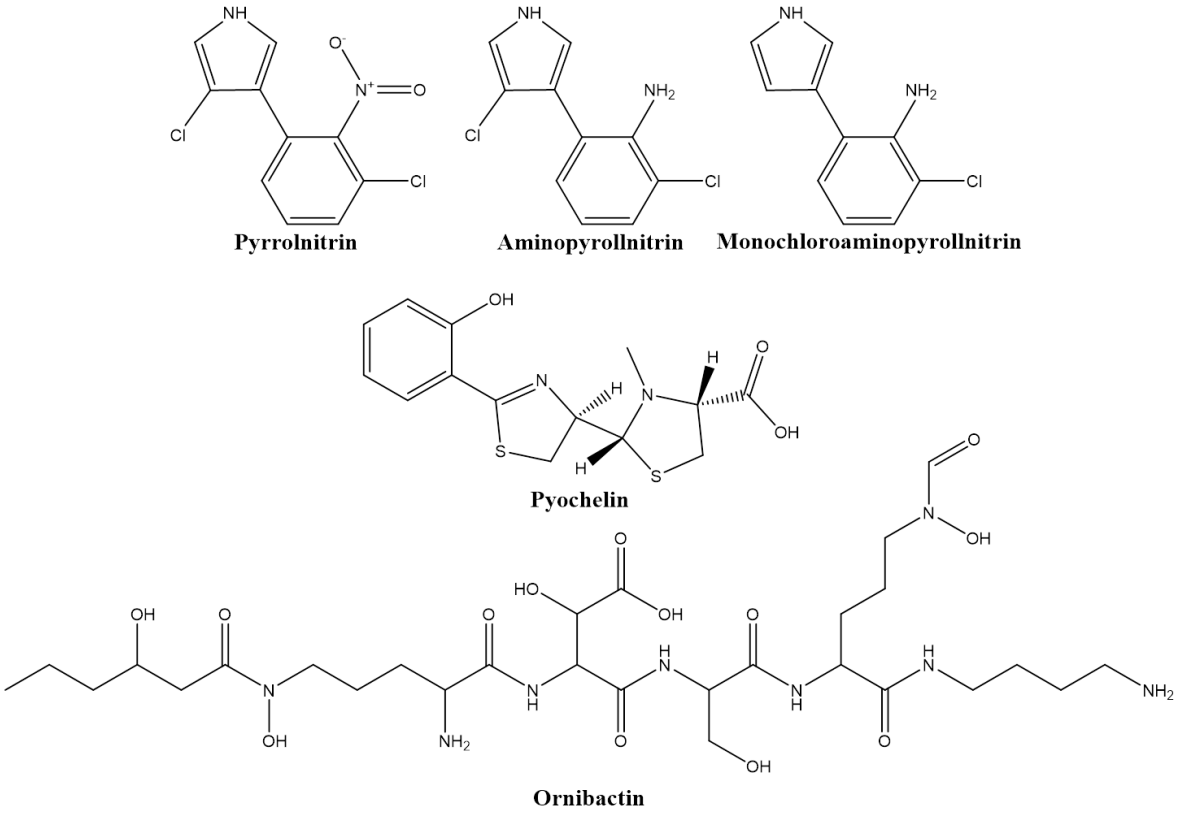

Figure 1.2: *Burkholderia* derived siderophores with reported antimicrobial activity

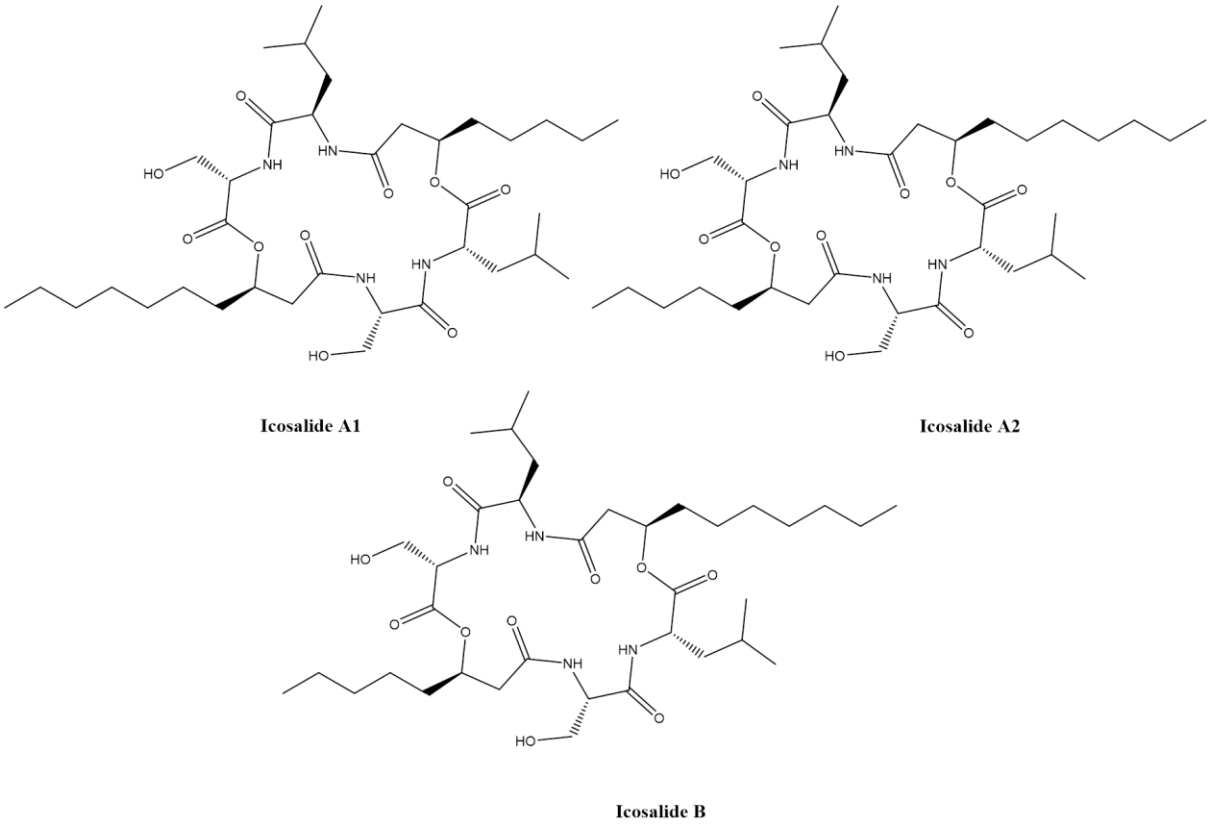

Figure 1.3: Icosalide variants produced by *B. gladioli* Lv-StA (HKI0739)

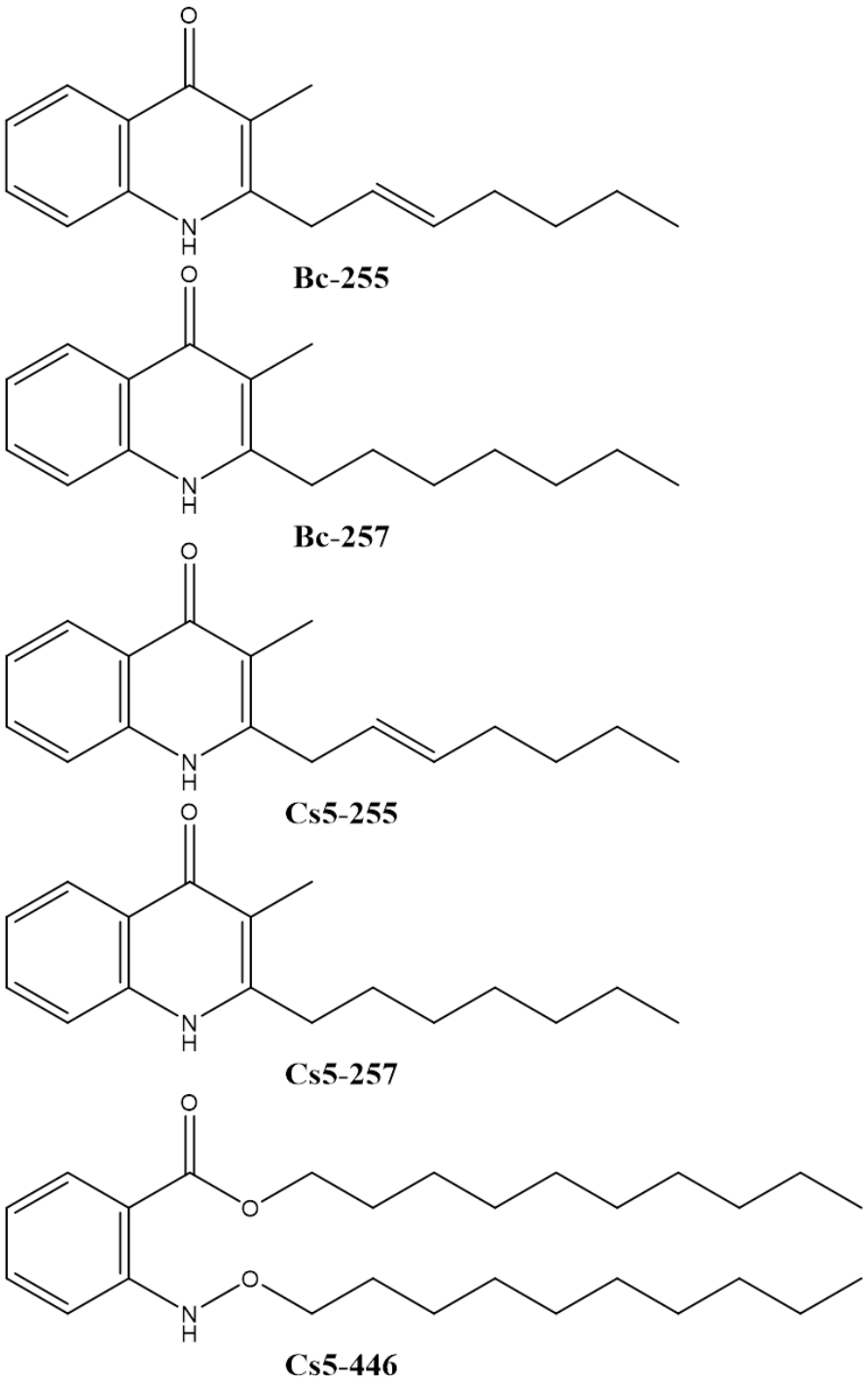

Figure 1.4: 2-alkylquinolone antimicrobials produced by *Burkholderia*. Although Bc-255 and Bc-257 have identical structures to Cs5-255 and Cs5-257, they are produced by different *Burkholderia* strains.

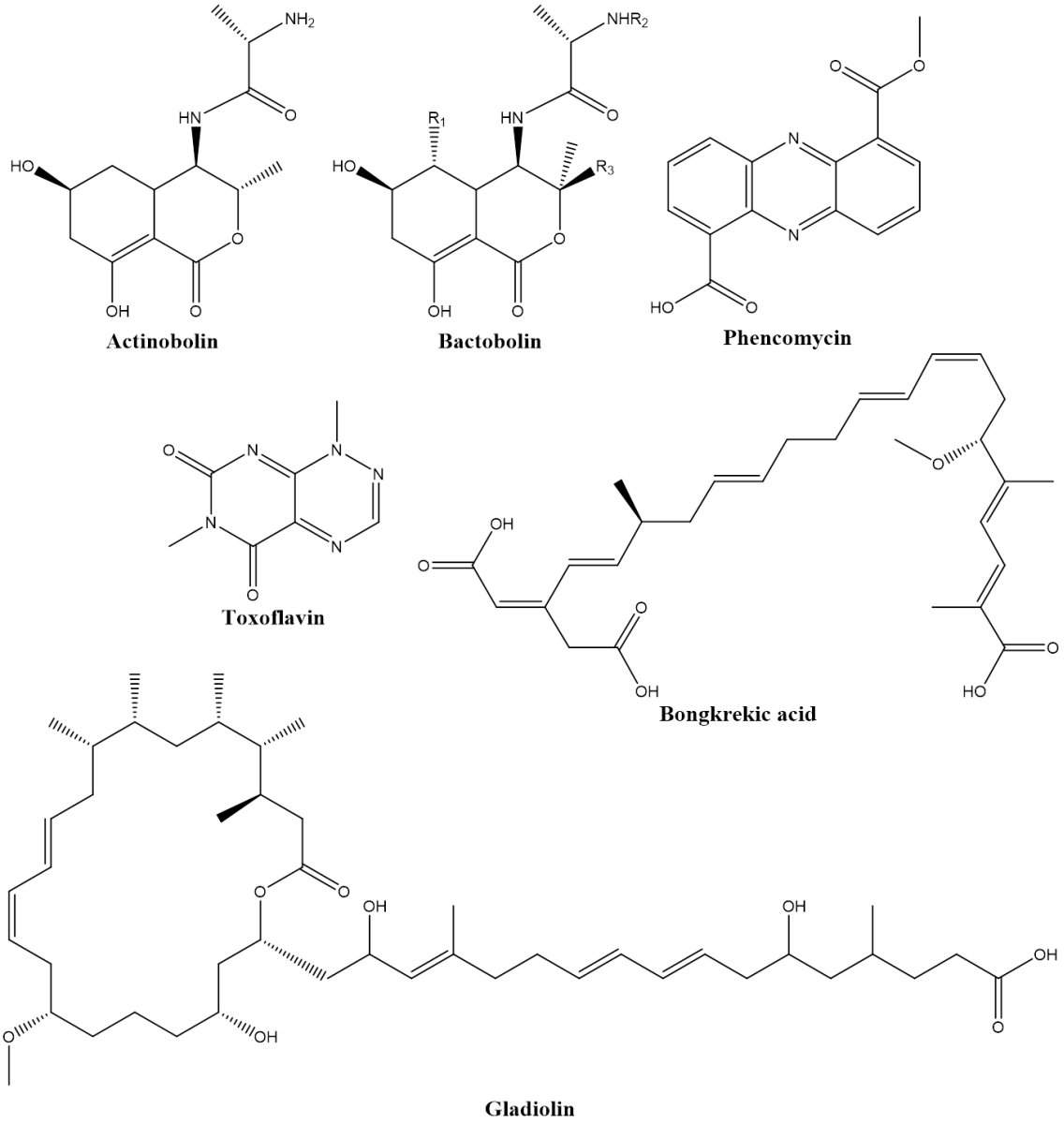

Figure 1.5: Additional antimicrobials produced by *Burkholderia* species

2. SIDEROPHORE PRODUCT ORNIBACTIN IS REQUIRED FOR THE BACTERICIDAL ACTIVITY OF *BURKHOLDERIA CONTAMINANS* MS14*

2.1 Overview

Burkholderia contaminans MS14 was isolated from soil in Mississippi. When cultivated on nutrient broth–yeast extract (NBY) agar medium, the colonies exhibit bactericidal activity against a wide range of plant pathogenic bacteria. Only a bacteriostatic compound with siderophore activity was successfully purified, which was determined by nuclear magnetic resonance (NMR) to be ornibactin. The isolation of the bactericidal compound has not yet been achieved, thus, the exact nature of the bactericidal compound is still unknown. In an attempt to isolate bactericidal compound, an interesting relationship between the production of ornibactin and the bactericidal activity of MS14 was characterized. Transposon mutagenesis resulted in two strains that lost its bactericidal activity; an insertional mutation in a nonribosomal peptide synthetase (NRPS) gene for ornibactin biosynthesis and a *luxR* family transcriptional regulatory gene. Co-cultivation of these two mutant strains resulted in the restoration of the bactericidal activity. Ornibactin is produced by most *Burkholderia* species and further consideration for its role in regulating secondary metabolites in other species should be investigated.

*Reprinted with permission from "The Siderophore Product Ornibactin Is Required for the Bactericidal Activity of *Burkholderia contaminans* MS14" by Foxfire A, Deng P, Xu J, Baird SM, Jia J, Delgado KH, Shin R, Smith L, Lu SE. 2017. Appl Environ Microbiol 83.

Identification of the antibacterial product from strain MS14 is not the key feature to this study, but rather the study demonstrates that ornibactin is involved in the bactericidal activity of MS14. Two mutants were isolated that had no bactericidal activity. Mutations were in a *luxR* regulatory gene and an ornibactin biosynthesis gene. Growing these mutants in proximity to each other restored bactericidal activity in the ornibactin mutant strain. Ornibactin should be further evaluated for its role in regulating secondary metabolite biosynthesis in other *Burkholderia* species.

2.2 Introduction

The genus *Burkholderia* is composed of Gram-negative, rod-shaped, motile, environmental versatile and non-spore-forming bacteria that have been identified in many diverse ecological niches (68). Currently, 88 species have been recognized in the genus *Burkholderia* (69). The bacteria have the ability to use a large array of carbon sources to synthesize secondary metabolites (41, 70). *Burkholderia cepacia* complex (Bcc) is a group of *Burkholderia* species that include soil isolates and opportunistic bacteria which cause lung disease in immunocompromised individuals (71). The Bcc group composed of 9 different genomovars and at least 18 different species (72). Conversely, some strains of *Burkholderia cepacia* are related to the promotion of plant growth and are considered to be plant growth-promoting bacteria (PGPB). For example, *B. cepacia* strains could protect crops from the damping-off diseases caused by *Pythium* species and *Rhizoctonia solani* (70). Interests in the use of *Burkholderia* species or their secondary metabolites in agriculture have increased. In addition, multiple antimicrobials

have also been identified being produced by *Burkholderia* species, for example occidiofungin (73), pyrrolnitrin (41), pyoluteorin (74) and AFC-BC11 (52).

Siderophores are small-molecule, ferric ion specific chelating agents secreted by bacteria and fungi growing under low iron stress. It scavenges iron from the environment and make it available to the microbial cell (75). Siderophores are bacteriostatic agents that can inhibit pathogenic microorganism's growth by depleting iron in the soil (76). Many siderophores are synthesized by NRPS and PKS pathways. NRPS and PKS are large multimodular enzymes, which are involved in natural product synthesis in many microorganisms (77). NRPS, involved in the biosynthesis of an oligopeptide, is grouped by active sites termed modules, in which each module is required for catalyzing one single cycle of product length elongation. The order and number of the modules of a NRPS protein are mainly followed by the "collinearity rule" (78).

Ornibactin is an NRPS product with siderophore activity. Ornibactin production in *Burkholderia cepacia* was shown to be critical for establishing an infection in a chronic respiratory infection murine model (79). Being able to sequester iron within the lung from iron-binding proteins, such as lactoferrin, is crucial for survival within the respiratory mucus (80, 81). There is a long understanding that the role of ornibactin in virulence is by providing a source of iron in iron restricted environments. Interestingly, the production of ornibactin appeared to also be important for bacterial adherence or colonization (79). In addition, the report shows that the absence of ornibactin production leads to a significant increase in the production of salicylic acid. This observation suggests that ornibactin production represses salicylic acid biosynthesis. The regulatory

basis for these observations still remains to be explored. However, the study does suggest that ornibactin may have another biological role other than sequestering iron.

