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Mechanism of Anti-Virulence Compound 187R Inhibiting Pseudomonas Aeruginosa Type III Secretion System

Liwei Fang
University of Wisconsin-Milwaukee

MECHANISM OF ANTI-VIRULENCE COMPOUND 187R INHIBITING *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM

by

Liwei Fang

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ABSTRACT

MECHANISM OF ANTI-VIRULENCE COMPOUND 187R INHIBITING *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM

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Liwei Fang

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Antibiotics have been widely used for treating bacterial infectious diseases. However, the rapidly emerging of antibiotic resistance has dramatically decreased the efficacy of antibiotics and poses a serious worldwide crisis. In addition, the cell components serving as antibiotics' targets are conserved in many different bacterial species, as a result, antibiotic treatments disrupt the host microbiota and negatively influence the hosts health condition. Therefore, new alternative strategies for fighting infectious diseases without causing antibiotic resistance and disturbing the host microbiota are needed. Type 3 secretion system (T3SS) is a highly conserved virulence factor presents in many different Gram-negative pathogens. It is required for pathogens such as *P. aeruginosa*, surviving and initiating infection in their hosts. Therefore, targeting the T3SS is a promising alternative strategy for developing new antimicrobial therapies without disrupting the hosts' microbial community. Here, we identified a potent T3SS inhibitor, designated 187R, which strongly inhibits the expression of *P. aeruginosa* T3SS. Our data suggests that 187R inhibits T3SS expression through reducing the T3SS master regulator ExsA at the post-translational level. The impact of this anti-virulence compound on the hosts' microbial community was also tested using *Arabidopsis thaliana* phyllosphere as a model. We demonstrates that compared to the traditional antibiotics, our T3SS inhibitor 187R can preserve the microbial community better than antibiotics.

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Chapter 1 : Introduction

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a facultative anaerobic, Gram negative, rod-shaped bacterium which belongs to the family of gammaproteobacteria. It is able to survive under a wide range of temperatures from 4°C to 42°C. The genome analysis of the PAO1 strain shows that many genes function as substrate uptake or catalysis, which enable the bacteria to use various substances as nutrients (1). The bacterium is also able to survive under anaerobic conditions either by utilizing nitrate or nitrite as alternative external electron acceptors or by fermenting arginine or pyruvate as its energy source (2). All these features allow *P. aeruginosa* to adapt to different types of environments such as soil and water.

P. aeruginosa is an opportunistic pathogen causing acute and chronic infections in immunocompromised patients (3). It is a major cause of nosocomial infections which affects 2 millions patients per year. Cystic fibrosis (CF) patients are the group most vulnerable to *P. aeruginosa*. The pathogen is the leading cause of morbidity and mortality in CF patients. In healthy individuals, the infection caused by *P. aeruginosa* tends to occur after skin or eye damage (4). Moreover, the bacteria is able to form robust biofilm on the surface of medical devices which causes biofilm-mediated infections such as ventilator-associated pneumonia and catherter-associated urinary tract infection (5). The biofilm is hard to remove from the medical devices and triggers chronic infections. The only solution for stopping the biofilm-associated infection is to replace the medical devices (6). Hospital-acquired infections caused by *P. aeruginosa* have become a serious concern as the pathogen is resistant to different types of antibiotics. As a result, the bacteria is hard to eradicate from the patients and causes serious health issues (7).

Pathogenesis of *P. aeruginosa*

P. aeruginosa has a variety of virulence factors that lead to successful infections. Previous research showed that *P. aeruginosa* isolates from patients with acute infections are generally motile and capable of secreting toxins that cause host cell damage (8). These studies indicate that motility and toxin secretion are important for *P. aeruginosa* acute infections. Flagellar and type IV pili are bacterial cell surface virulence factors that are responsible for twitching, swimming and swarming motilities (9). These virulence factors enable *P. aeruginosa* to search for optimal niches. Type IV pili and flagella also mediate the adhesion to the airway epithelia cells, which is the first step towards colonization and disease initiation (10).

The secretion systems are also crucial for the virulence of *P. aeruginosa.* This pathogen possesses five different classes of secretion systems, all of which enable the pathogen to secrete different types of toxins into the environment or into host cells (11). In *P. aeruginosa,* two Type 1 secretion systems (T1SS) named T1SS (Apr) and T1SS (Has) have been discovered (12). The first T1SS, T1SS (Apr), secretes alkaline protease protein which interferes with complement activation thereby preventing phagocytosis and the death of *P. aeruginosa* (13). The second T1SS, T1SS (Has) secretes the heme acquisition protein which is crucial for iron acquisition from the environment (14). Type 2 secretion systems (T2SS) are important for the virulence of *P. aeruginosa* as the system secretes toxins such as elastase and exotoxin A that are believed to cause extensive tissue damage during the infection. It has also been proven that the T2SS is highly similar to type 4 pilus assembly system (T4PS) and can assemble type 2 pseudopilus. The presence of type 2 pseudopilus on *P. aeruginosa* increases the bacteria's adhesive ability and elevates the virulence of the bacteria (15). Type 3 secretion system is a needle like structure that translocates the effector proteins (toxins) from bacteria cytoplasm into the host cell. The effector proteins cause host cell death and tissue damage (16). The Type 5 secretion system not only secrets toxins such as patatin-like proteins (PLPs) (17), but is also

critical for the cell to cell adhesion and biofilm formation (11). The type 6 secretion system (T6SS) delivers the toxin into other bacteria cells. T6SS of *P. aeruginosa* also helps it compete with the other bacteria and survive in the microbial community (18).

T3SS is a primary acute virulence factor

Type 3 secretion system (T3SS) is considered the primary acute virulence factor for *P. aeruginosa* (19). Previous research showed that in both chronic and acute infection models, the presence of fully functional T3SS is associated with increased mortality rates (20). The increased severity of disease is not only due to tissue damage caused by the T3SS effector protein, but also by the cytotoxicity towards the immune cells such as the macrophages (21). Additionally, *P. aeruginosa* T3SS is involved in killing amoebae. Since predation by phagotrophic protists is the major cause of bacterial populations decreasing in many ecosystems, a fully functional T3SS is required for the bacteria's higher survival rate in the environment (22).

Structure of T3SS

T3SS is a needle-like structure that directly translocates the effector proteins (toxins) from the bacterial cytoplasm into the host cells (23). This multi-protein complex apparatus is composed of a needle-like injectisome and a basal body (Fig. 1.1A). The needle-like structure anchors on the basal body, which extends through the inner membrane, peptidoglycan layer and outer membrane. The basal body and the needle structure is built up under the non-secreting condition. Once the bacteria make contact with the host cell, PopB, PopD and PcrV are secreted directly by the T3SS. These three proteins forms translocation pores on the host

cell plasma membrane which allows effector proteins (toxins) transferred directly into the host cell through the translocation apparatus into the host cytosol (24).

Effector proteins of P. aeruginosa *T3SS*

In *P. aeruginosa*, four effector proteins named ExoS, ExoT, ExoY and ExoU have been discovered. These four effectors normally are not all present in one single strain; either ExoS or ExoU are absent in different strains (25). Studies of the T3SS effector proteins showed that these four effectors have different enzyme activity. As the most potent T3SS effector, ExoU showed strong phospholipase/lysophospholipase activity and resulted cell lysis through destroying host cell membrane (25). Two other effector proteins ExoS and ExoT share highly similar amino acids sequences. Both are bifunctional proteins with an GTPase-activating N-terminal domain and adenosine diphosphate ribosyltransferase (ADPRT) C-terminal domain. After injection, these two effector proteins work together, manipulating the host cell signaling transduction and causes host cell rounding and cell apoptosis. ExoY is an nucleotidyl cyclase toxin that contains ATP binding domains (26). After injection into the host cell cytoplasm, ExoY is activated by host cell filamentous actin and increases the intracellular levels of several cyclic nucleotides (27). The increased intracellular levels of cyclic nucleotide induces the cell death and causes tissue damage (28).