In this study, we show that *B. contaminans* MS14 produces a bactericidal compound that has good activity toward Gram negative bacterial plant pathogens. The bacteriostatic compound ornibactin was isolated and confirmed by NMR. Two mutants that lack bactericidal activity were evaluated. One mutation was within a *luxR* regulator gene and the other mutation was within a nonribosomal peptide synthetase gene for ornibactin synthesis. It was shown that growing the ornibactin synthesis mutant in proximity to the luxR mutant restored MS14 bactericidal activity. These findings show that ornibactin is an important component for the production of the bactericidal secondary metabolite produced by *B. contaminans* MS14.

2.3 Materials and methods

2.3.1 Bacterial strains, plasmids and culture media

Bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* strain TransforMaxTM EC100DTM pir+ (Epicentre Biotechnologies, Madison, WI, USA) was used for plasmid rescue cloning and cultured in Luria-Bertani (LB) medium at 37^{o}C. Nutrient broth–yeast extract (NBY) agar medium (82) was used to culture *Burkholderia* strains and for plate bioassays of the antimicrobial activities evaluation. Potato dextrose agar (PDA, Difco, Detroit, MI) was used for plate bioassays to evaluate antifungal activities. Antibiotics (Sigma Chemical Co., St. Louis, MO), if applicable, were added to media as the following concentrations: trimethoprim (100 mg

ml^{-1}), kanamycin (100 mg ml^{-1} for *Escherichia coli*, 300 mg ml^{-1} for *Burkholderia* strains)

2.3.2 Bioassay for antimicrobial activities

B. contaminans MS14 and its mutants used in this study were evaluated for antibacterial activities against *Erwinia amylovora* 2029 and other pathogenic indicators using the NBY plate bioassays, similar to the protocol described by Scholz-Schroeder and colleagues (83). Briefly, MS14 and mutants were grown overnight in 5 ml of NBY liquid medium at 28^{o}C. Then the bacterial cells were collected by centrifugation and resuspended in sterile distilled water (SDW) to an optical density of 0.3 (approximately 2 $\times 10^{8}$ CFU ml^{-1}). 5-µl aliquots of bacterial suspension were inoculated onto the center of NBY plates. The plates were then incubated for 3 days at 28°C. Afterwards, the NBY plates were oversprayed with suspension of indicator pathogenic bacteria (OD420=0.3) and PDA plates were oversprayed with the indicator fungus (OD420=0.3), respectively. Inhibition zones were measured from the margins of bacterial colonies after 24 hours, with the size of the zone compared between *B. contaminans* MS14 and its mutants. To differentiate bactericidal activity from bacteriostatic activity, agar plugs (1 x 1 mm each) within the zones of inhibition to the indicator bacterium *Erwinia amylovora* 2029 were removed and grown on culture media. Bactericidal activity was indicated by no growth of the indictor bacterium. A minimum inhibitory assay of purified antimicrobial compounds was conducted as described previously (73).

2.3.3 Random mutagenesis

The EZ-Tn5 <R6Kcori/KAN-2>Tnp Transposome kit (Epicentre Biotechnologies, Madison, WI) was used to characterize the genes dedicated to antibacterial activity of *B. contaminans* MS14. *B. contaminans* MS14 are able to aquire kanamycin resistance on NBY plates through an EZ::TN transposon insertion into the genome, and mutants are able to be selected on the NBY plates supplemented with 300 μg ml^{-1} kanamycin. The mutants that exhibited reduced or no antibacterial activity against *Erwinia amylovora* were isolated. 16S rRNA and *recA* genes were cloned and sequenced, confirming that the resulting mutants were derivatives of strain *B. contaminans* MS14. Plasmid rescue cloning was performed according to the transposome kit instructions to generate plasmids pPD357 and pPD577 (Table 2.1). A portion of the Tn5 transposon sequence was amplified by PCR with the primers R6kF1 (5'- GGGTAGCCAGCAGCATCCT-3') and R6kR1 (5'- CATGATCGTGCTCCTGTCGTT-3') to confirm that the rescue plasmid contained the transposon sequence. The positive rescue clones were sequenced for further analysis. Sequence analysis was accomplished using the Lasergene Cloning suite version 12 (DNASTAR, Inc., Madison, WI). Genes were searched against the *B. contaminans* MS14 reference genome (84). BLASTn comparison of genomes was visualized by BRIG (85).

2.3.4 Analysis and isolation of the siderophore product

The wild-type strain MS14 was grown on modified NBY (487 mL distilled water, 2.5 g peptone, 1.5 g Todd Hewitt, 1.0 g Yeast Extract, 1.0 g K_2HPO_4 (anhydrous), 0.25 g KH_2PO_4, and 1.5% agar; 12.5 mL of 20% glucose and 0.5 mL of 1M $MgSO_4$ was added after autoclaving) agar plates overnight at 28°C. Colonies from the overnight NBY agar plate were stabbed into 500 mL of modified NBY soft agar (NBY with only 0.75 % agar). The inoculum in soft agar was placed at 28 °C for 4 days, and immediately frozen at -80°C. The media was then thawed in a 55°C water bath for 1 hour. The inoculum was then placed in 250 mL centrifuge bottles and centrifuged at 20,000 g for 30 minutes. The collected supernatant was pooled, mixed with 1 gram of poly-aromatic absorbance resin Diaion HP-20 and shaken for one hour. The resin was allowed to settle before decanting the supernatant and was resuspended in 10 mL of 50% acetonitrile:water. The extract was dried by lyophilization and resuspended in 1mL of 35% acetonitrile:water. The extracts were tested for siderophore and antibacterial activity by spotting 10 µL of the extract on a Chrome azurol S (CAS) plates or on an NBY plate overlaid with *Erwinia amylovora* 2029. RP-HPLC was done using a 4.6 × 250 mm C18 column (Grace-Vydac, catalog 201TP54) on a Bio-Rad BioLogic F10 Duo Flow with Quad Tec UV-Vis Detector system. Fractions were separated using a thirty minute gradient of 90:10 water:acetonitrile:0.1% trifluoroacetic acid to 20:80 water:acetonitrile:0.1% trifluoroacetic acid.

2.3.5 Structural determination by NMR of the siderophore product

A 2 mM sample of the purified bacteriostatic compound was prepared in (50:50) acetonitrile-d_3 (Cambridge Isotopes):H_2O. The NMR data were collected on a Bruker AVANCE III HD 600 MHz spectrometer and a Bruker AVANCE III HD 850 MHz spectrometer, using TCI cryoprobes on each spectrometer. The ^{1}H resonances were assigned according to standard methods (86) using COSY, TOCSY, NOESY and ^{13}C-HSQC experiments. NMR experiments were collected at 10°C. The carrier frequency was centered on the water resonance, which was suppressed minimally using standard presaturation methods. A 2.0 s relaxation delay was used between scans. The TOCSY experiment was acquired with a 60 ms mixing time using the Bruker DIPSI-2 spinlock sequence. The NOESY experiment was acquired with 400 ms mixing time. The parameters for collecting the HSQC spectrum were optimized to observe aliphatic and aromatic CH groups. The spectral sweep width for the TOCSY and NOESY was 11.35 ppm in both dimensions. The spectral sweep widths for HSQC were 11.35 ppm in the proton dimensions and 0 and 85 ppm for the carbon dimension. All 2D data were collected with 2048 complex points in the acquisition dimension and 256 complex points for the indirect dimensions, except for the HSQC which was collected with 2048 and 128 complex points in the direct and indirect dimension, respectively. Phase sensitive indirect detection for NOESY, TOCSY, and COSY experiments was achieved using the standard Bruker pulse sequences. ^{1}H chemical shifts were referenced to the residual water peak (3.33 ppm). Data were processed with nmrPipe (87) by first removing the residual water signal by deconvolution, multiplying the data in both dimensions by a

squared sinebell function with 45 or 60 degree shifts (for the ^{1}H dimension of HSQC), zerofilling once, Fourier transformation, and baseline correction. Data were analyzed with the interactive computer program NMRView (88).

2.3.6 Mass spectrometry of the siderophore product

The mass of the purified bacteriostatic product was confirmed on a Shimadzu matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) in both the linear and reflectron modes. The isolated compound was analyzed by electrospray mass spectrometry (ESI-MS) using a Micromass Q-TOF II mass spectrometer. The compound was dissolved in 50/50 acetonitrile/water (v/v) with 0.1% formic acid and injected into a 1 μL/min flow of the same solvent using a Harvard syringe pump. The flow was sprayed using the nano-LC interface. Tandem MS (MS/MS) was performed with singly charged ions using standard collision energy (34 V) and higher collision energy (50 V).

2.3.7 Plasmid construction for LuxR gene complementation

The intact *LuxR* gene was amplified using the primer pair LuxRF (5'-CTGAGGATCC ATTCAAACTAAACGAACGGGG-3') and LuxRR (5'-GACGAAGCTTTGGCTCAGCGC GTTTC-3'), in which the restriction endonuclease cutting sites BamHI and HindIII were added. The resulting PCR product containing the intact wild-type *LuxR* gene were digested using the respective enzymes, then cloned into the expression vector pMLS7 to generate plasmid pDP357-2, as previously described (89). Plasmid pDP357-2 was then electroporated into *B. contaminans* MT357 competent

cells to recover the wild-type characteristics. An empty vector was used as negative control. Single colonies were picked from NBY plates supplemented with trimethoprim (100 μg ml^{-1}) and kanamycin (300 μg ml^{-1}). The plasmid was successfully extracted from the colonies. Sequencing results confirmed the existence of the resultant plasmid pDP357-2. Plate bioassays were used to evaluate the antibacterial activity of the resulting cells.

2.4 Results

2.4.1 Antibacterial activity of MS14

Burkholderia contaminans MS14 has previously been shown to produce a potent antifungal named occidiofungin (90). In this study, zone of inhibition plate assays of strain MS14 grown on nutrient broth–yeast extract demonstrated significant bactericidal activity against a broad array of plant bacterial pathogens (Figure 2.1 and Table 2.2). Cultivation of agar plugs within the zones of inhibition from the indictor bacterium *Erwinia amylovora* did not yield in any viable colonies, supporting the classification of the MS14 antibacterial product as a bactericidal compound. *Xanthomonas citri* pv. *malvacearum,* one of a most destructive pathogen on cotton (91), is best inhibited by strain MS14 with a radius of 39 mm clear non-growing zone formed on the plate. *Pectobacterium carotovorum* WSCH1, which is the pathogen of bacterial soft rot on potato and other vegetables (92), *Ralstonia solanacearum,* which causes bacterial wilt of tomato and potato (93), were also significantly inhibited by strain MS14. The fire blight pathogen *Erwinia amylovora* of apple and pears (94) and bacterial panicle blight

pathogen *Burkholderia glumae* (95) were also highly sensitive to MS14 with a inhibition zone radius of 23 mm and 22 mm, respectively. Plates assays revealed that strain MS14 could significantly inhibit the gram-positive bacteria *Clavibacter michiganensis* subsp. *michiganensis,* the pathogen of a major tomato disease, tomato wilt and canker (96). However, another Gram-positive bacteria *Bacillus megaterium,* was not very sensitive to the growth of strain MS14 compared to other pathogenic bacteria tested. Overall the data indicate that the cell metabolites of strain MS14 have possible application as potent broad spectrum antibacterial agent against plant pathogens. Mutagenesis analysis of MS14 generated antibacterial-defective mutants MS14MT357 and MS14MT577 retained a similar antifungal pattern compared to the wild strain (Figure 2.6). The data indicate that the antibacterial mechanism is independent from the antifungal occidiofungin production.

2.4.2 Identification of genes involved in production of the antibacterial product

Mutants of strain MS14 created by EZ-Tn5 transposon insertion were obtained and were tested for antibacterial activity against our indicator strain of *Erwinia amylovora.* Two mutants that lost activity in the overlay assay were named MS14MT357 and MS14MT577. Plasmid rescue method obtained plasmids (pDP357 and pDP577) from the genomes of the mutants respectively. Plasmids details are shown in Table 1.1. BLAST analysis using the DNA sequence generated from the plasmids pDP357 rescued from the mutant MS14MT357 against the MS14 genome showed that the disrupted gene NL30_RS14390 is 671 bp in size *LuxR* family transcriptional regulator (Figure 2.7).

Sequence analysis of pDP577 revealed that the disrupted gene in mutant MS14MT577 is the gene locus NL30_RS14890, which is 9,662 bp in size (Figure 2.8) and encode a 3,219-aa peptide. The deduced peptide of NL30_RS14890 shares a 93% identity with the *orbI* gene in *B. cenocepacia* J2315, which is one of the two NRPS genes for siderophore ornibactin biosynthesis (97). Given the size of the gene product in the MS14MT577 mutant, complementation cannot be achieved. Furthermore, the possibility of any polar effects would be on downstream genes for the biosynthesis of an ornibactin related compound.

2.4.3 Complementation of the mutated LuxR type gene

The intact *LuxR* family transcriptional regulator gene was cloned into the *Burkholderia* expression vector pMLS7 using the primer pair LuxRF (5'-CTGAGGATCCATTCAAACTAAACGAACGGGG-3') and LuxRR (5'-GACGAAGCTTTGGCTCAGCGCGTTTC-3') with BamHI and HindIII enzyme digesting sequence added, respectively. The cloned *LuxR* genes were regulated by the S7 ribosomal protein promoter (98). The generated plasmid pDP357-2 was transformed into the mutant MS14MT357 to be expressed constitutively. Plate bioassays revealed that the antibacterial activities of these mutants against *Erwinia amylovora* had fully been restored to the wild-type level as compared with the strain MS14 (Figure 2.9). Considering EZ-Tn5 transposome was previously reported to have no polar effects leading to the inactivation of downstream genes (99) and *LuxR* gene complementation could fully restore MS14 antibacterial activities, we believe downstream genes were

unlikely to be effected by the insertional mutagenesis. The result showed that the *LuxR* family transcriptional regulator is essential for the observed bactericidal activity in strain MS14.

2.4.4 Isolation and characterization of products from MS14MT357 and MS14MT577

Wild-type strain MS14 and mutant strains MS14MT357 and MS14MT577 were cultured and extracted following an identical procedure. Extracts were run on a RP-HPLC column to determine differences within the isolated products. The wild-type MS14 and MS14MT357 had a comparable peak at the retention time of 18 minutes eluting in 64:36 water:acetonitrile (Figure 2.2). The mutant strain MS14MT577 did not produce a similar product at this retention time. In a minimum inhibitory assay, the fraction at 18 minutes was the only product that exhibited any inhibitory activity against *E. amylovora*, but this activity was clearly not bactericidal. The initial Diaion HP-20 extracts had bactericidal activity in a zone of inhibition plate assay, but the bactericidal activity was not recovered from any RP-HPLC fraction. The isolation of the bactericidal compound has not yet been achieved.

The bacteriostatic product was isolated from wild-type MS14 and the structure was characterized by COSY, TOCSY, NOESY, and HSQC NMR (Figure 2.10, Figure 2.11, Figure 2.12, and Figure 2.13) and mass spectrometry. NMR analysis revealed that the purified product contained TOCSY spin systems for a 3-hydroxyoctanoic acid (HOA), ornithine, aspartic acid (Asp), serine (Ser), and putrescine (Put) (Figure 2.3 and Table 2.3). Furthermore, nuclear overhauser effects (NOEs) were observed in the

NOESY experiment confirming the assigned position of each residue within the structure. NOEs were observed between the Orn(Nδ-OH)1λ to Asp(β-OH)2NH, Orn(Nδ-OH)1α to Asp(β-OH)2NH, Asp(β-OH)2α to Ser3NH, Ser3α to Orn(Nδ-OH)4NH, Orn(Nδ-OH)4δ to Formyl, Orn(Nδ-OH)4α to PutNH, and Ser3α to Put NH3+ (Figure 2.14 and Figure 2.15). The isolated product was structurally determined to be ornibactin-F with a mass of 737 Da. In addition, a chrome azurol S (CAS) plate assay was used to demonstrate that the isolated product had siderophore activity (Figure 2.16). The observed lack of the product in the MS14MT357 strain is to be expected given that the mutation is within the biosynthesis pathway for ornibactin.

2.4.5 Co-culture of MS14MT357 and MS14MT577 restores bactericidal activity

The isolated ornibactin-f product does not account for the bactericidal activity observed in the wild-type strain. This is also supported by the lack of bactericidal activity in the MS14MT357 strain that is capable of producing the same ornibactin product as wild-type MS14. Therefore, ornibactin is not directly responsible for the observed bactericidal activity. The relationship between ornibactin production and the LuxR family transcriptional regulator for the synthesis of the bactericidal compound was further evaluated using a plate overlay assay (Figure 2.4). The mutant strains MS14MT357 and MS14MT577 were spotted on a plate in the shape of a "V", in which the colonies at the bottom are comprised of a mixed culture. Siderophore bacteriostatic activity is observed around each colony of the MS14MT357 strain along the right side of the “V”, while the MS14MT577 did not inhibit the grown of the indicator strain along

the left side of the “V”. The bactericidal activity, which is observed by the clear zone of inhibition at the bottom of the “V”, is only present when the MS14MT357 and MS14MT577 mutants are grown in close proximity. The synthesis of both the LuxR family transcriptional regulator and ornibactin is required to produce the bactericidal compound. To further test this observation, the bactericidal and siderophore activity was tested with elevated concentrations of ferric iron. Increasing concentration of ferric iron has been shown to regulate the synthesis of ornibactin (100). If ornibactin is directly involved in the regulation of the bactericidal product, bactericidal activity should absent at the same ferric iron concentration that inhibits ornibactin biosynthesis. A concomitant loss of siderophore activity and bactericidal activity was observed in Diaion HP-20 extracts with increasing concentration of available ferric iron (Figure 2.17A&B). The loss of siderophore and bactericidal activity corresponded to the observed loss of the ornibactin product by RP-HPLC (Figure 2.17C). The data supports the observation that ornibactin is indirectly required for bactericidal activity of MS14. One possibility is that an ornibactin byproduct is responsible for the observed bactericidal activity. This would suggest that *luxR* gene NL30_RS14390 product is involved in the regulation of a product that modifies ornibactin. This scenario is unlikely, given that this product should presumably be isolated by the same extraction method used to isolate ornibactin. It is more likely that ornibactin has a regulatory role in MS14 and that it promotes the synthesis of a bactericidal secondary product. This is supported by the observation that bactericidal activity is only restored in the mutant deficient in ornibactin production and not in the *luxR* regulatory mutant strain.