The regulatory mechanism of *P. aeruginosa* T3SS

T3SS master regulator ExsA

The expression of *P. aeruginosa* T3SS regulon genes is under the control of the transcriptional activator ExsA (29). ExsA is an AraC family transcriptional regulator which contains a DNA binding domain (30). The master regulator ExsA directly binds to the DNA

consensus sequence (TNAAAANA) in the target gene promoter regions and activates the transcription of T3SS genes (31).

The activity and function of ExsA is post-translationally regulated by the ExsCEDA partner switch regulatory cascade (Fig. 1.1B) (32). Under non-secreting condition, anti-activator ExsD binds to the master regulator ExsA and form a 1:1 complex (33), the binding of ExsD to ExsA deactivates the master regulator ExsA (34). The activity of ExsD is modulated by anti-anti-activator ExsC. ExsC is post translationally regulated by the secret regulator ExsE. Under non-secreting conditions, ExsE binds to ExsC and prevents the anti-anti-activator binding to ExsD. Upon T3SS triggering by a low calcium level or contact to the host cell, ExsE is secreted out and frees the anti-anti-activator ExsC. ExsC then binds to the anti-activator ExsD, thus releasing ExsA and activating the transcription of T3SS (33).

Transcriptional regulation of ExsA

The transcription of *exsA* is a fine-tuned response to various of environmental changes and nutrient sources. The low calcium environment and presence of preferred carbon source can activate the transcription of T3SS master regulator ExsA, thus triggering the expression of T3SS genes (35, 36). The major regulatory pathways illustrated in Fig. 1. 2.

The expression of *exsA* can be activated through two different promoter regions (*exsC* promoter region and *exsA* promoter region) in *exsCEBA* operon. *ExsA* promoter region is controlled by Vfr (Virulence factor regulator), a homolog of *Escherichia coli* CRP (cAMP-activated global transcriptional regulator) transcriptional activator. In a low calcium environment, the adenylate cyclase CyaB (the major adenylate cyclase in *P. aeruginosa* required for the bacterial virulence) responds to the low calcium level and synthesizes cyclic-AMP. Vfr responds directly to the increasing intracellular cyclic-AMP levels. The Vfr protein binds directly to cAMP and undergoes conformational change. The Vfr-cAMP complex then activates *exsA* transcription via binding to *exsA* promoter region (37, 38).

ExsC promoter is ExsA dependent. The master regulator ExsA autoregulates its own expression via *exsC* promoter region (39). Besides ExsCEDA regulatory cascade, the transcription of *exsCEBA* is fine-tuned by the Cbr/Crc regulatory pathway. CbrA/B is a two-component regulatory system. The sensor kinase CbrA responds to non-preferred carbon sources such as mannitol and activates the response regulator CbrB. CbrB then triggers the gene expression of a non-coding regulatory RNA gene *crcZ*. The non-coding regulatory RNA CrcZ, RNA chaperone Hfq, and Catabolite repression control protein (Crc) form a complex and deactivate Crc activity (40). Since Crc positively regulates T3SS via enhancing the transcription of *exsCEBA* operon, deactivating Crc reduces the expression of T3SS. Although the gene expression profile analysis of *crc* mutant showed that Crc may regulate T3SS through various pathways, the underlying regulatory mechanism of Crc to T3SS is largely unknown. (36)

Post-transcriptional regulation of T3SS

RsmA (Repressor of Secondary Metabolism) is a Csr (Carbon storage repressor) family protein (41). In *P. aeruginosa,* RsmA is crucial for regulating different virulence factors such as T3SS, T6SS and motility (19, 42, 43). It is also the key regulator for switching the life style of *P. aeruginosa* from planktonic to biofilm through regulating the secondary messenger molecule cyclic-di-GMP (44).

The post-transcriptional regulator RsmA plays an important role in modulating T3SS. Previous research demonstrated that deletion of *rsmA* leads to a defect in *exsA* at the posttranscriptional level (45). The regulation of T3SS by RsmA is similar to the Cbr/Crc regulatory pathway. GacS/GacA two component system regulates the T3SS via activating the transcription of non-coding regulatory small RNAs RsmY and RsmZ. These two non-coding regulatory small RNAs directly bind to, and deactivate RsmA (46), decreasing the intracellular

levels of the master regulator ExsA and reducing the T3SS gene expression. There are also other regulatory components such as AlgZR two component system and MgtE which regulate T3SS via Gac/Rsm system. AlgZR two component system controls the alginate synthesis. Once this two component system is activated, the response regulator AlgR increase RsmY/Z levels through unknown mechanisms and decreases the expression of T3SS (45). The magnesium transporter MgtE can also inhibit T3SS via activating the GacS/GacA two component system and increasing the RsmY/Z level (47). These previous studies indicate the Gac/Rsm system is crucial for regulating *P. aeruginosa* T3SS.

The translation of ExsA mRNA relies on the RNA helicase DeaD which is encoded by the gene PA2840 in PAO1 strain (48). The deletion of *deaD* causes greatly reduced ExsA protein levels and results in defects in the virulence of *P. aeruginosa* (49). *P. aeruginosa* DeaD is an RNA helicase of the DEAD-Box protein family. This group of RNA helicases are present in a wide range of bacteria and archaea. In prokaryotic cells, DEAD RNA helicases either unwind short duplex RNA or stimulate the RNA degradation (50). Bioinformatic analysis and genetic experiments showed that 37nt of *exsA* mRNA 5'-UTR forms an inhibitory structure that prevents translation, however, RNA helicase DeaD relieves the structure and allows the translation of *exsA* mRNA (48).

Other regulatory components regulate T3SS

There are several other regulatory components that regulate T3SS - Quorum sensing and sigma factors RpoN negatively control the T3SS (51). A gene designated as *ptrB* (related to DNA damage) suppresses the T3SS under the stress of DNA damage (52), and the multi-drug efflux pump inhibits the T3SS (53). In addition, some environmental signals such as the oxygen and NO level can also affect the expression of *P. aeruginosa* T3SS (54, 55). However, the exact mechanism is unknown.

The crisis of antibiotic resistance

Antibiotic resistance has become a critical public health crisis worldwide. In some areas of the world, superbugs resistant to all existing antibiotics have been isolated (56). Therefore, alternative anti-infection therapy with new inhibitory mechanisms and lower occurrence of antibiotic resistance are needed. Futhermore, since lots of evidence shows that microbiota is crucial for the health of their host (57, 58), the preservation of microbiota during infectious disease treatments has become a major concern during the development of new anti-infection therapy.

Antibiotic resistance

Antibiotic therapy is the most effective treatment for bacterial and fungal infectious diseases. The current antibiotic therapy targets cellular components which are essential for cell survival (i.e. bacterial cell wall and bacteria ribosome), however, the strong selective pressure leads to the development of antibiotic resistance (59). Currently, antibiotic resistance has been found in nearly all commercially available antibiotics (56). Antibiotic resistance leads to higher medical cost, prolonged hospital stays and increased mortality. In the United States, antibiotic resistance causes more than 23,000 deaths per year (60). A recent study conducted in America demonstrated that from 2002 to 2014, the share of antibiotic resistant bacterial infections rose from 5.2% to 11%. The increased antibiotic resistance costs more than $2 billion annually in the nation (61).

P. aeruginosa is intrinsically resistant to antibiotics. Low cell wall permeability, outer membrane and efflux systems all contribute to *P. aeruginosa*'s innate antibiotic resistance. All of the major classes of antibiotics treating *P. aeruginosa* infection need to cross the bacterial cell wall to reach their target, however, the low cell wall permeability limits the amount of

antibiotics that can cross the cell wall. The outer membrane is another barrier that restricts antibiotic penetration. Moreover, *P. aeruginosa* has at least four different antibiotic efflux pump systems capable of efficiently exporting antibiotics from the cytoplasm. These three resistance mechanisms combine together and keep the intracellular antibiotic concentration at low levels. *P. aeruginosa* can also acquire resistance genes through other organisms or via mutation in chromosomal genes (62).

Human microbiota and antibiotic therapy

Microbiota refers to all the microbes inhabiting many sites in and on multicellular organisms (hosts). The microbiota contains host-specific microbes with various of functions. It is essential for the health of the hosts in several different ways. It provides nutrients to the hosts, represses pathogens and parasites, and stimulates the development of the host's immune system (63, 64). Recent studies done in fish models have proved that the life span of older fish can be extended after inoculation of healthy gut microbiota from young fish (65). Other investigations on the human microbiome suggest that the microbiota is associated with the severity of infectious diseases (66, 67).

The use of antibiotics, especially the broad spectrum antibiotics, affects a large amount of different bacteria species in microbiota and decreases the taxonomic richness, diversity and evenness of host-associated microbiota. As a result, negatively impact the health status of hosts. For example, disrupting the human microbiota can lead to some serious health problems such as gut microbial disorders correlating with *Clostridium difficile* infection (68) and inflammatory bowel disease (69). The recovery of microbiota after treatment with antibiotics can take years which affects long term health of the patients (70).

Targeting T3SS

As the T3SS is the primary acute virulence factor in *P. aeruginosa*, it is not only important for initiating and increasing the severity of the disease via the effector protein, but also critical for the survival of *P. aeruginosa* in the early stages of infection (71). Thus, inhibiting the T3SS is an excellent strategy for developing new anti-infection therapy. Several studies have shown that disarming the pathogen's T3SS reduces bacterial virulence and disease severity (20). Moreover, targeting the virulence factors of the pathogen has the potential advantage to minimize the damage to the host microbiota (72).

In our lab, we screened a compound library and looked for compounds which inhibits *P. aeruginosa* T3SS but not the bacterial growth. We discovered a potent *P. aeruginosa* T3SS inhibitor named 187R that can silence the T3SS. In this research, we determined the molecular mechanism of 187R inhibiting the T3SS. It has been long discussed whether anti-virulence drugs can preserve the microbiota better than antibiotics. In this research, we have also explore the impact of anti-virulence compound 187R on the microbiota of *Arabidopsis thaliana*, one of the hosts of *P. aeruginosa*.

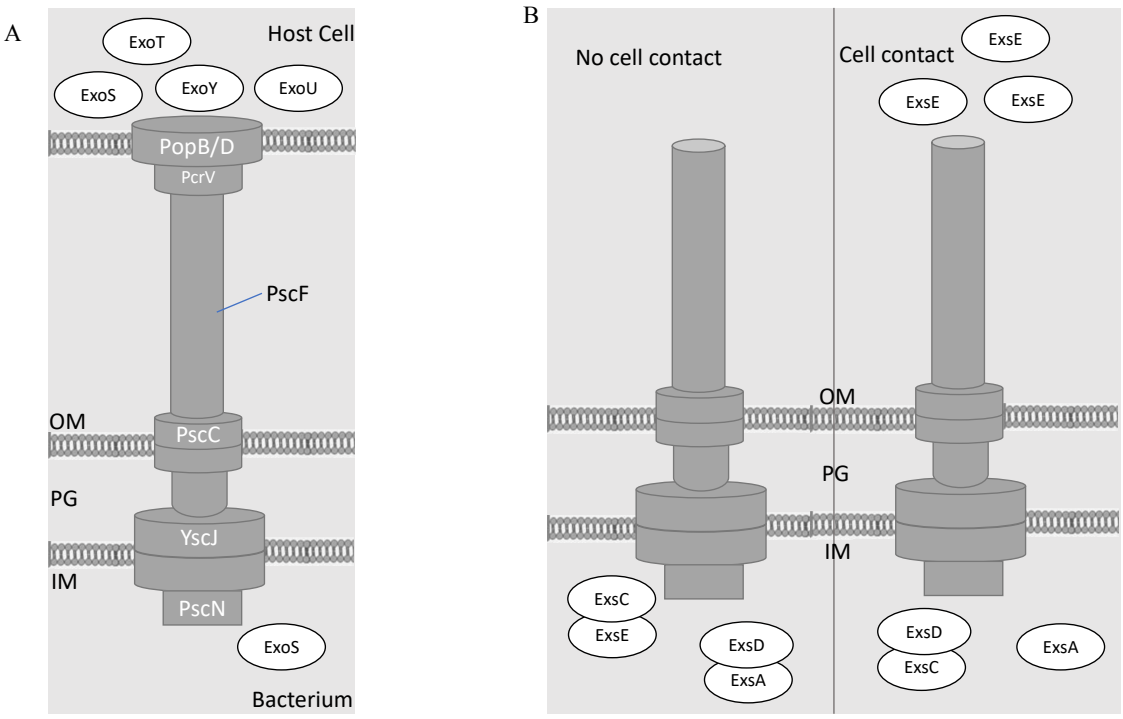

Figure 1.1 The structure and regulatory of T3SS (A) T3SS secretion system of *P. aeruginosa*. (B) ExsCEBA regulatory cascade. ExsA is the master regulator of T3SS. Under non-secretion conditions, ExsE binds to ExsC, ExsD binds to ExsA and deactivate ExsA. Once in contact with the host cell, T3SS secretes ExsE and releases ExsC. ExsC then binds to ExsD and frees ExsA, as a result, activating the T3SS gene expression. (Galle M. et, al., 2012) IM: inner membrane; PG: periplasm; OM: outer membrane.