2.4.6 Genetic architecture of ornibactin biosynthesis locus among Burkholderia *species*

Given the fact the relationship between the production of ornibactin and Bcc virulence remains unclear (101), the ornibactin biosynthesis locus is compared within *Burkholderia* species (Figure 2.5). *B. cenocepacia* is selected as the reference given the ornibactin biosynthesis locus is best described in strain J2315 (97, 102). Using this reference the ornibactin loci were identified from thirteen *Burkholderia* species, which includes the pathogenic species *B. multivorans* ATCC 17616, *B. mallei* ATCC 23344, *B. thailandensis* E264, *B. oklahomensis* EO147, *B. pseudomallei* 1026b and *B. pseudomallei* K96243 (103-106), the PGPB *B. lata* 383, *B. ambifaria* AMMD and *B. phytofirmans* PsJN (107-109), soil isolates *B. cepacia* GG4, *B. vietnamiensis* G4, *B. phymatum* STM815 and *B. xenovorans* LB400 (110-112). The comparison of ornibactin loci showed a high degree of conservation of the *orbl*, *orbJ*, *orbE* and *pvdA* genes, which are responsible for ornibactin biosynthesis and ornibactin export across cytoplasmic membrane. MS14 NRPS gene NL30_RS14885 and NL30_RS14890 share a 90% nucleotide identity with the *orbJ* and *orbI* genes in *B. cenocepacia* J2315 and deduced peptides share 93% and identity with those in J2315. Conversely, the genes from *orbS* to *orbB* and from *orbA* to *orbL,* which involved in ornibactin biosynthesis initiation, regulation, transportation and modification, show significant diversity within the studied *Burkholderia* genome. We also analyzed the plant pathogenic *B. glumae* BGR1 and *B. gladioli* BSR3 (113, 114), however the ornibactin biosynthesis locus is not identified from the two species.

2.5 Discussion

The findings from this study demonstrate that MS14 has antibacterial activities against a wide range of plant pathogenic bacteria that includes *Erwinia amylovora, Xanthomonas citri* pv. *malvacearum*, and *Clavibacter michiganensis* subsp. *michiganensis*. Random mutagenesis studies resulted in the identification of two mutants in MS14 that had a loss in antibacterial production. Both of these mutations occur within regions that are not directly involved with the biosynthesis of the bactericidal product. Bactericidal activity could be restored by growing the ornibactin NRPS mutant and the LuxR family transcriptional regulatory mutant in proximity, suggesting that ornibactin production is essential for the production of the antibacterial compound. We have also showed a significant amount of diversity within *Burkholderia* species for the regions within ornibactin biosynthesis that would influence regulation and transport. Given the genomic diversity of these regions, ornibactin presumably has evolved to have additional functional roles aside from iron sequestration and iron uptake.

The determined structure of the isolated siderophore from our analysis is the same as has been previously reported for ornibactin-F (115). The mass for this product is also similar to the reported mass for ornibactin-F, i.e. 737 Da (115). Ornibactin (100) is a tetrapeptide siderophore that was first reported being produced by *Pseudomonas* (116) and then by several *B. cenocepacia* strains (100, 117). The ornibactin gene cluster contains two core NRPS genes (97), each of which is comprised of an amino acid adenylation domain and a condensation domain. The two domains are core components of NRPS mechanism, that is involved in bioactive product biosynthesis in many

microorganisms (118). These domains are also conserved among the *Burkholderia* species that were compared (Figure 2.5).

A LuxR family transcriptional regulator and the synthesis of ornibactin are essential for the production of a bactericidal compound in *B. contaminans* MS14 under the tested culture conditions. A concentration of ≤15 μM ferric iron was enough to suppress ornibactin biosynthesis by members of the *Burkholderia cepacia* complex. Ornibactin production was shown to be suppressed with 100 μM ferric iron with a concomitant loss of bactericidal activity (Figure 2.17). Comparison of the ornibactin gene cluster with fourteen previously sequenced *Burkholderia* species, including plant-growth-promoting strains and mammalian pathogenic strain, indicated that the ornibactin biosynthesis gene cluster commonly exists among other *Burkholderia* species. The diversity within the regulatory and transport regions within the gene cluster for ornibactin biosynthesis supports additional regulatory roles for the compound within Burkholderia species. In the case of MS14, it does have a clear role for promoting the antibacterial activity of the strain. Given the requirement of ornibactin for the production of the bactericidal compound in MS14, ornibactin may also be crucial for the synthesis of other secondary metabolites or possible virulence factors within other bacterial systems. It is becoming clearer that ornibactin has a larger role than iron sequestration and uptake within some bacterial systems and that a better understanding of these alternative activities could possibly promote the isolation of novel secondary metabolites or promote a more complete picture of its role in bacterial virulence.

2.6 Conclusion

This study uncovered that ornibactin, primarily thought of as a siderophore, has a secondary function beyond iron uptake for a *B. contaminans* MS14 bacterium. Ornibactin plays a key role in the regulatory pathway of an antibacterial secondary metabolite, with its absence leading to a complete loss of production of this metabolite. This is an exciting discovery as it could potentially be applied to more virulent strains of *Burkholderia,* This could lead to a new means of therapeutics if it is explored further if its principals can be applied to eliminate virulence factors of these more dangerous strains.

Table 2.1: Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Sources
Escherichia coli		
Ec100D	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Str^R) nupG pir$^+$(DHFR)*	Epicentre Corporation
Burkholderia contaminans		
MS14	Wild-type strain	
MS14MT357	LuxR regulator gene::Tn5 derivative of MS14; Km^r	This study
MS14MT577	NRPS gene::Tn5 derivative of MS14; Km^r	This study
Plasmid		
pDP357	EZ-Tn5 carrying 1.2-kb genomic DNA of MS14MT357; Km^r	This study
pDP577	EZ-Tn5 carrying 0.9-kb genomic DNA of MS14MT577; Km^r	This study
pMLS7	Expression vector of Burkholderia; Tp^r	Lefebre & Valvano (98)
pDP357-2	pMLS7 carrying 828bp BamHI and HindIII fragment containing the intact LuxR gene; Tp^r	This study

Table 2.2: Antibacterial activities of *Burkholderia contaminans* MS14

Indicator Pathogenic Bacteria	Inhibition Zone Radius (mm)		
	MS14	MT357	MT577
Xanthomonas citri pv. *malvacearum* MSCT1	36±1.66	0	0
Pectobacterium carotovorum subsp. *carotovora* EC101	33±0.86	0	1±0.5
Ralstonia solanacearum 102	31±0.08	0	0
Pseudomonas syringae pv. *syringae* B301D	29±1.00	0	0
Erwinia amylovora 2029	23±0.85	0	0
Burkholderia glumae 291	22±0.08	4±1.55	5±1.30
Escherichia coli JM109	22±0.77	0	0
Clavibacter michiganensis subsp. *michiganensis* 1-07	17±0.07	0	0
Bacillus megaterium Km	2.5±0.04	0	0

Table 2.3: Chemical shift values for the siderophore product ornibactin

Residue	^{1}H	^{13}C	Residue	^{1}H	^{13}C
HOA-C-2	2.67/2.47	42.56	Ser^{3}-NH	8.05	
HOA-C-3	3.98	71.42	Ser^{3}-Cα	4.33	-
HOA-C-4	1.45/1.36	39.91	Ser^{3}-Cβ	3.82/3.75	64.13
HOA-C-5	1.25	34.58	$Orn^{4}(N^{\delta}$-OH)-NH^{3+}	8.14/8.04	
HOA-C-6	1.25	28.13	$Orn^{4}(N^{\delta}$-OH)-Cα	4.20/4.18	52.74
HOA-C-7	1.25	25.48	$Orn^{4}(N^{\delta}$-OH)-Cβ	1.68/1.60 1.73/1.66	25.54 22.87
HOA-C-8	0.85	16.65	$Orn^{4}(N^{\delta}$-OH)-Cγ	1.68/1.60 1.83/1.74	30.85
$Orn^{1}(N^{\delta}$-OH)-NH^{3+}	-	-	$Orn^{4}(N^{\delta}$-OH)-Cδ	3.48/3.17	53.14
$Orn^{1}(N^{\delta}$-OH)-Cα	4.08	56.09	Nδ-formyl		
$Orn^{1}(N^{\delta}$-OH)-Cβ	1.55	24.33	Put-NH	7.77	
$Orn^{1}(N^{\delta}$-OH)-Cγ	1.77	31.25	Put-C-1	3.14	41.79
$Orn^{1}(N^{\delta}$-OH)-Cδ	3.74/3.47	50.23	Put-C-2	1.47	28.65
Asp^{2}(β-OH)-NH	8.57	-	Put-C-3	1.53	27.36
Asp^{2}(β-OH)-Cα	4.89	59.70	Put-C-4	2.88	42.56
Asp^{2}(β-OH)-Cβ	4.60	74.38	Put-NH^{3+}	7.42	

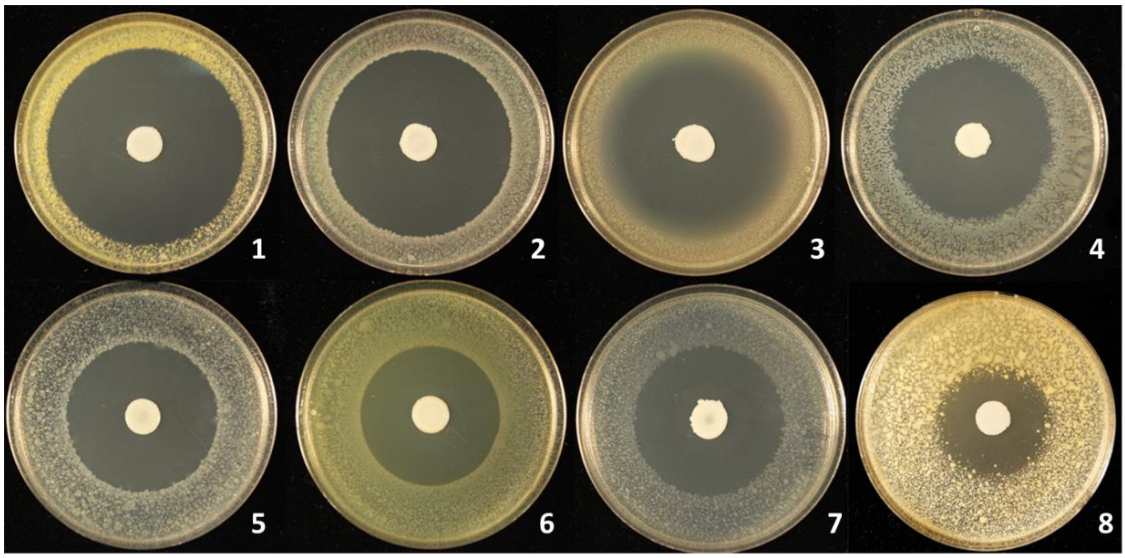

Figure 2.1: Antibacterial activity of *Burkholderia contaminans* MS14 against (1) *Xanthomonas citri* pv. *malvacearum* MSCT1, (2) *Pectobacterium carotovorum* WSCH1, (3) *Ralstonia solanacearum*, (4) *Pseudomonas syringae* pv. *syringae* B301D, (5) *Erwinia amylovora* 2029, (6) *Burkholderia glumae* 291, (7) *Escherichia coli*, and (8) *Clavibacter michiganensis* subsp. *michiganensis* Lu-01. 5-µl aliquots of bacterial suspension (OD_{420}=0.3) were inoculated onto the center of NBY plates. After the plates were incubated for 3 days at 28°C, the NBY plates were oversprayed with suspension of indicator pathogenic bacteria (OD_{420}=0.3). Inhibition zones were measured from the margins of bacterial colonies 24 hours later. Image courtesy of Dr. Shien Lu

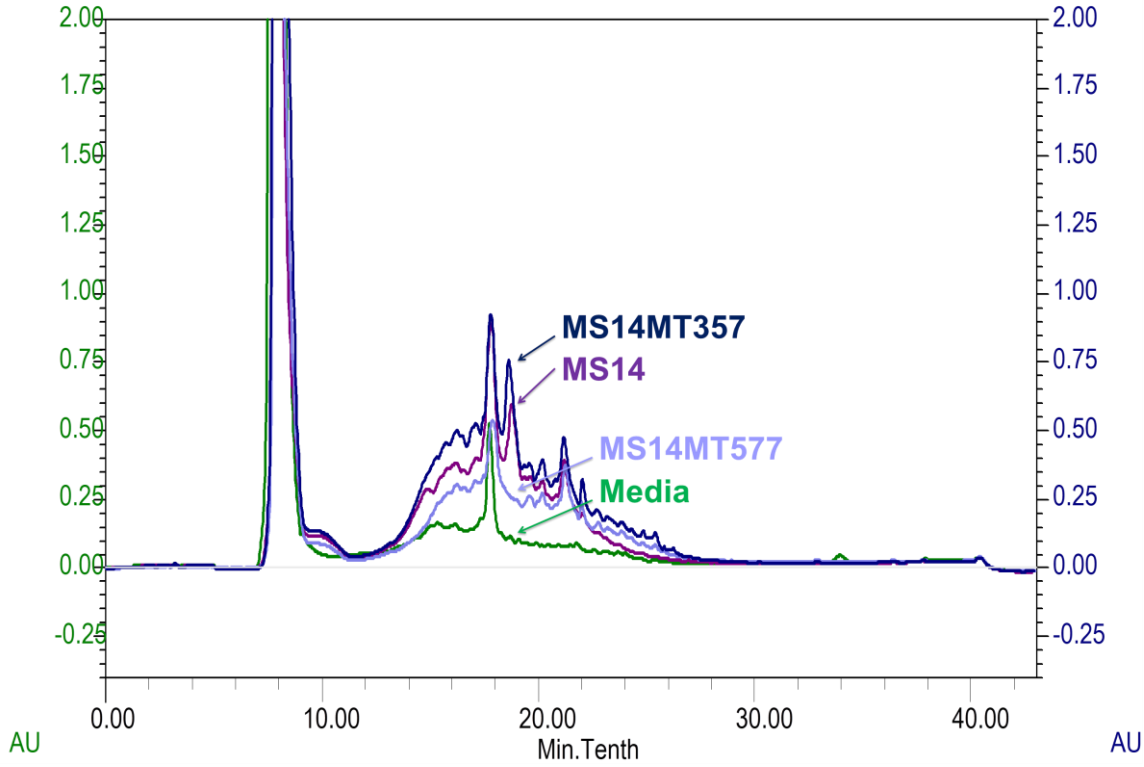

Figure 2.2: RP-HPLC chromatograms. Overlay of the chromatograms of the final purification step of the wild-type MS14 fraction, MS14MT357, and MS14MT577 at 220 nm using a 4.6 x 250 mm C18 column. Extracted media was run as a negative control.

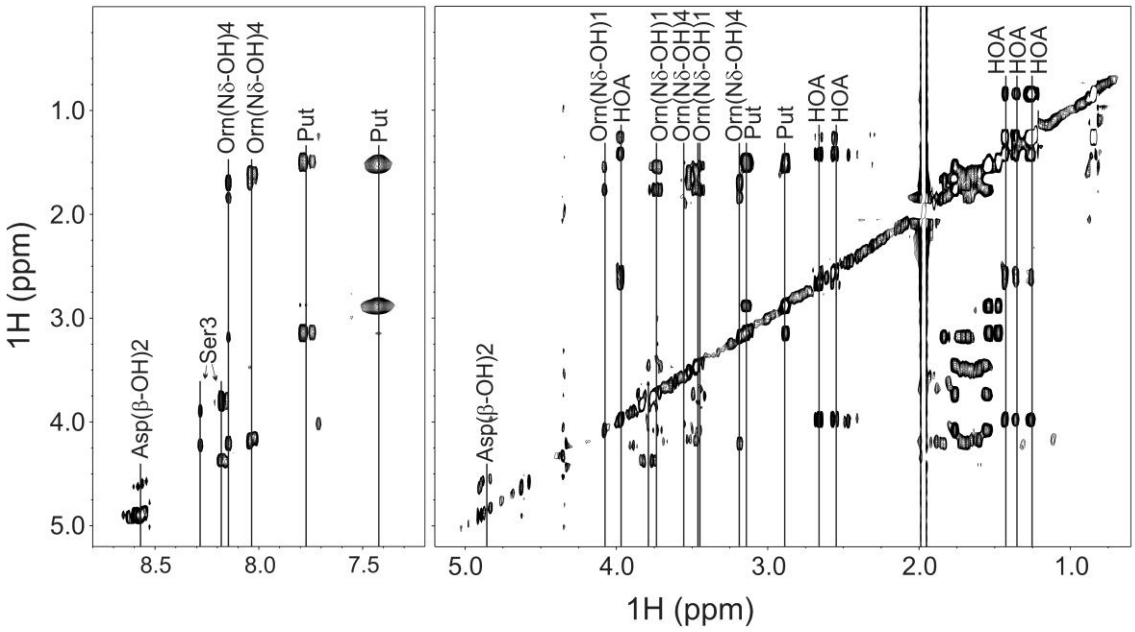

Figure 2.3: TOCSY spin system correlations of the siderophore product. Fingerprint region (NH correlations), alpha to side chain correlations and side chain correlations are shown. Abbreviations are: ornithine (Orn), putrescine (Put), and hydroxyoctanoic acid (HOA).

Figure 2.4: Bioassay for antibacterial activity. The two mutant strains MS14MT357 and MS14MT577 were spotted and grown overnight, heat killed, and overlayed with indicator strain *E. amylovora.* The bacteriostatic activity of ornibactin production in the MS14MT357 strain is visible by the observed growth reduction of the indicator strain. Bactericidal activity is not observed until the two strains come close together or are co-cultured together.