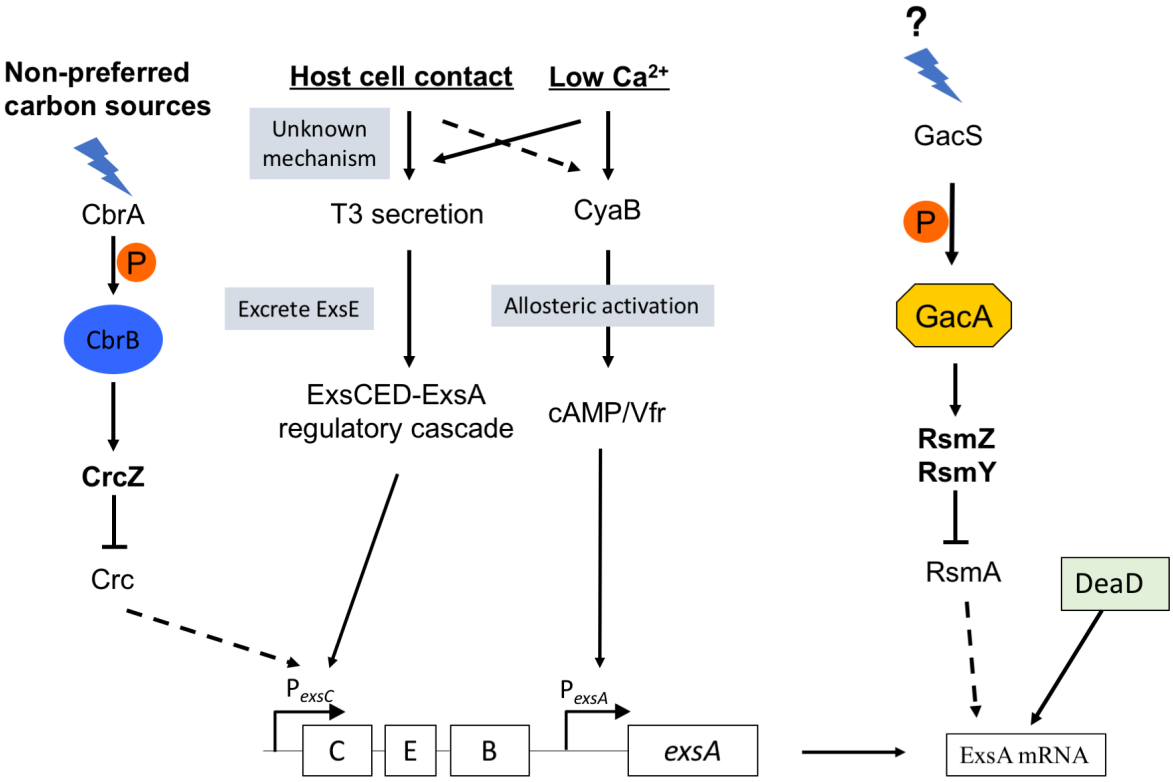

Figure 1.2 Major regulatory pathways modulating T3SS in *P. aeruginosa*. CbrA/CbrB two component system activates *CrcZ* non-coding small RNA and deactivates Crc which positively regulates T3SS. ExsCEDA regulatory cascade positively regulates T3SS by free ExsA. These two pathways activate the T3SS via activating the transcription of the *exsCEBA* operon through the *exsC* promoter. Vfr-cAMP responds to the increasing intracellular cAMP level and binds to the *exsA* promoter region and activates *exsA* expression. Similar to the Cbr/Crc pathway, two-component system GacS/GacA activates the transcription of two non-coding small RNA and deactivates the posttranscriptional regulator RsmA which positively regulates T3SS. DeaD is the RNA helicase required for stimulating the translation of ExsA mRNA. Dashed line indicates that the regulatory mechanism is not completely understood.

Chapter 2 : Discovery of new T3SS inhibitor and its mechanisms of T3SS inhibition

Abstract

Antibiotics have been widely used for treating bacterial infectious diseases. However, traditional antibiotic therapies target the essential cell components, and this strong selective pressure leads to the antibiotic resistance. To overcome or evade the emerging antibiotic resistance crisis, new alternative strategies for fighting the bacterial infection is needed. *Pseudomonas aeruginosa* is an opportunistic human pathogen resistant to a variety of commonly used antibiotics. According to World Health Organization (WHO) report, *P. aeruginosa* is one of the three 'top priority antibiotic-resistant pathogens' that pose the greatest threat to human health. Therefore, new antimicrobial agents with new inhibitory mechanisms are urgently needed for treating *P. aeruginosa* infection. Type 3 secretion system (T3SS) is the major virulence factor of *P. aeruginosa* required for the initiation of infection and bacterial survival in the epithelial airway. Therefore, targeting T3SS is a promising alternative strategy for new drug development. In this study, we identified a potent T3SS inhibitor, designated 187R, inhibit T3SS but not the growth of *P. aeruginosa* by screening a compound library. The compound greatly reduced the T3SS expression and attenuated *P. aeruginosa* T3SS-mediated cytotoxicity towards HeLa cells. Examining the regulatory components and pathways of *P. aeruginosa* T3SS with the presence of the 187R showed that 187R silences the *P. aeruginosa* T3SS through post-translationally decreasing protein level T3SS master regulator ExsA. The inhibitory mechanism of 187R on ExsA is independent of the known major T3SS regulatory pathways.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes acute and chronic infections in immunocompromised patients (3). A primary virulence factor for this pathogen is the type III secretion system (T3SS). T3SS is a needle-like structure that directly translocates

the effector proteins (toxins) from the bacterial cytoplasm into the host cells (23). In *P. aeruginosa*, four effector proteins named ExoS, ExoT, ExoY and ExoU have been discovered (25). These effector proteins manipulate signal transduction in the host cells, resulting in attenuated host immune responses and increased severity of disease (28).

P. aeruginosa T3SS consists of over 40 genes organized within 10 transcriptional units. The expression of all 10 transcriptional units are under the control of master regulator ExsA - an AraC family transcriptional activator (30). Two monomers of ExsA bind to the consensus sequence (TNAAAANA) in the target gene promoter regions to recruit RNA polymerase and activate the transcription of T3SS genes (31). The activity and function of ExsA is post-translationally regulated by the ExsCEDA regulatory cascade (32). While triggered by the low calcium level or contact to the host cell, ExsE is secreted which frees the anti-anti-activator ExsC. ExsC then binds to the anti-activator ExsD which releases ExsA to turn on the expression of the T3SS (33). ExsA also autoregulates the transcription of itself by binding to the promoter region of *exsCEBA* operon (39). Besides the ExsCEDA regulatory cascade, *exsA* is modulated by several other pathways including the Vfr-cAMP pathway, Cbr/Crc pathway, and RsmY/Z/A pathway (35, 55, 73). In a low calcium environment, Vfr (Virulence factor regulator) responds directly to the increasing intracellular cyclic-AMP levels and activates *exsA* transcription via binding to *exsA* promoter region (37, 38). Catabolite repression control protein (Crc) is another positive regulator that regulates *exsA* through enhancing *exsCEBA* expression (36). The activity of Crc is controlled by non-coding regulatory small RNA CrcZ (74). The expression of *crcZ* is triggered by the CbrA/B two-component system which senses and responds to non-preferred carbon sources such as mannitol. The non-preferred carbon sources stimulate the two component system and activates the transcription of *crcZ.* CrcZ RNA binds to Crc protein and deactivates the protein with the assistance of RNA chaperone Hfq (75, 76).

The post-transcriptional regulator RsmA also plays an important role in modulating the T3SS. Similar to the Cbr/Crc regulatory pathway, the GacS/GacA two component system regulates the T3SS via activating the transcription of non-coding small RNAs RsmY and RsmZ. These two small RNAs deactivate RsmA which is the posttranscriptional activator for T3SS (46). Although the regulatory mechanism is not well understood, previous research demonstrated that deletion of *rsmA* showed a defect in ExsA at the posttranscriptional level (45). Another important regulatory component stimulates the translation of *exsA* independent of RsmA is RNA helicase DeaD. DeaD unwinds 5' UTR of ExsA mRNA and allows the imitation of translation. (48). The deletion of *deaD* greatly reduced ExsA protein levels and resulted in defect in the virulence of *P. aeruginosa* (49).

Since Alexander Fleming discovered penicillin in 1928, antibiotic therapy has been widely used for treating bacterial infections. However, the current antibiotic therapy targets the cellular components which are essential for cell survival (i.e. bacterial cell wall and bacteria ribosome). The strong selective pressure leads to the development of antibiotic resistance (59). Currently, antibiotic resistance has been found in nearly all commercially available antibiotics (56). *Pseudomonas aeruginosa* is resistant to a variety of commonly used antibiotics such as carbapenem. According to World Health Organization (WHO) report, *P. aeruginosa* is one of the three 'top priority antibiotic-resistant pathogens' that pose the greatest threat to human health (77). New antimicrobial agents with new inhibitory mechanisms are urgently needed for treating *P. aeruginosa* infection. In addition, antibiotic therapy can alter human microbiota and affects host health status in the long term. In some cases, disturbing the human microbiota is associated with several serious diseases; for example, gut microbial disorder correlates with *Clostridium difficile* infection (68) and inflammatory bowel disease (69).