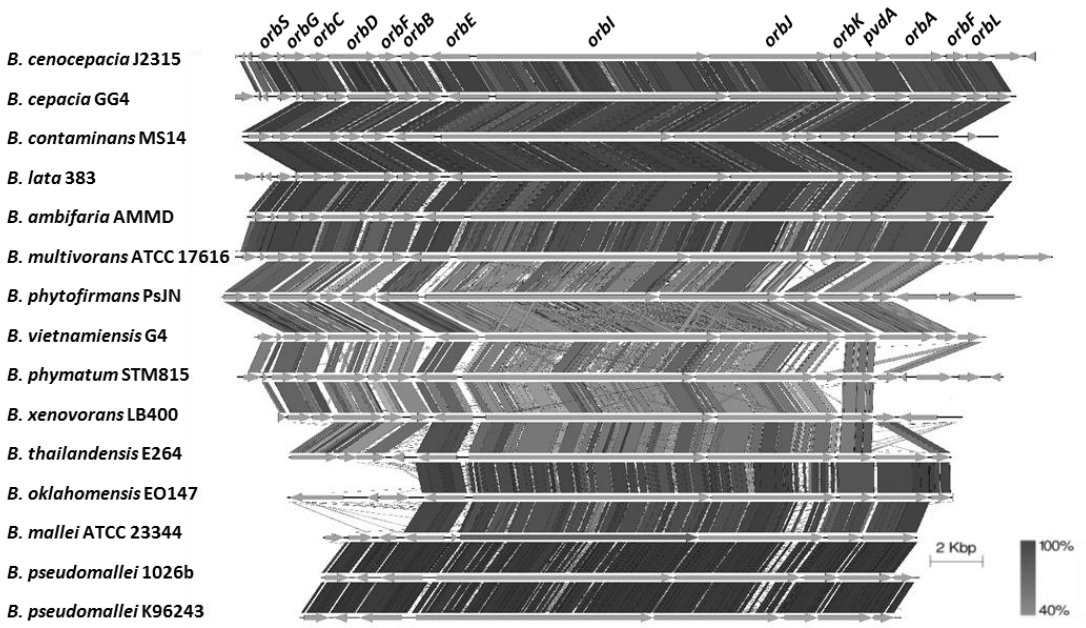

Figure 2.5: Ornibactin biosynthesis locus genetics of *Burkholderia* species. Image courtesy of Dr. Shien Lu

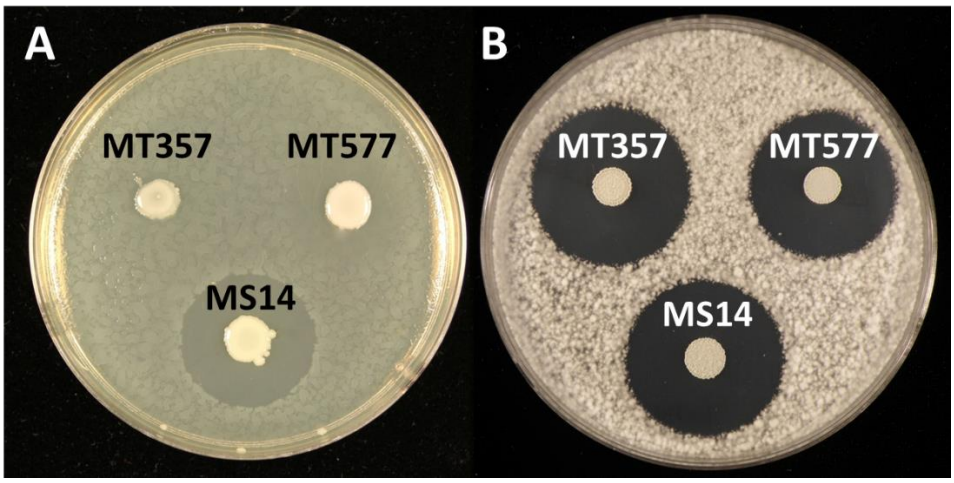

Figure 2.6: Antibacterial activity of *Burkholderia contaminans* strain MS14 against *Erwinia amylovora* was lost in MS14MT357 and MS14MT577 (A). Antifungal activity of *Burkholderia contaminans* strain MS14, MT357 and MT577 against *Geotrichum candidum* (B).

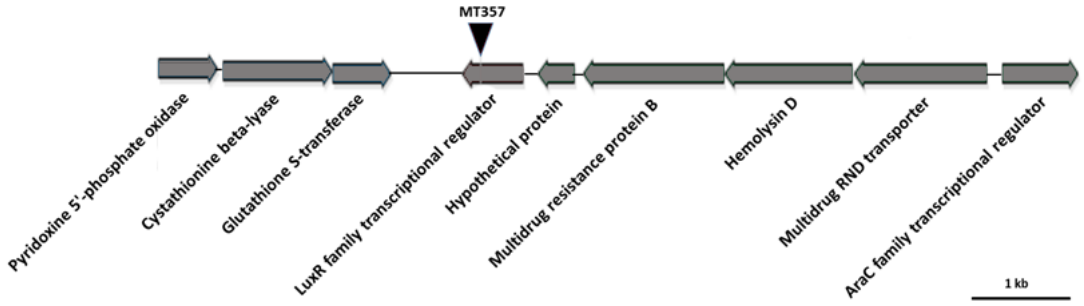

Figure 2.7: A 10-Kb genomic region of *Burkholderia contaminans* strain MS14 with the mutation location 357.

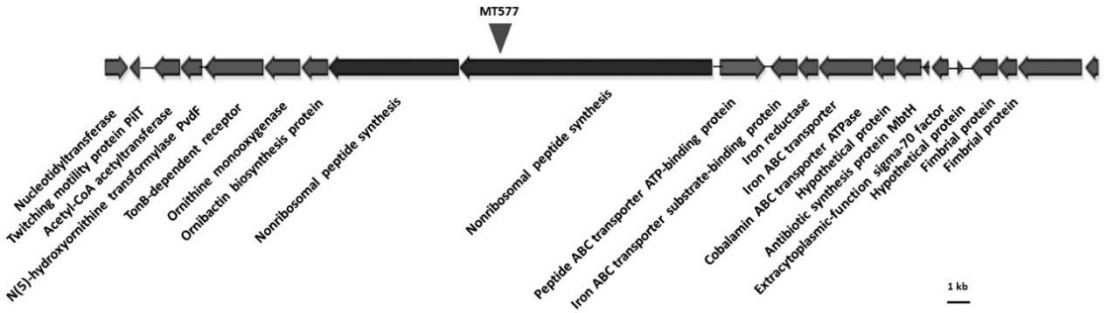

Figure 2.8: A 34-Kb genomic region of the *Burkholderia contaminans* strain MS14 with the mutation location 577.

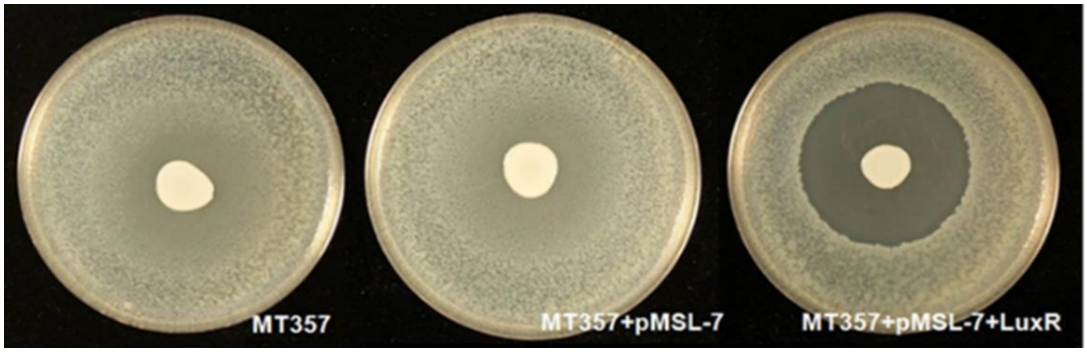

Figure 2.9: Constitutive expression of the LuxR homolog gene could restore the antibacterial activity against *Erwinia amylovora.*

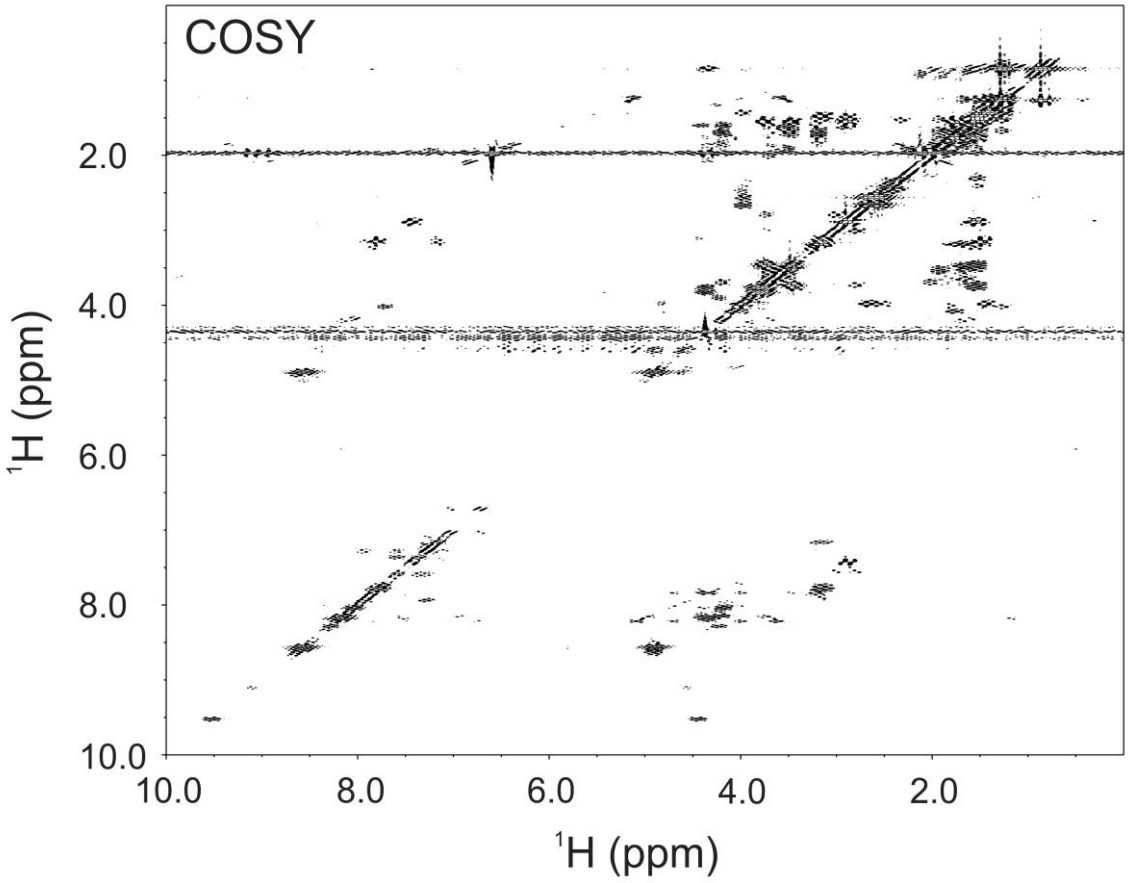

Figure 2.10: COSY60 NMR spectrum of Ornibactin recorded at 600 MHz in (50:50) acetonitrile-d3.

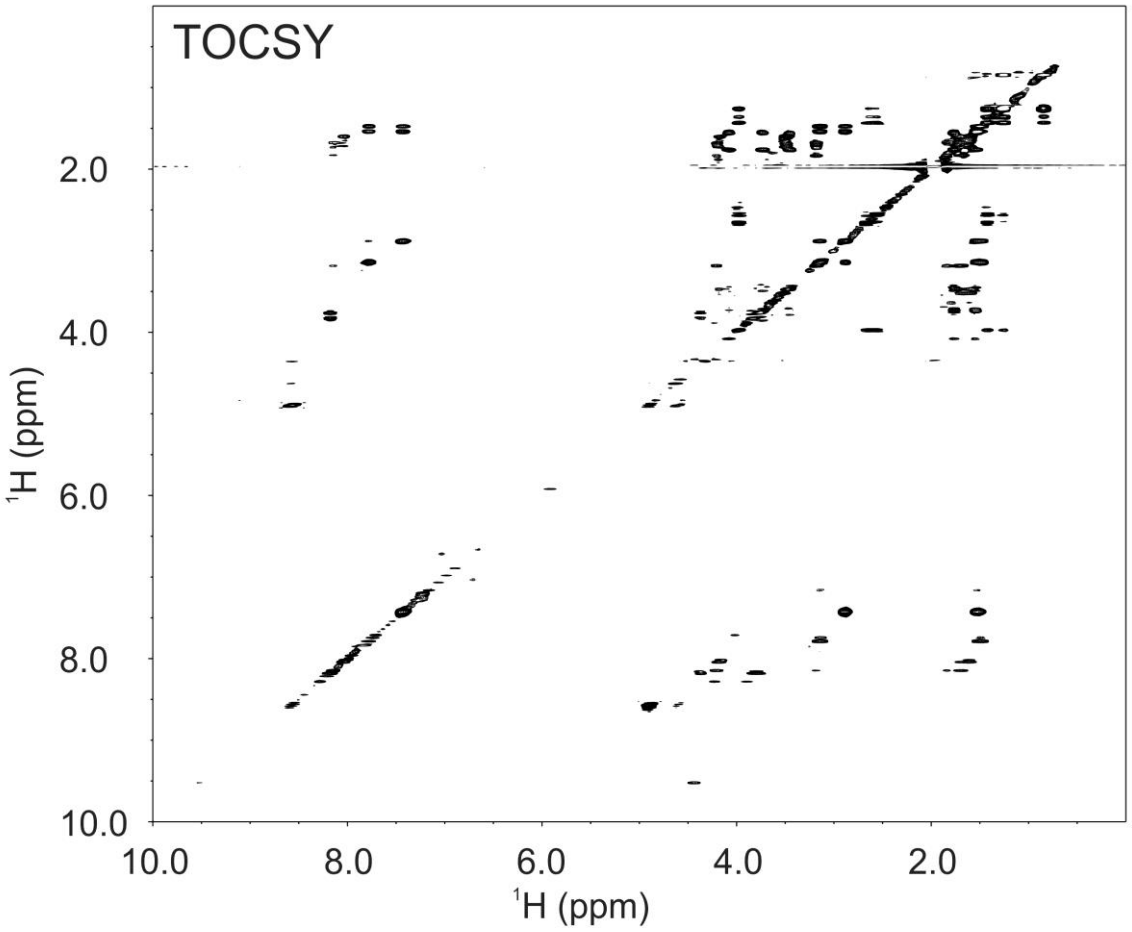

Figure 2.11: TOCSY60 NMR spectrum of Ornibactin recorded at 600 MHz in (50:50) acetonitrile-d3.

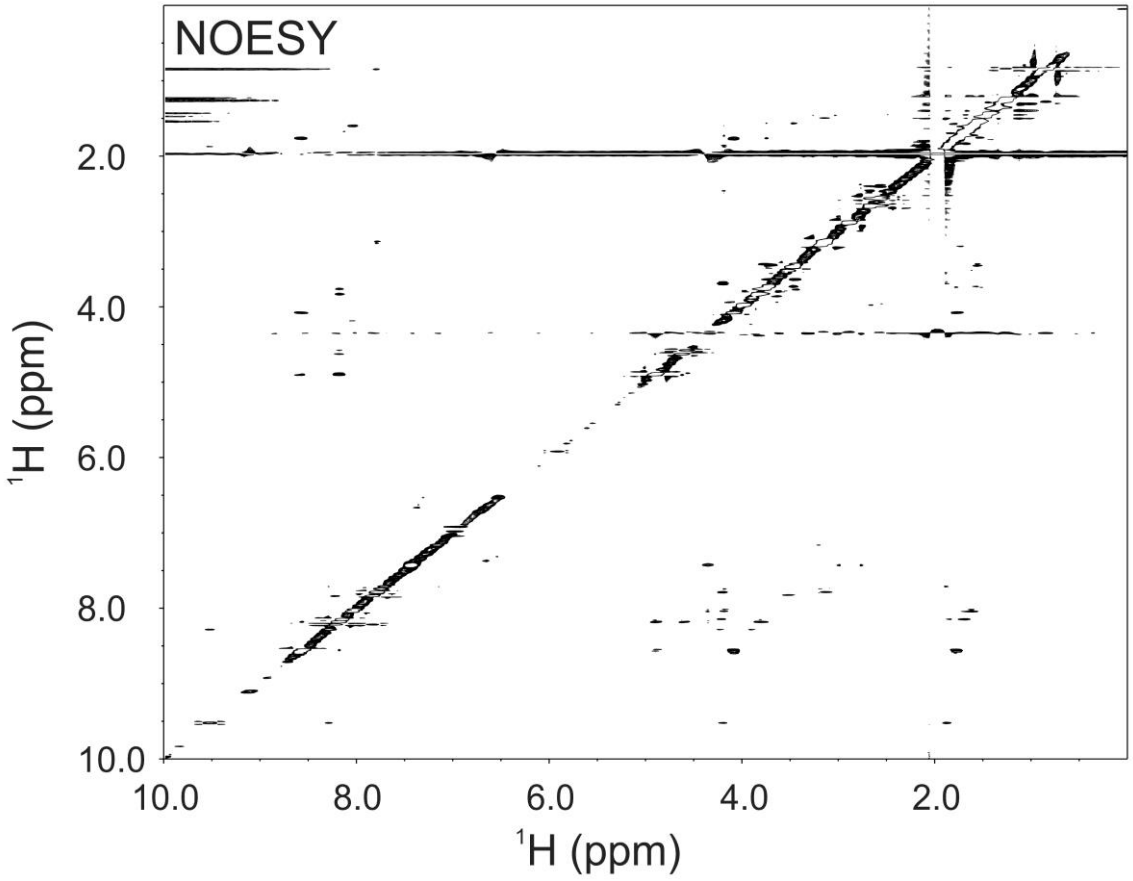

Figure 2.12: NOESY400 NMR spectrum of Ornibactin recorded at 600 MHz in (50:50) acetonitrile-d3.

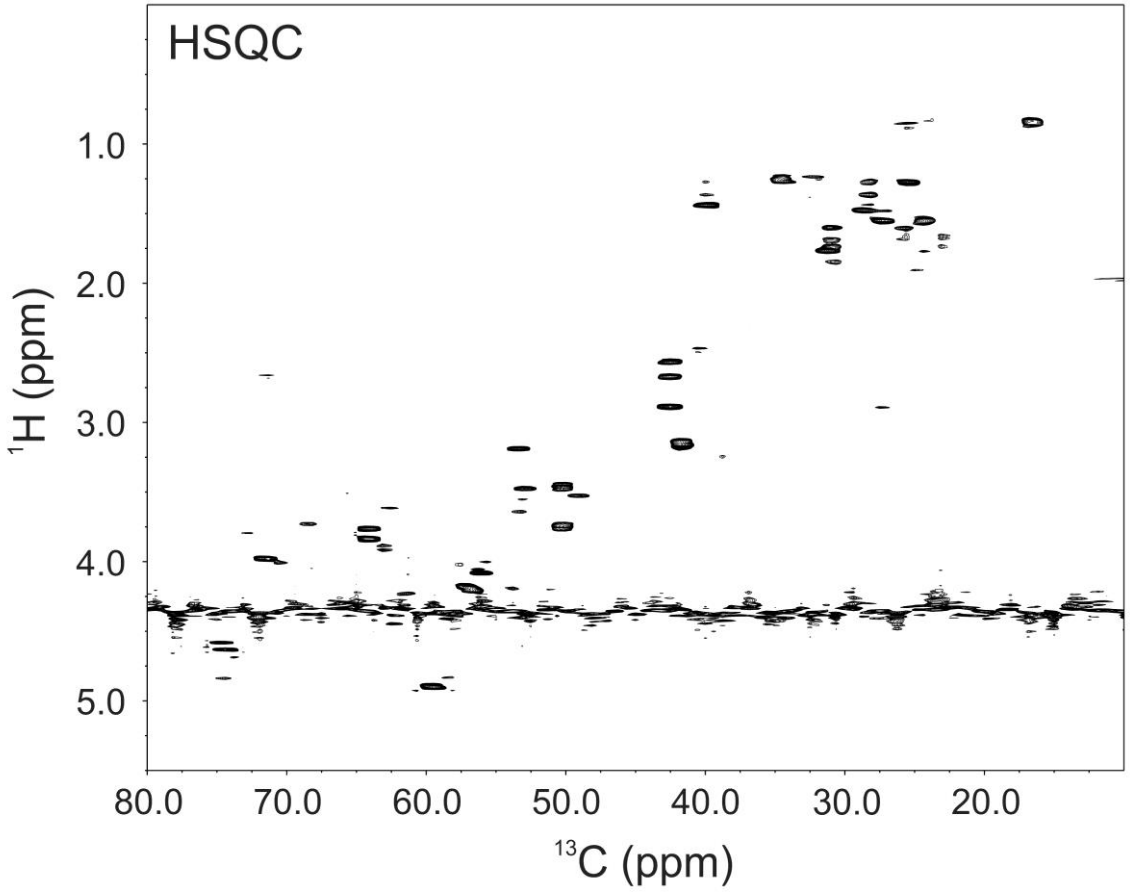

Figure 2.13: 13C-HSQC NMR Spectrum of Ornibactin recorded at 600 MHz in (50:50) acetonitrile-d3.