Therefore, alternative anti-infection therapies, which can preserve the microbiota and lower the occurrence of antibiotic resistance, are needed. T3SS is a major virulence factor in *P.*

aeruginosa. It is not only critical for initiating the infection but also responsible for killing the hosts' immune cells. Therefore, inhibiting T3SS decreases the bacterial survival in hosts and reduces the severity of the diseases (71). Moreover, targeting the virulence factors of the pathogen has the potential advantage of minimizing the damage to the host microbiota (72).

Various T3SS inhibitors, including several small-molecule inhibitors and a monoclonal antibody, have been developed during the last decade. These T3SS inhibitors target for different T3SS components, such as the extracellular structural components, effector proteins and intracellular regulatory proteins (78). One group of extracellular structural components used as targets of the inhibitors is the traslocon proteins. Previous research showed that the Phenoxyacetamide inhibitors and monoclonal antibody KB001-A target the needle structural protein PscF and needle tip protein PcrV, respectively (21, 79). The interaction of the compound and needle structural protein block the translocation of the effector proteins, greatly reducing the virulence of *P. aeruginosa.* The small molecule Pseudolipasin is able to inhibit the ExoU PLA_2 activity and ExoS ADP-ribosyltransferase activity respectively (80, 81). In vitro experiments have proved that these two anti-effector molecules neutralize the toxicity of T3SS effector and protect the CHO cells. However, these two strategies all have their limitations since 1) inserting the needle of T3SS itself can cause damage to the host cells, and 2) different *P. aeruginosa* strains carry different sets of effector proteins (23). An attractive target for developing T3SS inhibitors is the master regulator ExsA. Since all the *P. aeruginosa* T3SS genes are ExsA-dependent, inhibiting the synthesis or activity of ExsA would lead to the decrease or complete elimination of all T3SS gene expression.

In the present work, we screened a compound library and search for compounds inhibit *P. aeruginosa* T3SS but not the bacterial growth. We discovered a potent *P. aeruginosa* T3SS inhibitor named 187R. The compound strongly inhibits the T3SS while not affecting the growth of *P. aeruginosa*. In vitro HeLa cell infection assay suggested 187R reduced T3SS-mediated

cytotoxicity in the assay. Further research demonstrated that 187R dramatically decreased the protein level of ExsA through post-translational mechanism.

Materials and Methods

Bacterial strains, plasmids, primers, and growth condition.

Bacterial strains and plasmids used in this study are listed in Table 2.1. *E. coli* and *P. aeruginosa* were routinely cultured in LB medium at 37°C unless otherwise stated. LB supplemented with 200 mM NaCl and 10 mM nitrilotriacetic acid (NTA) were used as the T3SS inducing medium. Antibiotics were used at the following concentrations: 100 μg/mL of carbenicillin (Cb), 10 μg/mL of gentamicin (Gm), 10 μg/mL of tetracycline (Tc) for *E. coli* and 100 μg/mL of Cb, 50 μg/mL of Km, 50 μg/mL of Gm and 30μg/mL of Tc for *P. aeruginosa.* Primers used for PCR are listed in Table 2.2.

Screening for *P. aeruginosa* T3SS inhibitors

The *P. aeruginosa* strain PAO1 harboring *exoS-gfp* (Green fluorescent protein) transcriptional fusion reporter plasmid was used for screening the T3SS inhibitors. Overnight culture of PAO1 harboring the reporter plasmid at a 1:1000 was inoculated into T3SS inducing medium with 250 μM of the screening compound. *P. aeruginosa* treated with DMSO (solvent for screening compounds) was used as negative control. Bacteria were cultured at 37°C for seven hours before harvesting. The harvested bacterial cells were diluted in PBS and the GFP intensity was measured using fluorescence-activated cell sorter (FACS) flow cytometry (BD Biosciences, CA).

Western blotting

5μL of PAO1 overnight culture was inoculated into 5 mL T3SS inducing medium with 250 μM of the screening compound. DMSO was used as control. Bacterial cell pellets and supernatant were separated by centrifugation at seven hours post-inoculation. For measuring the ExoS and ExsA protein in the bacteria cell, the cell pellets were resuspended in 50 μL of PBS. 50 μL of 2X SDS-page buffer was added into each sample and followed by boiling for 10 minutes. For measuring the secreted ExoS protein level in the supernatant, the supernatant collected from the previous step was centrifuged again to remove any remaining cells. Trichloroacetic acid was added into the supernatant to reach the concentration of 10% and followed by centrifugation at 13,500 rpm for 30 min to collect the secreted protein. The protein pellets were resuspended in 2x SDS-page buffer. The protein sample from equal amount of cells were loaded on SDS page gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane and probed with a rabbit polyclonal antibody against ExsA or a chicken polyclonal antibody against ExoS. A mouse antibody against RNA polymerase was used as control for the total protein quantity.

HeLa cell cytotoxicity assay

T3SS-mediated cytotoxicity was determined by a cell lifting assay. HeLa cells (1×10^5) were seeded in each well of a 12 well plate and cultured for 18 hours at 37°C with 5% CO^2 in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, penicillin (100 Units/mL), and streptomycin (100 μg/mL). Before the infection assay, the HeLa cell culture medium was removed from the well and the cells were washed twice with PBS (Phosphate Buffered Saline). HeLa cell culture medium without antibiotic supplemented with 250 μM of compound or DMSO, was added into each well. Bacteria were cultured overnight in LB medium at 37 °C. After collecting the overnight culture by centrifugation. The bacteria cell pellet was washed with PBS and resuspended in DMEM. HeLa cells were then

infected with the bacteria at MOI (Multiplicity of infection) of 30. Three and half hours post infection, the medium were removed and the HeLa cells were stained by crystal violet after rinsing twice with PBS. The plate was washed twice with PBS and then 200 μL of 95% ethanol was added to the wells for dissolving the crystal violet. The OD490 of the ethanol solution with dissolved crystal violet was measured to determine the cells that were attached to the surface.

Mutant strain and reporter plasmid construction

To construct the *deaD* deletion mutant, a PCR fragment consisting of the flanking region of ~900 bp up and down stream of *deaD* were cloned into the HindIII and BamHI restriction site of pEX18GM. The plasmid was transferred to *P. aeruginosa* PAO1 strain by conjugal mating as previously described (82).

The GFP reporter plasmid was constructed by cloning promoter regions of *exsC, exsA, exoY* and *exoT* into pProbe-AT, a broad host range vector with a promoterless GFP (green fluorescent protein) (83), respectively. The promoter activity was monitored by measuring GFP intensity using flow cytometry as described earlier.

For constructing the *tssA1'-'lacZ*, *exsA'-'lacZ* and *exsCEBA'-'lacZ* translational fusion reporter plasmid, 5'UTR and first 25 codons of *tssA1* gene, 5'UTR and first 52 codons of *exsA,* region between *exsC* transcriptional start site to 52 codons of *exsA* were amplified by PCR, respectively, and cloned into the translational *lacZ* fusion vector PSW205 (84). The forward oligonucleotide primers for constructing these two translational fusion reporters were engineered to include an EcoRI restriction site and a *lacUV5* promoter. Since there is no *lacI* gene in *P. aeruginosa,* IPTG inducing is not necessary. The expression of the translational fusion reporters were measured by β-Galactosidase activity as previously described (51).

qRT-PCR analysis

The mRNA levels of *exsA* were measured by qRT-PCR. Bacterial cells cultured in T3SS inducing medium for 7 h were harvested. RNeasy mini kit (Qiagen, Hilden, Germany) was used to isolate the total RNA. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA level of *exsA* gene was quantified by qRT-PCR using SYBR green master mix (Applied Biosystems, CA). Data were analyzed using a Relative Expression Software Tool (85). The expression level of *rpsL* was used as an endogenous control for data analysis (36).

Motility assay

Swarming. Swarming plates were prepared as previous described (86). 187R (250 μM) or DMSO were adding into the medium. *P. aeruginosa* from overnight LB agar was inoculated on the plate. After overnight incubation at 30°C. The diameters were measured.

Twitching. LB broth solidified with 1% (wt/vol) agar was used for twitching motility. *P. aeruginosa* was stab inoculated to the bottom of the petri-dish. 187R (250 μM) or DMSO was added into the medium. After inoculation for 16 hours at 37°C, the medium was removed from the plate. The zone of motility at the agar/Petri dish interface was measured after staining with crystal violet.

Biofilm assay

Quantification of biofilm was performed in 96 well microtiter plates. Bacterial cells from overnight cultures were inoculated at 1:100 dilution into T3SS inducing medium or ABTGC medium (87). The plate was incubated overnight at 37°C without shaking. Biofilms were stained with 0.1% crystal violet and were washed with water to remove unbound dye, and the

crystal violet was dissolved in 95% ethanol and the absorbance at 570 nm was measured. For each treatment, six independent experimental repeats were performed.

Minimal inhibition concentrations (MICs)

Determination of the minimum inhibitory concentration (MIC) for antibiotics was performed in LB broth as previously described (88).The lowest antibiotic concentration that showed no growth was considered as the MIC.

β-Galactosidase Assays

β-Galactosidase assays were carried out in triplicate. The activity was determined as described (89). The samples were collected by centrifuge. The cell pellets were rinsed and resuspended in PBS to washed off the compound. The expression levels of the reporter plasmids were determined by normalization of β-Galactosidase activity (OD_{420}) to the bacterial growth (OD_{600}).

Statistical Analysis

Two-tailed student's t-test was used to assess the inhibition of 187R on the T3SS gene expression. One way analysis of variance (ANOVA) combined with Tukey-HSD were applied for multiple comparisons. The test was performed by statistic software R (90).

Results

Screening inhibitors of *P. aeruginosa* T3SS

To discover the compound inhibit T3SS but not the growth of *P.* aeruginosa, we screened a small molecule compound library. The compound library was consisted by salicylic acid, its

precursors and their analogues. A total of 264 small molecule compounds were screened for identifying the inhibitors of *P. aeruginosa* T3SS.

For monitoring the expression of *exoS* under the T3SS inducing condition (200 mM NaCl and 10 mM nitrilotriacetic acid), a GFP reporter carrying the *exoS* promoter region (59) was used in the screening process. Each compound was dissolved in DMSO and was added into the medium at a concentration of 250 μM. Bacteria cells were harvested seven hours post inoculation and the Mean Fluorescence Intensity (MFI) was measured by flow cytometry. The MFI of DMSO (vehicle dissolve the compound) treated reporter strain was used as control. Among all the compounds screened, compound 187 strongly inhibited the expression of *exoS* (90% inhibitory at concentration of 250 μM) in contrast to the DMSO treatment. Further analysis of two different 187 conformational isomers showed that only 187R inhibits the *exoS* while 187S does not (Fig. 2.1A). Also, no inhibition of bacterial growth was observed upon the addition of 250 μM of compound 187R (Fig. 2.1B). The dose-dependent effectiveness of 187R on *P. aeruginosa exoS* promoter activity was examined in a T3SS inducing medium supplemented with different concentrations of 187R. As shown in Fig. 2.1C, the minimal concentration of 187R required to reach its maximum inhibition of the *exoS* promoter activity is 35 μM.

187R impaired T3SS expression and reduced the virulence of *P. aeruginosa*

To further examine the effect of 187R on *P. aeruginosa* T3SS, the expression levels of two effector encoding genes, *exoY* and *exoT*, were measured. The results indicated that the promoter activities of both genes were decreased when exposed to 187R (Fig.2.2A and B). In addition, a Western blot was performed to determine the ExoS protein (48.3kDa) level in the cell pellet and the supernatant. An anti-ExoS polyclonal antibody was used in the Western blot. Similar to previous studies the anti-ExoS polyclonal antibody also cross-reacted to the ExoT (59, 91)

showing another band at around 48kDa. As shown in Fig. 2.2C, consistent with the *exoS* and *exoT* promoter activity, ExoS and ExoT protein levels were reduced in the presence of 187R in both bacteria culture supernatant and cell pellets.

Previous reports showed that *P. aeruginosa* T3SS translocates effector proteins into the cytoplasm of host cells and causes cell rounding, lifting, and death (92, 93). The infected cells detach from the monolayer culture, which allows us to evaluate the efficacy of T3SS inhibitors by a HeLa cell lifting assay (93). Briefly, the HeLa cells were infected with *P. aeruginosa* at a multiplicity of infection (MOI) of 30 in the presence of 187R. We added DMSO as a control. After incubation for three and half hours, the cell culture was rinsed twice with PBS and the living cells attached to the surface were stained with crystal violet. After removing the unbound crystal violet, 200 μL of ethanol was added. The dissolved crystal violet was measured by absorbance at a wavelength of 490 nm. In this assay, we used PAK strain since 1) it has higher and earlier T3SS expression than the PAO1 strain (94); 2) its T3SS repressed by 187R. A T3SS defect strain, *pscJ* mutant strain, was used as a control. *pscJ* is a gene which encodes a lipoprotein component of the T3SS basal substructure of the needle complex. It is required for the assembly of a functional T3SS (26). Consistent with the previous research (71), deletion of *pscJ* greatly decreased virulence compared to wild type PAK. In addition, adding 187R significantly reduced the cytotoxicity of PAK wild-type while showing no T3SS-mediated cytotoxicity to HeLa cells. The cell morphology after *P. aeruginosa* inoculation also confirmed that T3SS-mediated cell rounding greatly reduced when 187R was supplemented (Fig. 2.3B-D). In conclusion, these results demonstrated 187R is a potent *P. aeruginosa* T3SS inhibitor.

187R did not affect Vfr-cAMP pathway

Transcriptional regulator protein Vfr (virulence factor regulator) activates *P. aeruginosa* T3SS through cyclic AMP dependent pathway. Previous research demonstrated that Vfr-cAMP

complex autoregulates *vfr* gene expression by directly binding to its own promoter region (35). To test if 187R inhibits T3SS through Vfr-cAMP pathway, *vfr* promoter activity was measured in the present of 187R. *vfr* mutant was used as a control in the experiment. In agreement with the previous research, mutation of *vfr* greatly reduced *vfr* promoter activity, indicating that Vfr is involved in autoregulation (Fig. 2.4A). However, in the wild-type strain, 187R did not alter the *vfr* promoter activity compared to the DMSO control (Fig. 2.4A). This result suggested that 187R does not inhibit *P. aeruginosa* T3SS through Vfr-cAMP pathway. To further confirm this conclusion, we tested the *exsA* promoter activity in the presence of 187R. Previous research states that Vfr-cAMP complex directly binds to T3SS master regulator *exsA* promoter region and activates the transcription of *exsA*. The low *exsA* promoter activity in *vfr* mutant was observed as previously described (38), which proved that Vfr positivly regulates *exsA* promoter activity. However, we found that there were no significant changes in *exsA* promoter activity when treated with 187R (Fig. 2.4B). These results suggested that 187R did not affect the Vfr-cAMP pathway.