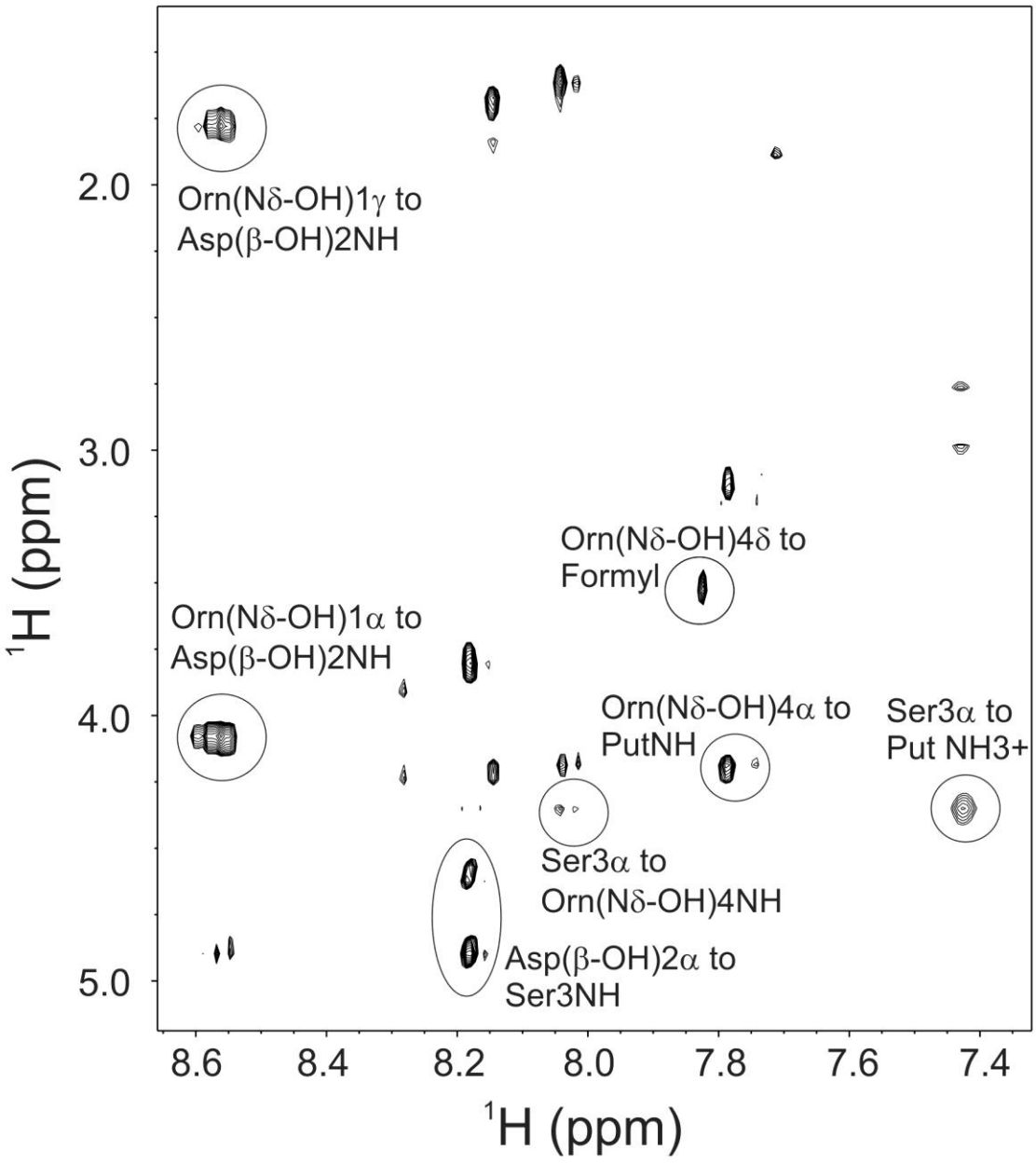

Figure 2.14: Sequential NOE contacts in ornibactin-F found in NOESY spectra.

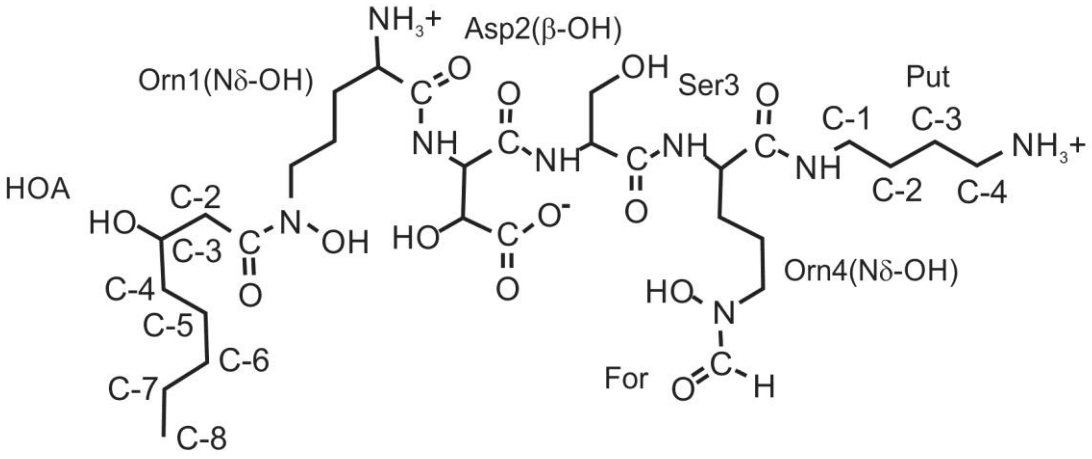

Figure 2.15: Covalent structure of ornibactin-F. The position of each amino acid is labeled in the tetrapeptide. The location of the 3-hydroxyoctanoic acid (HOA), putrescine (Put), and Nδ-formyl are demarcated.

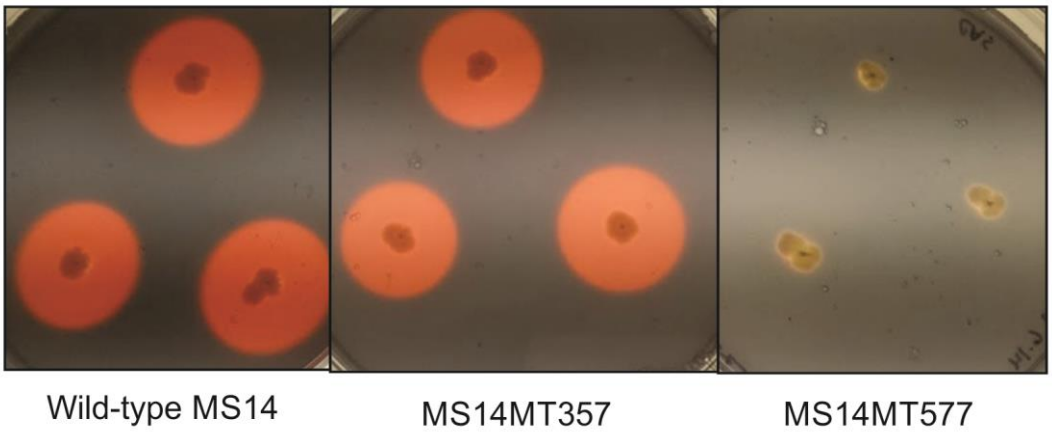

Figure 2.16: Chrome azurol S (CAS) plate assay. The wild-type MS14 and MS14MT357 strains have a clear zone of siderophore activity, while the MS14MT577 has lost this activity due to the absence of ornibactin production.

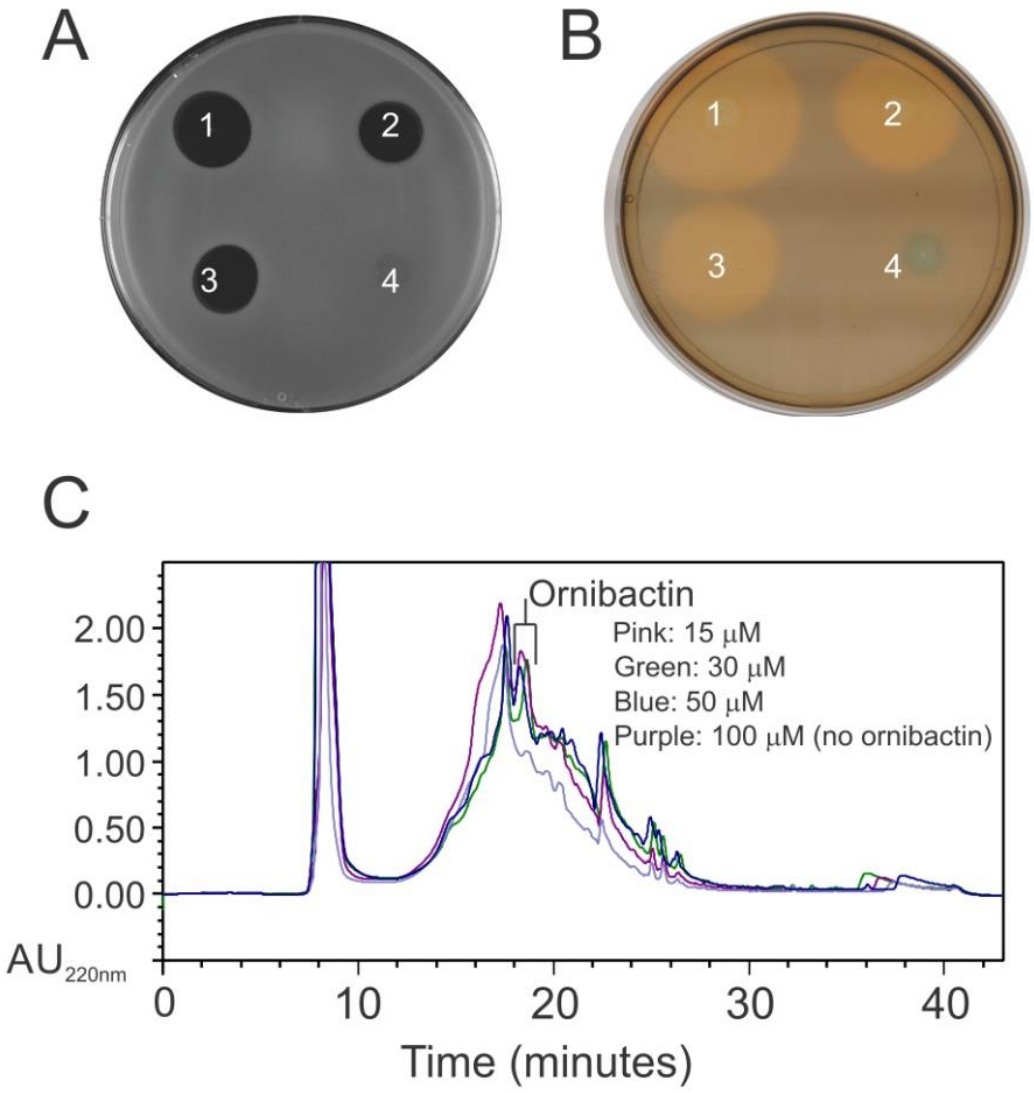

Figure 2.17: Bioactivity with supplemented ferric iron. **A.** Antibacterial activity of Diaion HP-20 extracts against *Erwinia amylovora* with cultures grown in 15(1), 30(2), 50(3), and 100(4) μM ferric iron. **B.** Chrome azurol S (CAS) plate assay of Diaion HP-20 extracts with cultures grown in 15(1), 30(2), 50(3), and 100(4) μM ferric iron. **C.** RP-HPLC chromatograms of Diaion HP-20 extracts of cultures grown in 15(1), 30(2), 50(3), and 100(4) μM ferric iron. No siderophore or bactericidal activity is observed in cultures grown with 100 μM ferric iron.

3. BIOPHYSICAL CHARACTERIZATION OF THE BACTERICIDAL COMPOUND PRODUCED BY *B. CONTAMINANS* MS14

3.1 Overview

There is an urgent need for the discovery of novel antibiotics for the treatment and prevention of infectious diseases in humans, animals, and plants. *Burkholderia contaminans* MS14 is known for its ability to produce a potent antifungal compound named occidiofungin. This bacterium has previously been shown to produce several compounds that have bactericidal properties; it also produces a bacteriostatic agent, ornibactin-F. Ornibactin-F is also involved in the bactericidal properties of this strain. This bactericidal activity was first observed in deferred antagonism assays while culturing MS14 in nutrient-rich media. The bactericidal activity was more selective toward Gram-negative organisms vs Gram-positive, and with no activity against fungi. In this report, we cover the work regarding the isolation and characterization of an unknown, novel antibacterial compound. This compound, while unknown and unnamed, is likely a novel antibiotic given similarity to a previously described polyketide biosynthetic gene cluster for malleilactone and the biophysical and bioactivity differences between malleilactone and the compound produced by MS14.

3.2 Introduction

The genus *Burkholderia* is composed of Gram-negative, rod-shaped, motile, environmentally versatile and non-spore-forming bacteria that have been identified in many diverse ecological niches (68). Currently, 88 species have been recognized in the genus *Burkholderia* (69). The bacteria have the ability to use a large array of carbon sources for various metabolic purposes, including synthesizing secondary metabolites (41, 70). *Burkholderia cepacia* complex (BCC) is a group of *Burkholderia* species that include soil isolates and opportunistic bacteria which may cause lung disease in immunocompromised individuals (71). Alternatively, some strains of the BCC are related to the promotion of plant growth and are considered to be plant growth-promoting bacteria. One member of the BCC with plant-growth promoting and antimicrobial activity is *Burkholderia contaminans* strain MS14. Of the members of the both *Burkholderia* genus and the BCC, *B. contaminans* MS14 is the most studied strain describing many facets of the antimicrobial agents produced. The confirmed antimicrobial secondary metabolites that are produced by MS14 are the antifungal occidiofungin (73) and the siderophore ornibactin (36), which has bacteriostatic bioactivity in addition to its normal role in iron acquisition. There is also evidence that an additional antibacterial metabolite is produced, and is regulated by the presence of ornibactin. This additional product can be observed in deferred antagonism assays, with lethal activity against many Gram-negative and Gram-positive indicator bacteria (Figure 3.1) (36). Ornibactin appears to be involved in the regulation of the production of the bactericidal compound. A mutant strain that is incapable of producing ornibactin is

capable of producing the bactericidal compound when grown in the presence of supplemented ornibactin (36). With the activites of occidiofungin and ornibactin already known, it was hypothesized that the new antibacterial compound was a polyketide.

Polyketides are a diverse group of natural compounds comprised of alternating carbonyl and methylene groups. While a number mycotoxins produced by fungi fall into the category of polyketides (119), there are also many that have been found to have beneficial antibacterial activity. Polyketides have been organized into different classes, primarily based on their structure and the manner in which they are synthesized. Type I polyketide synthetases (PKS) are linearly arranged and covalently fused catalytic domains within large multifunctional enzymes. These are produced by both bacteria and fungi. Type II polyketides include a dissociable complex of monofunctional enzymes, and are exclusively produced by bacteria. Type II polyketides include one of the more well-known group of broad-spectrum antibiotics, tetracyclines. Finally, Type III polyketides are commonly associated with chalcones, an organic compound frequently employed as a defense mechanism in plants. As one might infer, Type III polyketides are most often produced by plants, however there are some produced by bacteria and fungi (120). Polyketides make up a number of well-documented and effective antimicrobials: in addition to the previously mentioned tetracyclines, the antibacterials erythromycin and azithromycin, as well as the antiparasitic ivermectin, are just some of the examples of polyketide antibiotics commonly in use today. An example of a polyketide produced by *Burkholderia* would be malleilactone. The enzymes that make malleilactone are encoded by the *mal* gene cluster, which is highly conserved amongst different *Burkholderia*

species. While having minor antimicrobial activity on its own, it is cytotoxic to mammalian cells, as well as being a virulence factor for *B. pseudomallei*, the causative agent of melioidosis (121, 122). Because of the presence of bactericidal activity in *B. contaminans* MS14, separate from the bioactivity produced by occidiofungin and ornibactin, experiments were performed with the goal of isolating the bactericidal product for further characterization.