187R did not inhibit T3SS through Cbr/Crc pathway

P. aeruginosa regulates T3SS through the Cbr/Crc pathway in which Crc (catabolite repression control protein) serves as a positive regulator of T3SS and modulates the transcription of *exsCEBA* (36). Therefore, if 187R inhibits T3SS through the Cbr/Crc pathway, *exsC* promoter activity should reduce. Consistent with the previous results, in a *crc* mutant strain, *exsC* promoter activity greatly reduced compared to the wild type (Fig 2.5A). However, we found that the *exsC* promoter activity was not altered when treated with 187R (Fig. 2.5B). Crc represses the translation of *amiE,* which encodes an aliphatic amidase (74). Using the *amiE'-'lacZ* translational fusion reporter, we found that *amiE'-'lacZ* expression did not show significant differences between DMSO and 187R treated wild type strains (Fig. 2.5C). Similar

to previous research, there was a significant increase of *amiE'-'lacZ* expression in the *crc* mutant strain (74). In conclusion, these results confirmed that 187R did not inhibit T3SS via the Cbr/Crc pathway.

187R increases the RsmY level in *P. aeruginosa*

RsmY and RsmZ small RNAs play crucial roles mediating various cellular processes including T3SS in *P. aeruginosa* (45). We first tested the mRNA levels of two regulatory small RNAs RsmY and RsmZ via qRT-PCR. As shown in Fig. 2.6, *rsmY* RNA level increased dramatically (four-fold increase in 187R-treated bacteria) while *rsmZ* RNA level has a moderate decreased in the presence of 187R. This result suggested that *rsmY* could be the potential target causing the inhibition of T3SS.

187R inhibits *P. aeruginosa* T3SS not through RsmY/Z/A regulatory pathway

Since *rsmY* small RNA inhibits T3SS by adjusting the activity of RsmA protein, we tested the activity of RsmA. Previous studies showed that increased RsmA activity elevates T3SS and represses T6SS at the post-transcription level. As a repressor for T6SS, RsmA binds to the GGA motif of *P. aeruginosa* T6SS mRNAs to repress the translation of T6SS genes. The *tssA1* is a gene that encodes a T6SS structural protein. It has a GGA motif and its translation is repressed by RsmA (95, 96). To evaluate the RsmA activity, a *tssA1'-'lacZ* translational fusion reporter was constructed by cloning the 5' UTR and the first 25 codons of *tssA1* into pSW205. The *tssA1'-'lacZ* translational fusion reporter is driven by *lacUV5* promoter. If 187R inhibits T3SS via RsmY/Z/A regulatory pathway, an increasing *tssA1'-' lacZ* expression level will be observed in the presence of 187R. However, as shown in figure 2.7A, the expression level of *tssA1'-'lacZ* decreased moderately in the presence of 187R, suggesting elevated RsmA activity. This data demonstrated that the 187R did not inhibit T3SS through RsmY/Z/A regulatory pathway. To further confirm the increased *rsmY* small RNA in the presence of 187R did not

inhibit the T3SS expression, we checked the T3SS expression under a *rsmYrsmZ* double mutant background within the presence of 187R. The result showed that 187R still strongly inhibits the *exoS* promoter activity (Fig. 2.7B), which confirmed that 187R did not inhibit T3SS through RsmY/Z/A regulatory pathway.

187R inhibits T3SS not through reducing Lon protease activity

Since the increasing RsmY did not affect T3SS, we examined the other regulatory components that can increase RsmY RNA levels and are capable of inhibiting T3SS independent of RsmA. One possible cellular component that may cause the increase of *rsmY* but not *rsmZ* RNA and inhibits the T3SS independent of RsmY/Z/A regulatory pathway is the Lon protease (97). Lon protease is an important enzyme involved in the protein quality control in microorganisms and required for full virulence of *P. aeruginosa* (98). Previous research suggested that Lon protease not only positively regulates T3SS through unknown mechanisms, but also negatively regulates the *rsmY* mRNA level through adjusting the protein level of RNA chaperon Hfq (99). If the compound 187R suppressed the T3SS through inhibiting Lon protease, the RNA chaperon Hfq would increase and elevate the *rsmY* but not *rsmZ* RNA level (100). Since Lon protease represses the expression level of quorum sensing autoinducer synthetase *rhlI* (101), we first performed qRT-PCR to find out if the mRNA level of *rhlI* is increased in the presence of 187R. The qRT-PCR results showed that *rhlI* mRNA levels decreased while adding 187R in the medium (Fig. 2.8). Lon protease is also involved in fluoroquinolone class antibiotic resistance. Mutation of *lon* resulted a four times increasing of ciprofloxacin susceptibility compared to the wild type (102). If 187R inhibits Lon protease, we would observe the reduction of ciprofloxacin MIC while adding 187R. However, the MIC of ciprofloxacin did not change after adding 187R (Table 2.3). These results demonstrate that 187R does not inhibit T3SS through Lon protease.

187R inhibits T3SS not through increasing the secondary messenger c-di-GMP level

Besides the Lon protease, another potential candidate that may cause the increasing *rsmY* (but not *rsmZ*) mRNA level is the diguanylate cyclase (DGC) HsbD. HsbD positively regulates *rsmY* transcription through increasing the intracellular c-di-GMP level (103). C-di-GMP is an important intracellular secondary messenger that regulates multiple virulence factors. T3SS is one of the virulence factors modulated by c-di-GMP. Although the mechanisms how c-di-GMP regulates T3SS is not completely understood, c-di-GMP negatively regulates T3SS. To measure the c-di-GMP level in the presence of 187R, we examined the *cdrA* promoter activity, which is positively regulated by the intracellular c-di-GMP level (104). Our result showed that *cdrA* promoter activity did not increase in the presence of 187R (Fig. 2.9A). In *P. aeruginosa*, increasing intracellular c-di-GMP represses swarming motility but promotes twitching motility (103). In order to further confirm that intracellular c-di-GMP level was not affected by 187R, the twitching and swarming motility of *P. aeruginosa* was examined in the presence of 187R. Our results showed that twitching motility (Fig. 2.9B-C) did not change while adding 187R in the medium. These results suggested that 187R did not inhibit the T3SS through increasing intracellular c-di-GMP level.

187R inhibits T3SS through post-translationally regulating the master regulator ExsA

For elucidating the impact of 187R on master regulator *exsA*, Western blots of ExsA was performed using anti-ExsA antibody. Western blot of ExsA protein showed that the ExsA protein level decreased dramatically in the presence of 187R (Fig. 2.10A). Since our results also indicated that *exsA* and *exsC* promoter activity was not altered by 187R (Fig. 2.4B & Fig. 2.5B), we investigated the inhibitory mechanisms of 187R on *exsA* at post-transcriptional level. We measured the *exsA* mRNA in the presence of 187R or DMSO using qRT-PCR. As shown

in Fig. 2.10B, the ratio of mRNA level in the presence of 187R and DMSO is close to one, revealing the similar *exsA* mRNA level in the presence of 187R or DMSO.

An alternative hypothesis to account for 187R decreasing ExsA protein level could be that 187R hampered the translation of *exsA* mRNA. To test this hypothesis, we first tested if 187R affects the activity of RNA helicase DeaD. DeaD is the major RNA helicase that stimulates *exsA* translation independent of RsmA (48). To examine whether 187R inhibits the T3SS via DeaD, we first determined the T3SS expression level under the *deaD* mutant background. The *exoS-gfp* transcriptional fusion reporter was introduced into the *deaD* mutant strain. Consistent with previous research, ExsA-dependent *exoS* gene expression was extremely low in the *deaD* mutant, indicating the T3SS expression was abolished in this mutant (Fig. 2.11A). Therefore, we were unable to compare the T3SS expression between 187R and DMSO treatments under *deaD* mutant background.