While there are numerous extraction methods available, there are a number of approaches that are particularly helpful when attempting to isolate proteinaceous compounds such as the antimicrobial compounds frequently isolated in the Smith Lab (123). When considering different methods of chromatography, size-exclusion, ion-exchange and reverse-phase are applicable for this function. As the name implies, size-exclusion chromatography separates compounds based on size. This separation is achieved through the use of a resin or beads in the stationary phase that contains many pores or channels. These pores are small enough to where smaller molecules are able to easily enter and thus will have to travel through these pores as they make their way to the bottom of the column, increasing their retention time. Because larger molecules are unable to fit through these pores, they pass by, needing only to travel around the larger beads that comprise the resin. This method allows for larger compounds to elute more rapidly, leading to separation based on size (123). Ion-exchange chromatography behaves in a similar principal to separation based on polarity, but rather than the stationary phase binding to the compound through hydrophilic or hydrophobic interactions, the stationary phase contains either positively or negatively charged ions

which will then bind to the compound of interest. Compounds in the mobile phase that are unable or only capable of weak interactions with the stationary phase are easily washed and eluted off while the compounds with strong electrostatic interaction remain on the column. Elution of the bound compounds can be achieved through the introduction of counter ions, which will compete for binding sites with the stationary phase, allowing for the compound to elute (123). Alternatively, the pH of the compound can be altered, which will affect its charge and thus will allow it to elute. Ion-exchange chromatography utilizes two principals, either cation-exchange or anion exchange. When the compound of interest is positively charged, cation exchange is more appropriate to use, and it incorporates a negatively charged stationary phase. For negatively charged molecules such as peptides or proteins, anion-exchange resins are used. The positively charged stationary phase will bind to negatively charged moieties, until they are eluted using the aforementioned methods (123). While normal-phase refers to chromatography that utilizes a hydrophilic stationary phase, reverse-phase chromatography uses a column that is hydrophobic in nature, inverting the polarity of the stationary and mobile phases involved in the separation. The end result of reverse-phase chromatography is that more non-polar compounds will bind to the stationary phase through hydrophobic interactions. Elution of these compounds involves the use of an aqueous polar mobile phase, which is then made increasing nonpolar with a water miscible organic solvent. Separation based on polarity does not have to incorporate a mobile or stationary phase. Liquid-to-liquid extractions separate compounds based on polarity between an aqueous polar phase and an organic nonpolar phase. For this particular extraction, the transfer of compound

typically moves from the aqueous phase to the organic phase through direct contact. After separation, the organic phase can then be further purified to isolate the now transferred compound (123).

What follows covers the work regarding the partial isolation and characterization of an unknown, novel antibacterial produced by wild-type *Burkholderia contaminans* MS14. The efforts to isolate the bactericidal compound of MS14 utilized all of the particular separation methods described above. Crude extracts containing the bactericidal compound were used in these isolation methods. Crude antimicrobial extracts were derived from culturing MS14 in nutrient-rich media that was shown to yield bactericidal activity in deferred antagonism assays against primarily Gram-negative organisms. From what has been learned in this study, the unknown compound is likely a novel antibiotic. For brevity, the unknown antimicrobial product being made by *Burkholderia contaminans* MS14 will be referred to as NAn-C (Novel Antibacterial Compound).

3.3 Materials and methods

3.3.1 Bacterial strains used and preparation of the crude extract containing bactericidal activity

The wild-type *B. contaminans* MS14 strain was grown on modified NBY (487 mL distilled water, 2.5 g peptone, 1.5 g Todd Hewitt, 1.0 g Yeast Extract, 1.0 g K_2HPO_4 (anhydrous), 0.25 g KH_2PO_4, and 1.5% agar; 12.5 mL of 20% glucose and 0.5 mL of 1M $MgSO_4$ were added after autoclaving) agar plates overnight at 28°C. Colonies from the

overnight NBY agar plate were stabbed into 500 mL of modified NBY soft agar (NBY with only 0.75 % agar). The inoculum was incubated at 28 °C for 4 days, and then immediately frozen at -80 °C. The culture was then thawed in a 55 °C water bath for 1 h, then transferred to 250 mL centrifuge bottles and centrifuged at 20,000 x g for 30 minutes. The collected supernatant was pooled, mixed with 1 gram of poly-aromatic absorbance resin Diaion HP-20 and shaken for one hour. The resin was allowed to settle before decanting the supernatant and was resuspended in 10 mL of 50% acetonitrile:water. The extract was dried by lyophilization and resuspended in 1 mL of 35% acetonitrile:water. The extract was tested for antibacterial activity, as described below, before being used in subsequent extraction experiments.

3.3.2 Procedure for overlay and chrome azurol S (CAS) bioassays

The extracts were tested for siderophore and antibacterial activity, by spotting on a Chrome Azurol S (CAS) plate or on an NBY plate overlaid with *Erwinia amylovora* 2029, respectively. These experiments also allowed a means to visualize separation efficiency between the antibacterial component and the siderophore ornibactin. CAS plates were prepared in accordance to the established protocol by Schwyn & Neilands (124, 125). Bioassay plates used in overlay assays were made using a suspension of *E. amylovora* 2029 in liquid NBY broth at an OD_{600} of 0.2. This suspension was added to molten NBY top (0.75%) agar (200 µL of bacterial suspension per 5 mL top agar), which was then mixed and poured onto a fresh NBY plates and allowed to set.

To perform the assays, both the prepared CAS plates and *E. amylovora* 2029 overlay plates were treated in the same manner: 10 µL of extract was spotted directly on the plate and allowed to air dry in a biosafety cabinet, and were incubated overnight at 28 °C. The following day, the CAS plates were observed for signs of chelation indicated by a color shift from blue to yellow-orange in the location of the spot, and the *E. amylovora* plates were observed for antibacterial activity by measuring the zones of inhibition.

3.3.3 HPLC, size exclusion, and anionic exchange isolation techniques for NAn-C

RP-HPLC was done using a 4.6 × 250 mm C18 column (Grace-Vydac, catalog 201TP54) on a Bio-Rad BioLogic F10 Duo Flow with Quad Tec UV-Vis Detector system. A 1:10 dilution of extract in 35% acetonitrile containing 0.1% TFA at a final volume of 1 mL was injected for each run. Fractions were separated using a thirty minute gradient of 90:10 water:acetonitrile:0.1% trifluoroacetic acid to 20:80 water:acetonitrile:0.1% trifluoroacetic acid at a flowrate of 1 mL/minute.

Size exclusion chromatography was performed using a sample of crude extract in 35% acetonitrile:water. The sample (1 mL) was loaded onto a 0.7 x 20 cm Flex-Column (Kimble-Chase) packed with Sephadex G-10 resin from GE Healthcare Life Sciences. A void volume of 3 mL was determined using Dextran Blue (Spectrum Chemicals) and collected in two separate 1.5 mL microcentrifuge tubes, with subsequent fractions collected in 1.5 mL microcentrifuge tubes at a volume of 1 mL for each fraction. Upon completion, each collected fraction was lyophilized via speed-vac until completely dry,

then reconstituted in 65:35 water:acetonitrile:0.1% trifluoroacetic acid. The reconstituted extracts were tested for siderophore and antibacterial activity, as well as the efficiency of separating the siderophore and bactericidal activity, by spotting 10 µL of the extract on a CAS plates or on an NBY plate overlaid with *Erwinia amylovora* 2029.

Anionic exchange resin was subsequently used to isolate NAn-C. Several 1 mL fractions of the active crude extract was combined with 0.5 mL each of a 50% slurry of Amberlite IRA-410 chloride resin (Dow Chemical/Sigma Aldrich) in separate microcentrifuge tubes, shaken for 60 minutes and then pipetted to new microcentrifuge tubes. To test for successful adsorption to the resin the extracts were tested for antibacterial activity by spotting 10 µL of the extract on an NBY plate overlaid with *Erwinia amylovora* 2029. The remaining Amberlite resins were then washed with different solutions: 2M NaCl, a pH 3 solution and a pH 5 solution.

3.3.4 Dialysis of NAn-C and siderophore ornibactin

A volume of 750 µL of new extract was pipetted into a Biotech cellulose ester (CE) dialysis cartridge (MWCO 500-1000 Daltons; Spectrum Chemical Mfg. Corp. New Brunswick, NJ; Product No. 888-10729). The cartridge was then placed in a beaker to float in 1 L of distilled water. The extract was allowed to dialyze for a total of 72 hours, while stirring. The extract was tested for siderophore and antibacterial activity, as well as separation efficiency, by spotting 10 µL of the extract on a Chrome azurol S (CAS) plates or on an NBY plate overlaid with *Erwinia amylovora* 2029. These tests were performed using samples of extract from T-0, T-24, T-48, and T-72 hour timepoints. To

test for successful passage of the antibacterial product and to ensure it did not bind to the membrane, the entire volume of dialysate water was lyophilized and reconstituted in 750 µl of 65:35 water acetonitrile:0.1% trifluoroacetic acid. This reconstituted dialysate was tested for siderophore and antibacterial activity, as well as separation efficiency, by spotting 10 µL of the extract on a CAS plates or on an NBY plate overlaid with *Erwinia amylovora* 2029.

3.3.5 Isolation of the antibacterial product through Kupchan extraction

Isolation of the antimicrobial product through Kupchan extraction followed a modified protocol for the isolation of malleilactone, as described by Biggens *et al* (121). The wild-type *B. contaminans* MS14 strain was grown in modified NBY (487 mL distilled water, 2.5 g peptone, 1.5 g Todd Hewitt, 1.0 g Yeast Extract, 1.0 g K_2HPO_4 (anhydrous), and 0.25 g KH_2PO_4; 12.5 mL of 20% glucose and 0.5 mL of 1M $MgSO_4$ was added after autoclaving) overnight at 28°C. 10 mL was inoculated into each of six flasks of 1 L modified NBY incubated at 28°C, 200 rpm, for 48 hours. The 6 L culture was extracted twice with an equal volume of ethyl acetate and dried *in vacuo*. This crude ethyl acetate extract was initially partitioned using a modified Kupchan Scheme (Figure 3.9). The extract was resuspended in 500 mL of 90% methanol and then extracted 4 times with 500 mL of hexanes. The remaining methanolic mixture was diluted to 2 L of 60% methanol, split into 1 L fractions and each liter was extracted 4 times with 1 L dichloromethane. All three fractions (hexanes, dichloromethane, & methanol) were dried *in vacuo*. The protocol as described successfully partitioned malleilactone in the

dichloromethane fraction, however as it was uncertain if the antimicrobial product was malleilactone, all extracts (including the ethyl acetate extract) were resuspended in 5 mL of 35% acetonitrile:water. These extracts were tested for antibacterial activity by the overlay assay described above as was the aqueous phase that remained after each extract was similarly dried and resuspended.

3.3.6 Biophysical characterization of the antibacterial product

The antibacterial product was tested for stability under high and low pH, high temperature, and direct UV exposure for two hours, with time points being taken at 15, 30 ,60 and 120 minutes. For the pH tests, the protocol from Ellis et. al was adapted (126). NAn-C extract was dried in separate 1.5-ml microcentrifuge tubes labelled pH 5 to pH 9. The dried samples were resuspended in 1 ml of RPMI 1640 medium. Using stock solutions of 6 M HCl and 6 M NaOH, the pHs of the samples were adjusted accordingly, and then samples were left at room temperature for 2 h. At the 15, 30, 60 and 120 minute time points, the pH were readjusted to pH 7.0 and bioassays were performed. To test heat stability, 500 µL of extract was mixed with 500 µL of distilled water in a 1.5 mL microcentrifuge tube and incubated in a 65 °C water bath. To expose a sample to UV light, 500 µL of extract was mixed with 500 µL of distilled water in a 1.5 mL microcentrifuge tube, and allowed to rest on an ultraviolet light box (265 nm) used for viewing electrophoresis gels.

10 µL of all of these samples were taken at 15, 30, 60 and 120 minutes and spotted on an E. amylovora 2029 overlay plate, which was prepared as previously

described above. After allowing to air dry, the plate was then incubated overnight at 28 °C and was checked for growth inhibition the following morning.

3.4 Results

3.4.1 Separation of ornibactin from the antibacterial product through HPLC

At this stage in the discovery process, it was well established that the bactericidal activity was closely tied to the presence of ornibactin. Because ornibactin also has bioactivity in the form of bacteriostatic growth inhibition, it was important to separate the two compounds. Attempts to separate the two compounds by HPLC were unsuccessful. As described in the methods, NAn-C did not bind to the C18 resin and remained in the flowthrough fraction, indicating that NAn-C is a polar compound (Figure 3.2). We then turned to other separation methods.

3.4.2 Separation of ornibactin from the antibacterial product through size-exclusion chromatography

A column packed with Sephadex G-10 resin was used to separate the bactericidal NAn-C and bacteriostatic ornibactin products present in the crude extracts of *B. contaminans* MS14. In order to determine separation efficiency between these antibiotics, an overlay assay and an iron chelation assay was performed (Figure 3.3). Fractions spotted on a Chrome azurol S (CAS) plate will change from blue to a yellow-orange color, if the fraction contains an iron chelator, such as ornibactin. By comparing the activity of each fraction by spotting 10 µl on an overlay assay and 10 µl on a CAS

plate, it did appear that a small amount of separation was achieved: ornibactin eluted slightly earlier than the bactericidal compound, with the majority of chelation occurring in fractions 5 and 6, while the major bactericidal activity was centered around fraction 7. Unfortunately, the fractions that contained the highest amount of bactericidal activity still eluted with ornibactin. With the commercially available size-exclusion resins I was unable to resolve NAn-C from ornibactin, so a new approach was used.

3.4.3 Isolation of the antibacterial product through ion-exchange

We next attempted to separate NAn-C and ornibactin by anion exchange chromatography. The initial results were promising. Upon exposure to the resin, the MS14 extract lost most of its bactericidal activity, indicating that NAn-C was successfully bound to the resin (Figure 3.4). The remaining activity in the extract appeared to be bacteriostatic in nature, as it did not yield a clear zone of inhibition. This suggests that ornibactin remained in the extract, or that the extract still contained a sub-lethal concentration of NAn-C. Unfortunately, bactericidal activity could not be recovered from the resin even after exposure to elution buffers containing 2M NaCl or at pH 5.0 or pH 3.0.

3.4.4 Separation of ornibactin from the antibacterial product through dialysis

Although the previous attempt to separate NAn-C and ornibactin by size exclusion chromatography was unsuccessful, the results suggested that the bactericidal NAn-C product is likely smaller in size than ornibactin. Dialysis was used to determine

whether NAn-C could be separated from the other known antibacterial products. Rather than using the standard dialysis tubing techinique a dialysis cassette was used because it allows for good flow of the dialysate through the double-sided membrane. Samples of the extract were taken from the cassette at T-0, T-24, T-48 and T-72 and plated in an overlay assay in order to determine when the bactericidal compound successfully dialyzed through the membrane. The samples were also plated on CAS plates to determine if ornibactin was retained in the cartridge or had dialyzed through the membrane. The bactericidal compound dialyzed within 24 hours, with only bacteriostatic activity remaining in the extract (Figure 3.5). While this would initially seem promising, ornibactin was also able to pass through the membrane in the same amount of time, enough to where partial bioactivity could be attributed to its presence. To test whether NAn-C passed through the dialysis membrane, the dialysate (500 mL water) was lyophilized and reconstituted with 35% ACN into an volume equal to the original volume of the extract placed into the cartridge. This sample was tested on an overlay assay and a clear bactericidal zone of inhibition was still present, indicating that the compound passed through the membrane and was not degraded or bound to the dialysis membrane. The results of the deferred antagonism assay also showed that ornibactin dialyzed at a slower rate than NAn-C indicating that NAn-C is likely smaller than ornibactin (737 Da), consistent with the results from the size-exclusion chromatography. However, because ornibactin dialyzed with NAn-C, this method was also unsuitable for isolation.

3.4.5 Isolation of the antibacterial product through Kupchan extraction

Unpublished RNA-seq and site directed mutagenesis work from Dr. Shien Lu's group supports NAn-C being a small polyketide product (Dr. Shien Lu, personal communication). The RNA Seq data from wild-type and NAn-C deficient strains of *B. contaminans* MS14 provided a means to predict the biosynthetic genes involved in the production of NAn-C. Site directed mutagenesis of two predicted polyketide synthetases within this gene cluster further confirmed the importance of these products for the synthesis of NAn-C (Figure 3.6). The predicted biosynthetic gene cluster in *B. contaminans* MS14 has a 76% DNA identity to a MAL gene cluster in *B. pseudomallei* K96243, the biosynthetic gene cluster responsible for the synthesis of malleilactone (122). The authors suggested that the compound likely functions as a siderophore and it only has a demonstrated bacteriostatic property, suggesting that while NAn-C may be similar in structure there are differences that grants in bactericidal activity. Following the established Kupchan extraction protocol (121) that was also used for isolating malleilactone from spent *Burkholderia* media, attempts with MS14 spent media did not yield the same results. Using this protocol, *B. contaminans* MS14 spent medium was successively washed with non-polar solvents, which in the original protocol would result in the product of interest being isolated in the final wash of dichloromethane. However, the bioactivity attributed to NAn-C remained in the aqueous phase, with only minor biostatic activity appearing in the ethyl acetate wash (Figure 3.7). These results suggest that the NAn-C compound produced by MS14 is not the same compound as malleilactone and that NAn-C is a highly polar compound. It is likely that the

differences in sequence identity between the predicted biosynthetic gene cluster of NAn-C and malleilactone contribute to differences in the final product being produced. Another key difference between malleilactone and the unknown product of MS14 is that malleilactone does not exhibit bactericidal activity, while NAn-C does.

3.4.6 Biophysical characterization of the unpurified antibacterial product NAn-C

Even without the successful purification of NAn-C, some of its properties could be elucidated. Aqueous extracts containing NAn-C were exposed to high and low pH, ultraviolet light, and high (65°C) temperature (Figure 3.8). Samples were taken after 15, 30, 60 and 120 minutes and plated against the Gram-negative indicator strain, *E. amylovora*. After 2 hours of exposure to 65°C, the sample appeared to retain most of its inhibitory activity. It does appear that some activity was lost after two hours of exposure to high and low pH, while most of the activity was retained at 60 minutes. However, in the sample exposed to UV light, the bactericidal activity was lost in as little as 15 minutes. Although some activity remained, complete loss of inhibitory activity occurred between 30 and 60 minutes of UV exposure. These results suggest that NAn-C is temperature and pH stable. However, the compound is UV sensitive.

3.5 Discussion

Despite not being able to successfully isolate the antibacterial compound, NAn-C, important information was learned. *B. contaminans* MS14 is producing a small antibacterial compound, presumably a hybrid PKS NRPS molecule based on data from

Dr. Lu's group. Size exclusion chromatography and dialysis studies performed indicates that this compound is likely smaller than ornibactin (737 Da). Based on information learned from the HPLC and Kupchan extraction approaches, the antibacterial compound is very polar. Additional biophysical characterization studies on NAn-C, suggests that it is stable at acidic and basic pH, and high temperature conditions, while being sensitive to UV exposure. The high polarity, small size, and UV light sensitivity, contributed to the difficulty in purifying the antibacterial compound.

Because it is well established that MS14 produces the potent antifungal occidiofungin, effort was made to ensure that the bioactivity that was being observed was due to a new product, rather than a secondary level of activity resulting from the production of occidiofungin. To test this, additional deferred antagonism assays were performed on the Gram-negative bacterium, *E. amylovora*. In this assay, *B. contaminans* MS14 exhibits strong bactericidal activity when grown on complex, nutrient-rich media (data not shown). However, when plated on minimal media, no such activity was observed. The same deferred antagonism assays were performed using the fungus *Geotrichum candidum*. In these particular assays, fungicidal activity was present due to the production of occidiofungin only on the minimal media plates. The lack of antibacterial activity on minimal media plates demonstrates that, even though occidiofungin is produced by MS14, it has no activity against a Gram-negative indicator *E. amylovora*, and thus the bactericidal activity observed is probably due to the new product, NAn-C. From here the primary goal was to purify the bactericidal product or at a minimum to separate the product from ornibactin. It was previously shown that a

mutant strain that could no longer make ornibactin was also unable to produce the bactericidal activity. Separating the bactericidal compound from ornibactin would support the notion that ornibactin is important for regulating the production of NAn-C and that it is not involved in the observed bactericidal activity.

A PKS biosynthetic gene cluster was identified by Dr. Lu's group (Dr. Lu, personal communication) and confirmed to be responsible for the observed bactericidal activity by mutagenesis and complementation studies. The predicted biosynthetic gene cluster is expected to make a small polyketide product. According to sequence identity, the small polyketide product is likely structurally similar to malleilactone (Figure 3.10). However, NAn-C probably contains UV sensitive conjugative double bonds and additional polar groups, which contribute to differences in NAn-C solubility and UV stability compared to malleilactone. Our data further supports that the biosynthetic gene cluster in MS14 is making a distinct, different compound from malleilactone, which is produced by *B. pseudomallei* K96243.

While the isolation of NAn-C was unsuccessful, modifications to the previous methods might allow it to be isolated. In particular, the size exclusion chromatography could be attempted using a longer column. Additionally, a weaker ion-exchange resin could be employed, possibly allowing the compound to unbind from IRA-410. Regarding the HPLC an additional test could be performed on the flowthrough: although NAn-C did not bind ornibactin should have. Testing the flowthrough for the presence (or lack) of ornibactin could have indicated an additional means of separation.

3.6 Conclusion

Utilizing NAn-C in future studies as a potential therapeutic is still possible. The groundwork has been laid for future researchers to continue the work to isolate and characterize the unknown compound. For *B. contaminans* MS14, the methods previously outlined for its cultivation are likely sufficient for producing amounts necessary for further study, with only the methods of isolation needing to be refined. With the benefit of data collected and hindsight, an aspect that cannot be overlooked is an issue with its initial extraction: by using a resin with hydrophobic properties in its initial extraction, it's likely that much of the antibacterial compound remained in the aqueous supernatant of the initial cultures. It is possible that the amounts of NAn-C that were able to be observed after extraction with the HP-20 resin were found due to non-specific binding to the resin, or possibly paired with a separate compound that had no trouble binding. With all the new information learned about the NAn-C compound, some of the previous attempts to purify it can be used again, albeit with altered parameters that could lead to more successful isolation. For example, a weaker ion-exchange resin could be used. Alternatively, HPLC can be attempted again. If using reverse phase, the column can be used to remove impurities from the flowthrough in a rather quick protocol. There also is the option of utilizing normal-phase HPLC, which would allow the hydrophilic NAn-C to remain in the stationary phase. Currently, we do know more about the likely physical features of the compound that will enable a more selective approach for isolation.

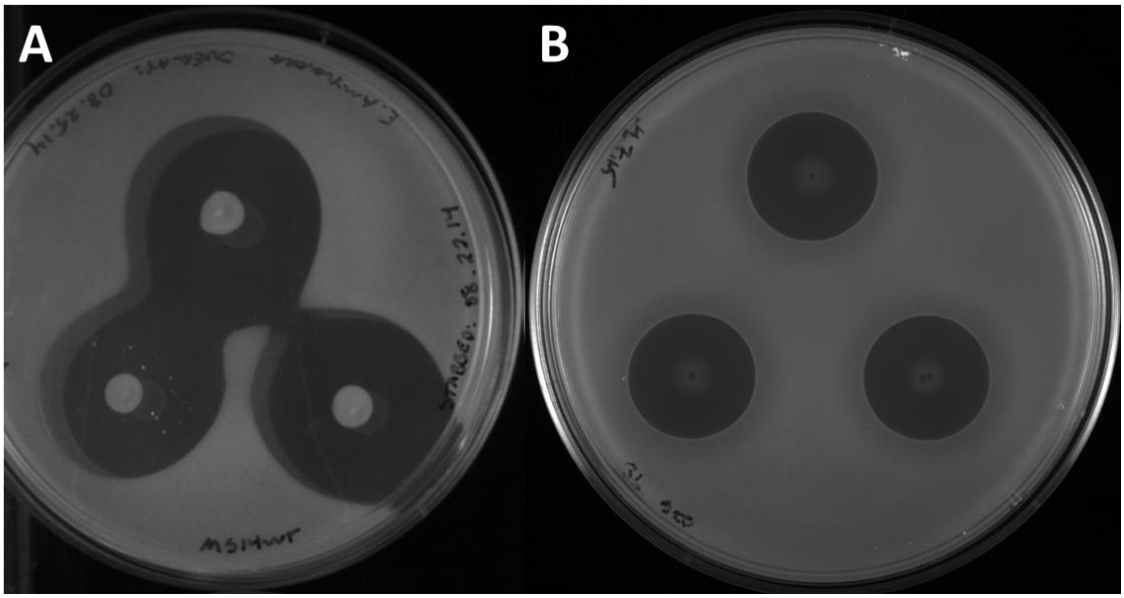

Figure 3.1: Bioassays showing antibacterial activity derived from *B. contaminans* MS14. A) A deferred antagonism assay using of *B. contaminans* MS14 against *E. amylovora* 2029. B) An overlay assay using *B. contaminans* MS14 media extracts against the indicator strain *Erwinia amylovora*. Bactericidal activity was confirmed by removing agar plugs within the zones of inhibition and plating them on fresh plates (data not shown). No growth was observed after 48 hours of incubation.

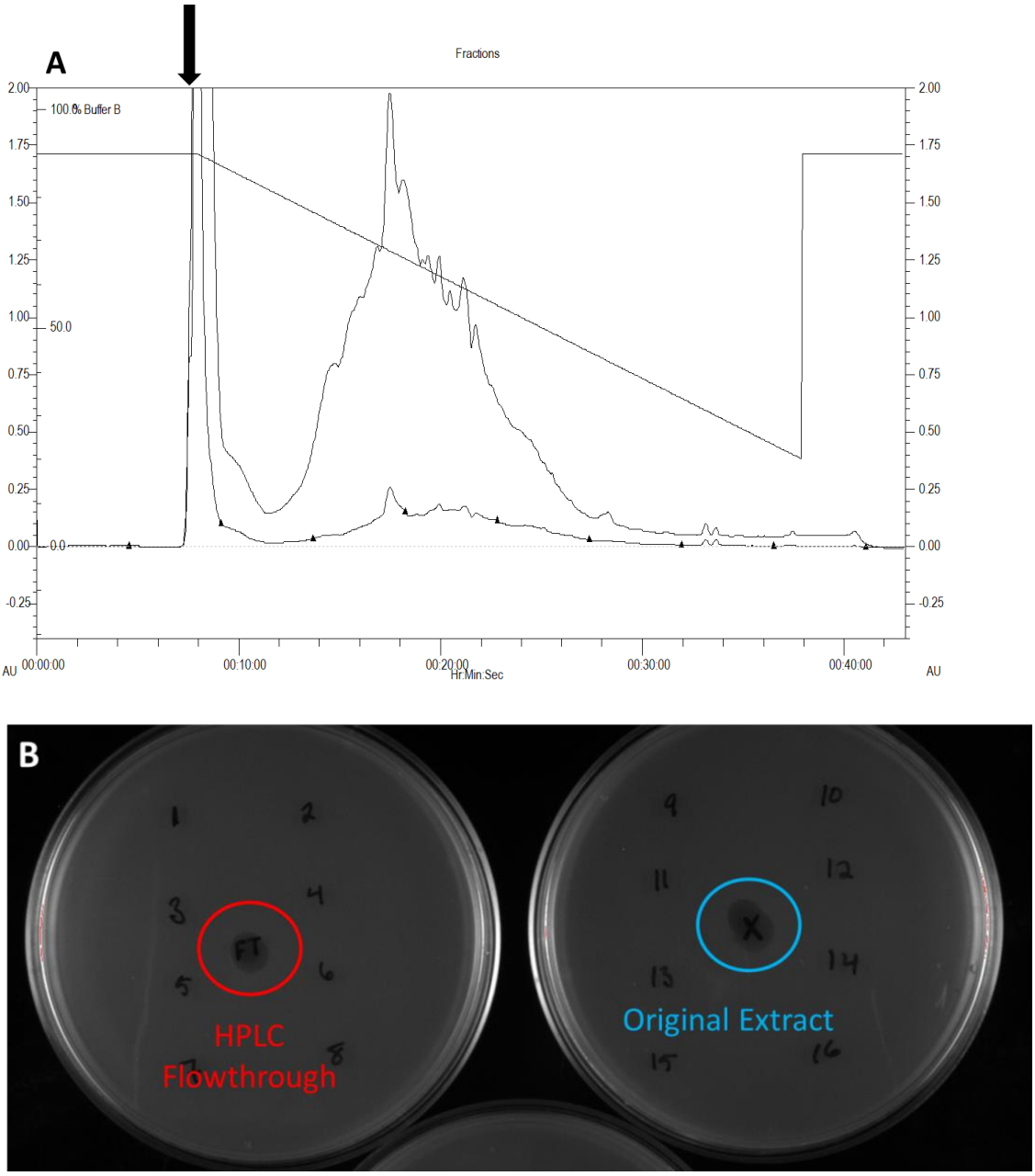

Figure 3.2: Retention of *B. contaminans* MS14 media extracts on reverse Phase C18 HPLC column. A) Chromatogram of a reverse phase HPLC using a gradient of 95-20 % water +0.1% trifluoracetic acid, on a 4.6 x 250 mm Agilent C-18 column. Starting with the flowthrough fraction (demarcated with an arrow), fractions were collected in 1 minute intervals. B) Overlay assay of *B. contaminans* MS14 extract (blue circle) and the HPLC fractions against indicator strain *Erwinia amylovora.* The HPLC flowthrough (red circle) is the only fraction that retained the inhibitory activity. The blue circle demarcates the inhibitory activity of the original extract material.

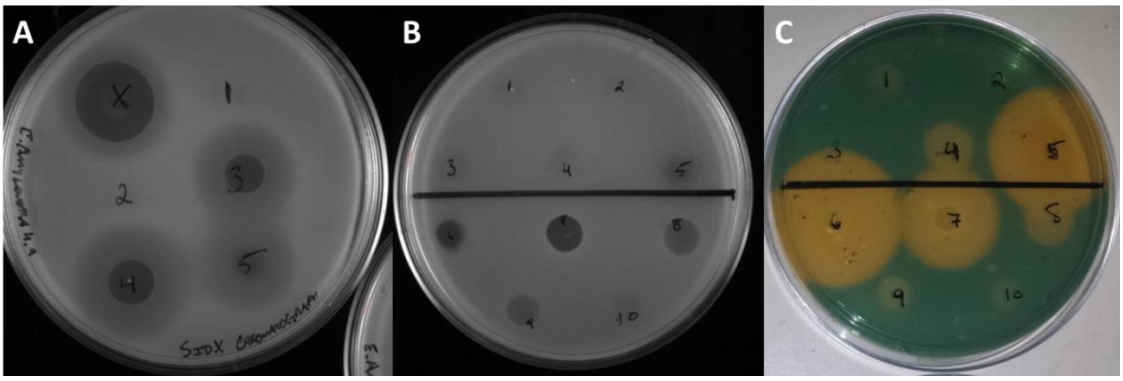

Figure 3.3: Size-exclusion chromatography (Sephadex G-10) of *B. contaminans* MS14 media extracts. A) *B. contaminans* MS14 extract was loaded onto column and 1 ml samples were collected following the determined void volume. Sample fractions were tested for inhibitory activity using an overlay assay against *E. amylovora.* B) *B. contaminans* MS14 extract was loaded onto column and 0.5 ml samples were collected following the determined void volume. Sample fractions were tested for inhibitory activity using an overlay assay against *E. amylovora*. C) The same 0.5 ml sample fractions from plate B were additionally tested for siderophore activity on a chrome azurol S plate. Note that all the fractions that demonstrated bioactivity has strong iron chelation present in the corresponding fractions on the CAS plate, indicating the presence of ornibactin along with the bactericidal compound, and a low separation efficiency.

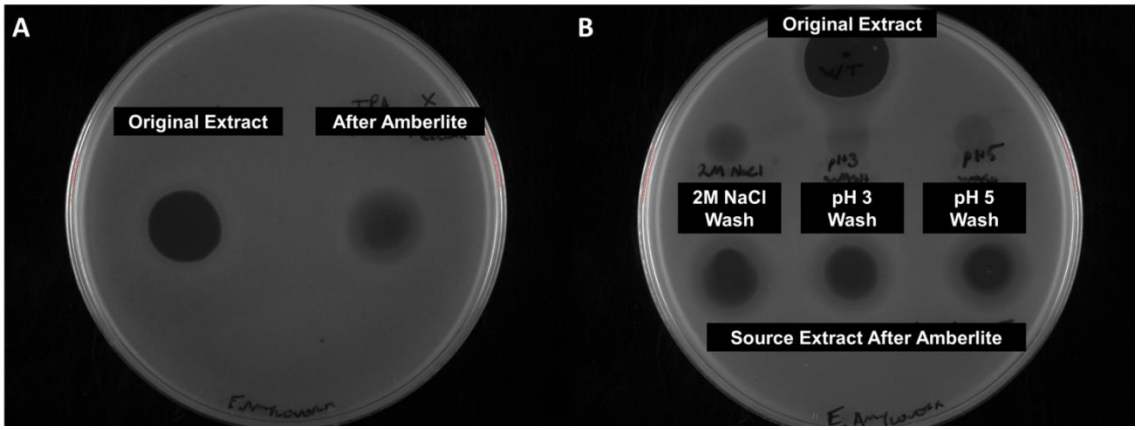

Figure 3.4: NAn-C extraction and isolation attempt using Amberlite IRA-410 Resin. A) 1mL of *B. contaminans* MS14 extract was combined with 0.5 mL slurry of Amberlite IRA-410, an anion exchange resin, and vigorously agitated for an hour. 10 uL of both the original and the Amberlite-exposed extract were tested against the indicator strain *E. amylovora* in an overlay assay. B) Attempts to extract the bactericidal compound from the Amberlite resin included washes with 2M NaCl, a pH3 and a pH5 buffer wash. These washes were tested for activity against the indicator bacterium *E. amylovora* in an overlay assay, along with post-amberlite exposed extracts.

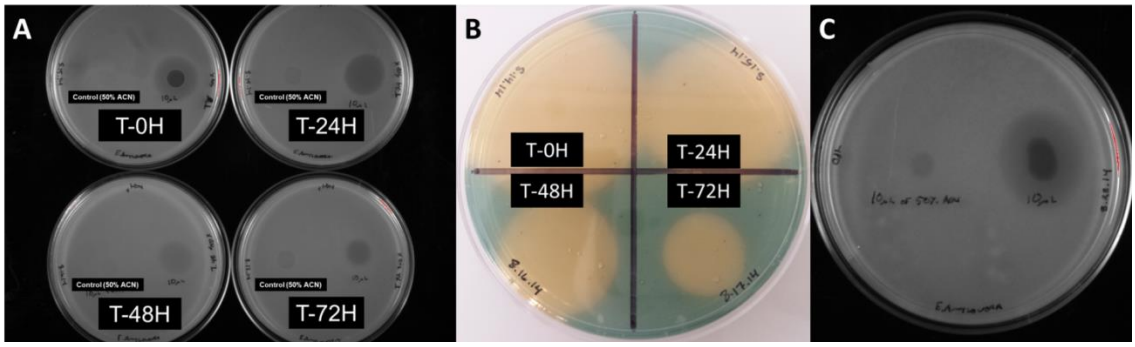

Figure 3.5: Dialysis of *B. contaminans* MS14 extract. A) *B. contaminans* MS14 extract was dialyzed using a CE cartridge (MWCO 500-1000 Da) for 72 hours in distilled water. At the 24, 48, and 72-hour time points, 10uL of the extract was withdrawn from the cartridge and stored to be tested later against the indicator bacterium *E. amylovora* in an overlay assay. Although bactericidal activity is observed at T-0, loss of bactericidal activity was observed at 24 hours, indicating that all of the bactericidal compound passed through the dialyzing membrane. The bacteriostatic activity of ornibactin is able to be observed at all time points. B) CAS plate corresponding to samples shown in image A showed that ornibactin diffused much slower across the membrane than NAn-C. Siderophore activity was still present in the 72-hour extract sample. C) After 72 hours of dialysis, the distilled water dialysate was lyophilized and reconstituted to a volume equal to the original dialyzed extract, then tested against the indicator bacterium *E. amylovora* in an overlay assay. Bactericidal activity was observed, indicating that NAn-C was successfully recovered. This indicates that the compound did not bind to the membrane and that the loss of activity was due to its rapid passage through the membrane.

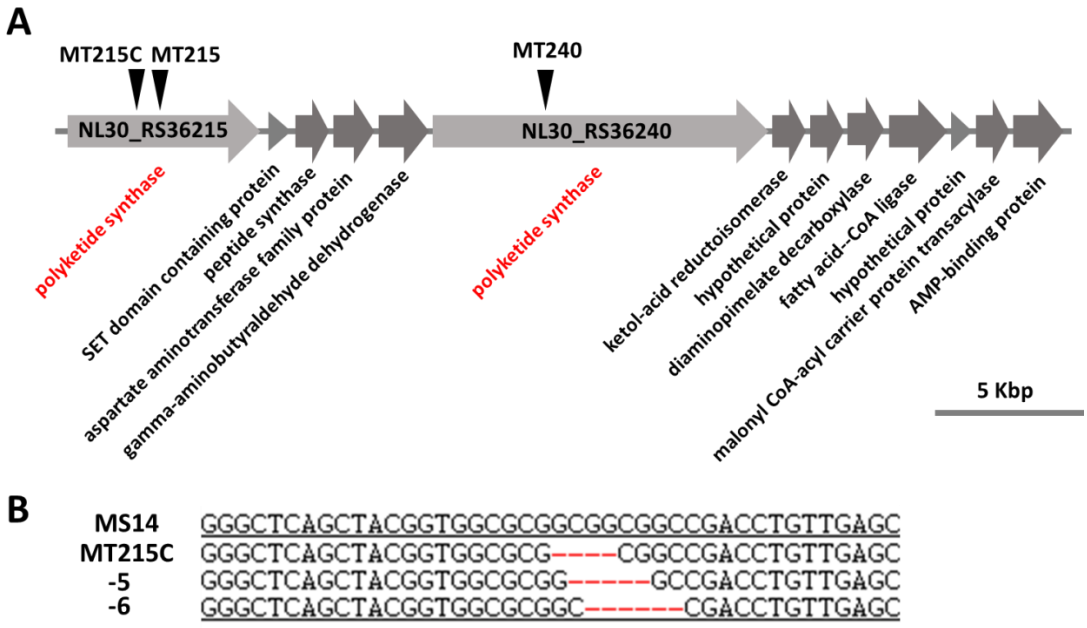

Figure 3.6: A: The 36 Kbp MS14 polyketide biosynthesis gene cluster. B: CRISPR-Cas9 mutagenesis sequence analysis at the targeting site of the gene NL30_RS36215 in the MT215C, MT215C-5, and MT215C-6 mutant strains. The figure was provided by Shien Lu's group (unpublished data).

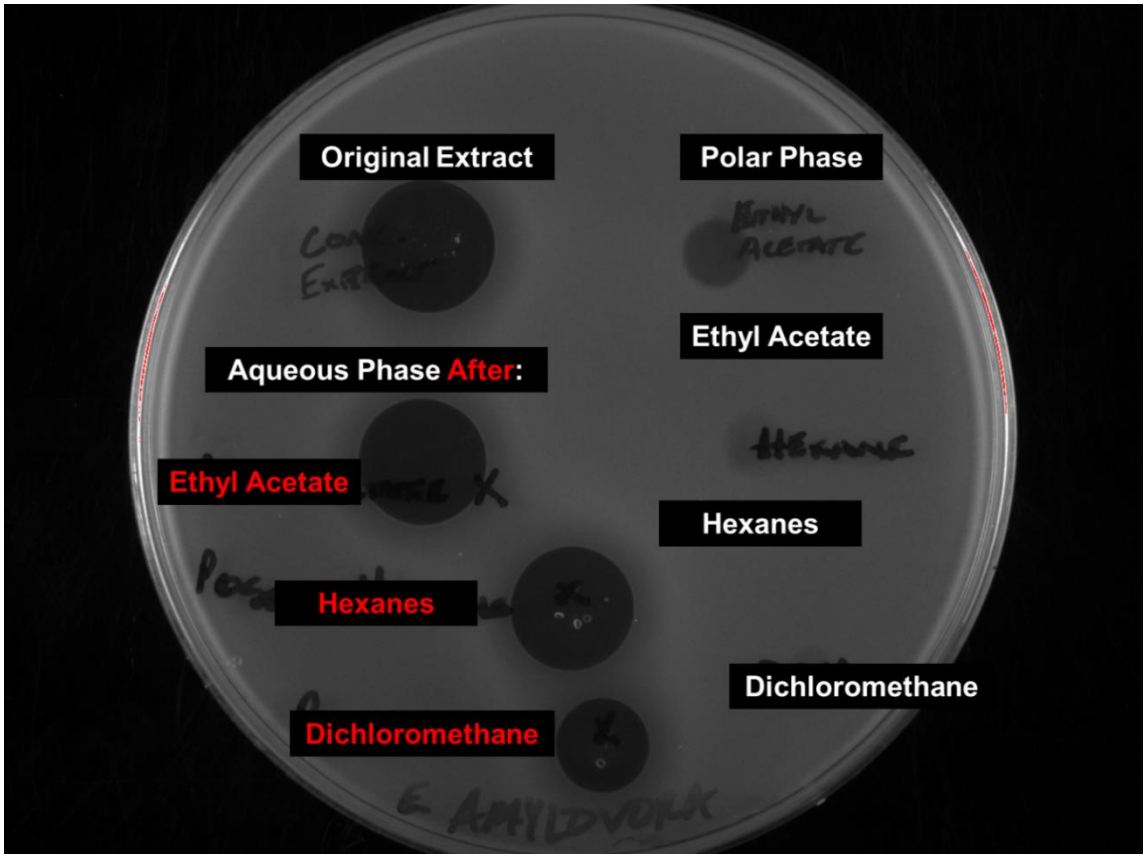

Figure 3.7: Kupchan extraction of NAn-C in the MS14 extract. A) Liquid to liquid extractions of cell-free *B. contaminans* MS14 spent media were performed following the protocol for malleilactone purification. In each extraction, successive washes of the aqueous extract phase were performed with non-polar solvents, in descending order. Following the extractions, all phases were evaporated/lyophilized and concentrated and tested for bactericidal activity against the indicator bacterium *E. amylovora* in an overlay assay. When compared to the original extract, all bactericidal activity remained in the aqueous phase with no activity being observed in any of the non-polar phases.

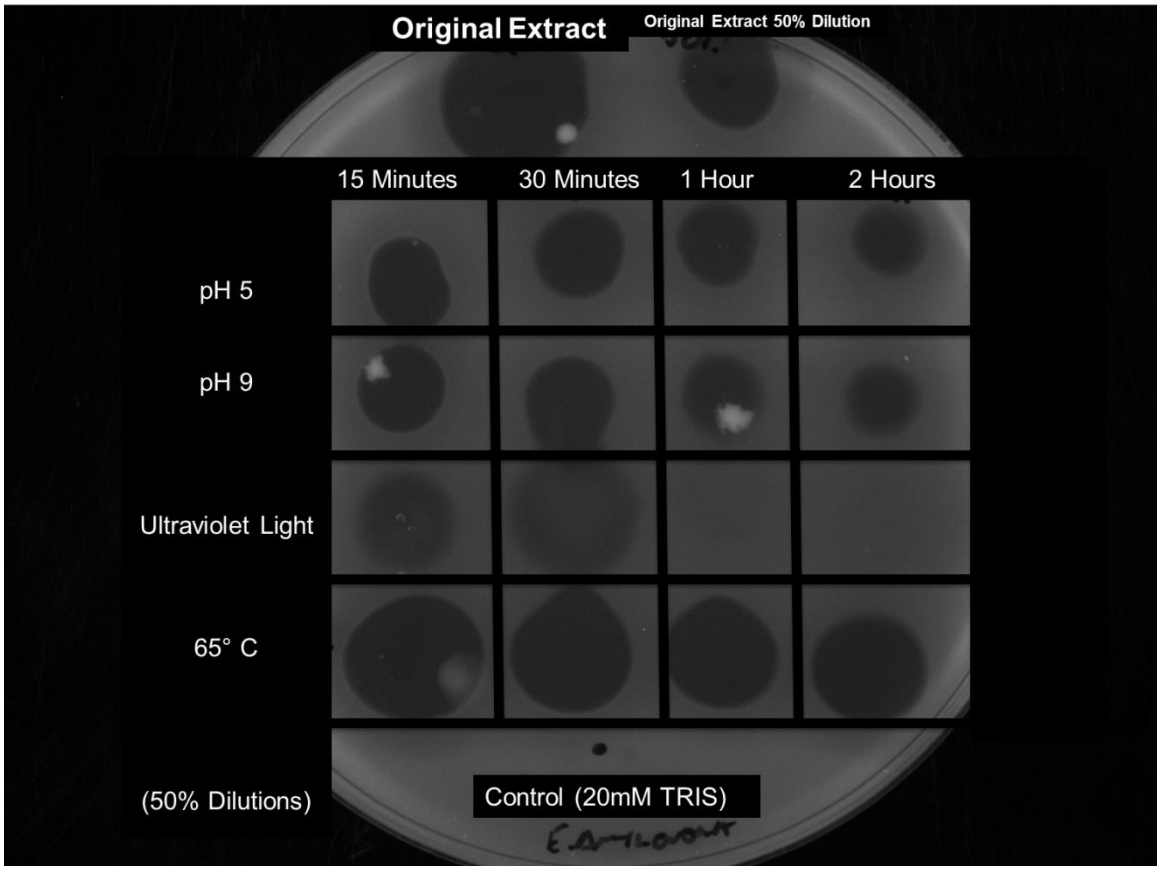

Figure 3.8: NAn-C stability assays using the MS14 extract. A) *B. contaminans* MS14 extract was exposed to acidic, basic, direct UV and 65°C conditions for two hours, with time points collected after 15, 30, 60 and 120 minutes and tested against the indicator bacterium *E. amylovora* in an overlay assay. Antibacterial activity was able to be observed in all samples tested, barring the sample exposed to ultraviolet light, which had attenuated activity after only 15 minutes. Complete loss of activity for this sample occurred somewhere between 30 to 60 minutes of exposure. Activity was persistent in all other variables tested, indicating that the NAn-C product of MS14 is highly stable in a variety (pH and high temperature) of other conditions.

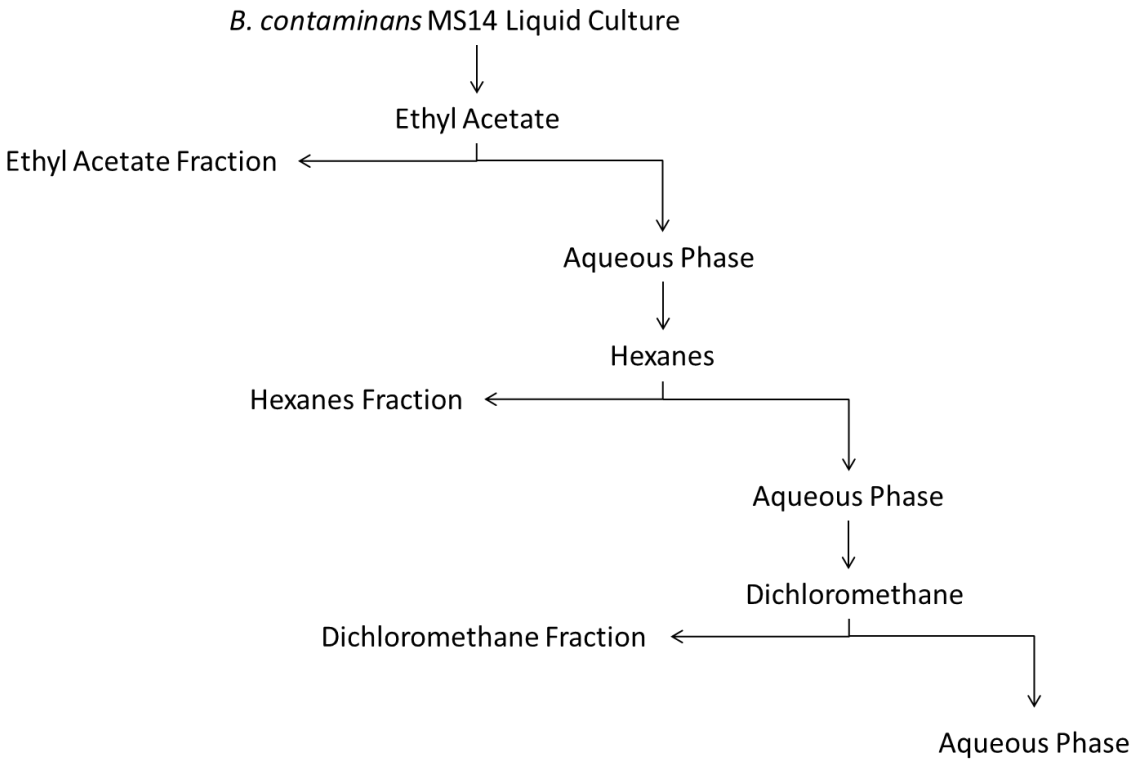

Figure 3.9: Diagram representing the modified Kupchan extraction. This protocol was adapted from the method used to extract malleilactone from *B. pseudomallei*. In the original protocol, malleilactone was found in the dichloromethane fraction, and it was predicted that NAn-C would be as well.

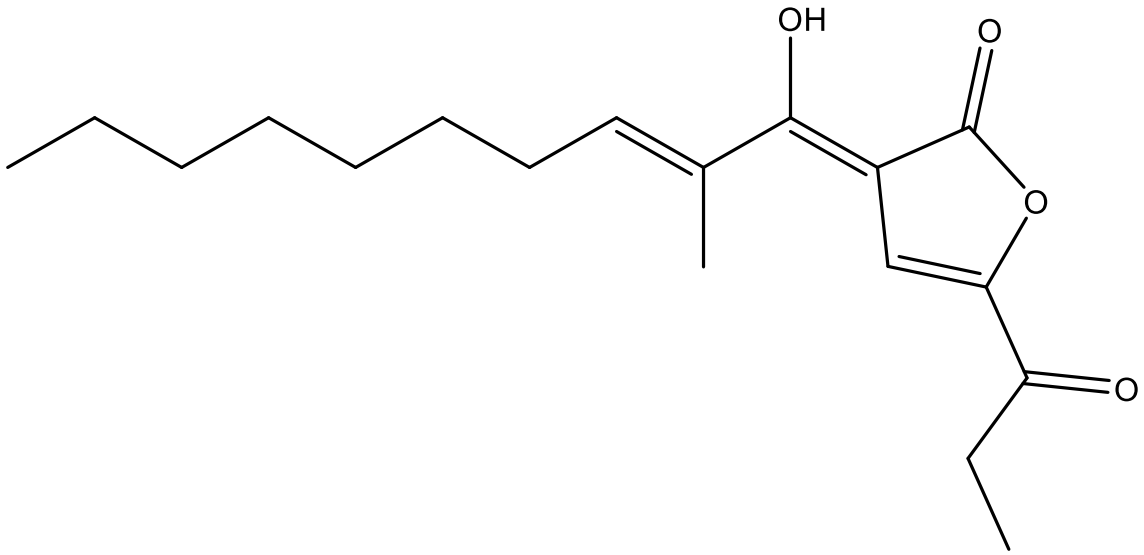

Figure 3.10: Malleilactone

4. CONCLUSION

The studies outlined in this thesis have highlighted the potential of the *Burkholderia* genus for the discovery of novel antimicrobial compounds. My thesis highlights the potential of *Burkholderia contaminans* MS14 to make novel antimicrobial metabolites, i.e. occidiofungins, ornibactin, and a novel bactericidal compound (NAn-C). There is a need for new treatment options as the ever-increasing prevalence of antibiotic-resistant pathogens, coupled with the declining rate of discovery for new antibiotic treatment options, could lead to a global crisis if not addressed. Further studies in this interesting genus could potentially lead to the development of novel therapeutics for treating infectious diseases.

Antibiotic resistant pathogens represent a substantial threat to nations worldwide, imposing loss of life, human suffering, and a drain of healthcare resources due to increased treatment cost and extended recovery times (2, 127). Antibiotic resistance also impairs the ability of agricultural systems to respond to blights amongst crops and outbreaks in livestock populations, posing a threat to regional and potentially global food supply lines. Due to the perceived high risk of investment into antibiotic research, investment into novel antibiotics by the world's largest pharmaceutical companies has fallen dramatically since its peak in the late 1980's; in 2020, only 1 of the 35 companies conducting research in antibiotic clinical development ranked among the top 50 pharmaceutical companies by sales (128). The need for novel antibiotics and funding for their research has been declared urgent by the World Health Organization, World

Organization for Animal Health, Food and Agriculture Organization of the United Nations, and the Organization for Economic Co-operation and Development in their joint report filed in 2017 (129, 130). If the discovery rate of novel antibiotics does not rebound, the impact of antibiotic resistance will continue to grow.

The use of bacterial and fungal-sourced medicines extends back to antiquity, however mass production of antibiotics from natural sources for commercial medicinal use is a concept that only goes back to the 20th century. These sources have brought civilization-changing medicines, with many treatments still prescribed today, such as the glycopeptide vancomycin, the macrolide erythromycin, or the most well-known beta-lactam antibiotic, penicillin. However, for every new antibiotic that makes it to clinical use, there are countless other that do not. The complications that arise with discovering new natural drug products are multi-faceted and are present at every stage of development. For example, identifying and locating producing species, establishing efficient culturing and purification procedures, and learning the spectrum of activity or mechanism of action can take several years to complete. Furthermore, identifying lead candidates for animal efficacy and toxicological studies often leads to the understanding of drug limitations. These studies further lead to efforts at synthesizing novel and more effective analogs and specialized formulations. Despite all of the knowledge and tools currently available for research, 1 in 5 drugs that make it into clinical trials still fail FDA approval. This is why it is crucial to identify potentially useful reservoirs of novel products that could have clinical implications and the *Burkholderia* genus appears to be an untapped resource.

With regards to antimicrobial production, *Burkholderia contaminans* MS14 is one of the most well studied strains of *Burkholderia*. Occidiofungin, which is produced by this strain, has been shown to be a potential therapeutic for fungal and parasitic infections (131), as well as having potent activity against several cancer cell lines (132). Several naturally occurring variants of occidiofungin are produced by the bacterium. Interestingly, the biosynthesis of occidiofungin involves two distinct thioesterases that lead to the formation of conformationally distinct products (133). Additional studies are needed to determine the differences and potential application that these naturally occurring analogs of occidiofungin for the treatment of infectious diseases and cancer. This stain also has a demonstrated bactericidal activity, in which the compound for the activity remains unknown. Remarkably, a novel function for a siderophore has been characterized in this strain. It has been shown to be essential for the production of an unknown polyketide antibiotic, suggesting that the siderophore is involved in the regulation of the product and not just being important for iron sequestration (36).

With the highly conserved genome amongst the *Burkholderia* genus, observations gleaned from one species can likely be applied to other species within the genus. There is evidence to support that secondary metabolite production can be impeded with slight manipulation to the host environment (36), suggesting that virulence factors in other more infectious species may also be attenuated in this manner. A better understanding of the environmental factors leading to the regulation of possible virulence factors may likely be learned from studying gene regulation within avirulent strains. This has broader applications than just *Burkholderia*, as it is also closely related

to the *Pseudomonas* genus and techniques learned could be applied to its species as well, such as *Pseudomonas aeruginosa*, the primary colonizer of cystic fibrosis patients (19).

With the rate of multi-drug resistant infections increasing, and the decrease in new antibiotic discovery, a more detailed examination of the members within the *Burkholderia* genus is more important than ever. Despite having two members tied to infectious diseases, many ot the hundreds more have been shown to have beneficial effects with growth promotion in crops, or be strong candidates for further studies aimed at isolating and characterizing the products being made. One of the larger challenges is overcoming the stigma associated with the more virulent members of the genus, as this causes some trepidation with regards to its widespread study. However, with new avenues needed for novel therapeutics, it is inevitable that *Burkholderia* genus will be studied more thoroughly. If successful, additional efforts studying *Burkholderia* could lead to the development of new antifungals and antibacterials for treating medically relevant infectious diseases. Further, the wide spread use of *Burkholderia* species may find its most promising use in promoting a sustainable food supply. The prevention of plant diseases caused by fungal diseases, bacterial diseases, and pathogenic nematodes may be accomplished by using tailored strains of *Burkholderia*. The genus requires additional attention towards identifying novel metabolites and research efforts aimed toward understanding their application for plant and human diseases.

My work has laid the foundation for future studies that could lead to new insights into novel therapeutics: there is a new potent antibacterial present in the production of NAn-C. Additionally its manufacture being closely tied to the production of ornibactin,

and its subsequent elimination when ornibactin production is halted, opens up the question if other secondary metabolites can be manipulated in a similar manner. Specifically, the question remains if these concepts can be applied to virulence factors of other species that also rely on ornibactin as it is shown to have a role beyond iron-sequestration. The possibility of using a novel approach such as this to attenuate virulence is exciting indeed.

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