An alternative strategy for testing whether DeaD is affected by 187R is observing the growth of bacteria at low temperature. *P. aeruginosa deaD* is a homologue of *E.coli deaD* which is essential for low temperature growth (50). In order to confirm *deaD* plays the similar role in *P. aeruginosa*, we measured the growth of *P. aeruginosa deaD* mutant strain under low temperature conditions (16°C) in both T3SS inducing medium and non-inducing (LB) medium. Similar to the *E.coli deaD* mutant, *P. aeruginosa deaD* mutant showed a growth defect at 16°C but not 37°C (Fig. 2.11B-D). Therefore, if 187R inhibits *exsA* translation by reducing DeaD activity, a reduced growth at 16°C would be observed when 187R was added into the growth medium. However, adding 187R did not affect the growth of the wild type strains at 16°C in both T3SS inducing and non-inducing conditions (Fig. 2.11C-D). As a result, we conclude that 187R does not inhibit T3SS through affecting the RNA helicase DeaD.

The transcription of *exsA* can be activated by ExsA through the *exsC* promoter region and Vfr-cAMP through the *exsA* promoter region, therefore, generating *exsCEBA* polycistronic

mRNA and *exsA* mRNA. Since the regulatory mechanisms of these two different mRNAs have not been completely understood, two different translational fusion reporters, *exsCEBA'-'lacZ* and *exsA'-'lacZ,* were constructed. As show in the Fig. 2.12A, *exsCEBA'-'lacZ* translational fusion reporter was constructed by cloning the region between the transcriptional start site of *exsC* to 52 codons of *exsA* into pSW205. *exsA'-'lacZ* translational fusion reporter was constructed by cloning the 5' UTR of *exsA* and 52 condons of *exsA* into pSW205 (Fig. 2.12A). These two *exsA'-'lacZ* and *exsCEBA'-'lacZ* translational fusion were driven by foreign *lacUV5* promoter. In the presence of 187R, the expression of *exsA'-'lacZ* and *exsCEBA'-'lacZ* translational fusion reporter were not reduced (Fig. 2.12B-C). In conclusion, the results above suggested that compound 187R suppresses ExsA protein but it is not through decreasing the transcription and translation of *exsA*.

187R inhibits T3SS not through nitrate reductase (*NirS*)

We also studied whether 187R inhibits the T3SS via nitrate reductase NirS which is required for the maximal virulence of T3SS (54). Previous study showed supplementation with sodium nitroprusside (SNP) as NO donor restored T3SS in a *NirS* mutant (54). Therefore, if the compound inhibits T3SS via *NirS,* supplementation with SNP as an NO donor would restore the T3SS with the presence of 187R. As shown in Fig. 2.13, supplementation with SNP did not restore the T3SS expression in the presence of 187R. In addition, the *NirS* mutant only showed decreased T3SS-mediated cytotoxicity when the MOI = 0.1. In our HeLa cell lifting assay, we used much higher MOI (MOI=30) for our assay and 187R showed significant inhibition of T3SS-mediated cytotoxicity. Therefore, *NirS* is not the target of 187R.

187R inhibits T3SS not through ppGpp

5'-diphosphate-3'-diphosphate (ppGpp) is another bacteria secondary messenger that regulates the virulence of bacteria. Although the regulatory mechanisms is not clear, this

secondary messenger is also involved in motility, biofilm formation and T3SS. In a ppGpp deficient strains, the swarm motility and biofilm formation are greatly reduced. Since we did not observe a reduced swarming motility in the presence of 187R, the compound probably did not affect the T3SS by reducing ppGpp level. Our biofilm formation assay (Fig. 2.14A-B) also found that the biofilm formation was not altered by 187R in both T3SS-inducing medium and ABTGC medium. These results suggested that 187R did not inhibit the T3SS by decreasing ppGpp.

Discussion

P. aeruginosa is resistant to many commonly used antibiotics. Since the T3SS is essential for initiating the acute infection and responsible for bacterial survival in epithelial airway (71), it has been considered as a potential alternative target for the development of new antimicrobial therapies. In this study, we screened a compound library to look for the anti-virulence compounds that inhibit *P. aeruginosa* T3SS but do not inhibit the growth of the bacteria. We found a potent T3SS inhibitor, 187R, that can suppress the *P. aeruginosa* T3SS. Our data suggested that the compound reduces the T3SS-mediated cytotoxicity towards HeLa cells. Further research proved that adding the compound did not inhibit T3SS through the major regulatory pathways including Cbr/crc, Vfr/cAMP and RsmY/Z/A regulatory pathway. We also confirmed that 187R did not inhibit T3SS through Lon protease, diguanylate cyclase (DGC) HsbD, or nitrate reductase NirS. The impact of the compound 187R on the master regulator ExsA was also assessed. Our data demonstrated that 187R dramatically reduced protein level of ExsA without inhibiting *exsA* gene expression and translation. These results indicate that there may be an unknown regulatory component that post-translationally regulates ExsA protein levels independent of the major regulatory pathways.

In this study, supplementation of 187R greatly reduced the protein level of ExsA and dramatically decreased the expression of ExsA-dependent genes such as *exoS* and *exoY*. However, the reduced ExsA protein level did not decrease the *exsC* promoter activity and *exsA* mRNA level. The phenotype was probably caused by the high binding affinity of ExsA to *exsC* promoter region (29). Therefore, the promoter activity of *exsC* was not affected by the decreased ExsA protein level. In addition, we found that although adding 187R elevates the RsmY RNA level, the increased RsmY RNA level did not sequester the RsmA and resulted defected *exsCEBA'-'lacZ* expression. A plausible explanation for this observation is that 187R may increase the protein level of the RNA chaperone Hfq. While Hfq and RsmA shared overlapping binding sites on *rsmY* small RNA, the binding affinity of Hfq to *rsmY* small RNA is higher than RsmA to *rsmY* small RNA. As a result, although the binding of Hfq to RsmY increased the RNA stability, it prevents RsmA from binding to *rsmY* small RNA (105). A further study on the effect of 187R on Hfq is needed to confirm the above hypothesis.

P. aeruginosa uses various planktonic virulence factors to initiate the infection in tissues (106). When the bacterium is under stress or unfavorable conditions, it turns off the planktonic virulence factors and switches the lifestyle from planktonic to biofilm. Eventually, the bacterium establishes the biofilm-based chronic infection. Clinically, treating the biofilm-associated infections is a tremendous challenge due to its higher tolerance to antibiotics and lower susceptibility to the host immune response (107). While *P. aeruginosa* shifts from planktonic lifestyle to biofilm, some regulatory components switch acute virulence factors and biofilm formation. For example, RsmA, the positive T3SS posttranscriptional regulator, negatively regulates biofilm formation through various regulatory pathways including controling intracellular c-di-GMP level (44). Previous research conducted in a murine lung infection model showed that although disrupting *rsmA* reduced the acute virulence factors, the mutant showed increased biofilm formation and increased pulmonary inflammation in a

chronic infection model (44, 108). Therefore, while screening the anti-virulence compounds like T3SS inhibitors, it is important to avoid the compounds impacting the cellular components that inversely control acute virulence factors and biofilm formation such as RsmA. Our data suggests that 187R did not inhibit T3SS via reducing RsmA activity or increasing c-di-GMP level. Further experiments showed that 187R did not promote the biofilm formation compared to the DMSO control (Fig. 2.14). Our results suggest that while 187R strongly inhibits the T3SS of *P. aeruginosa,* the compound does not increase biofilm formation.

Taken together, our research suggested that the compound 187R inhibits *P. aeruginosa* T3SS through reducing ExsA protein level at post-translational level. After examining the current known mechanism, we found that 187R did not inhibit T3SS through major known regulatory pathways or components. These results indicate that there is an unknown T3SS regulatory pathway way that regulates ExsA protein at the post-translational level. Moreover, 187R has not only shown inhibition to T3SS, further research suggested that 187R does not promote the other virulence factors such as motility, c-di-GMP and biofilm formation. Overall, 187R is a potential compound for further drug development.

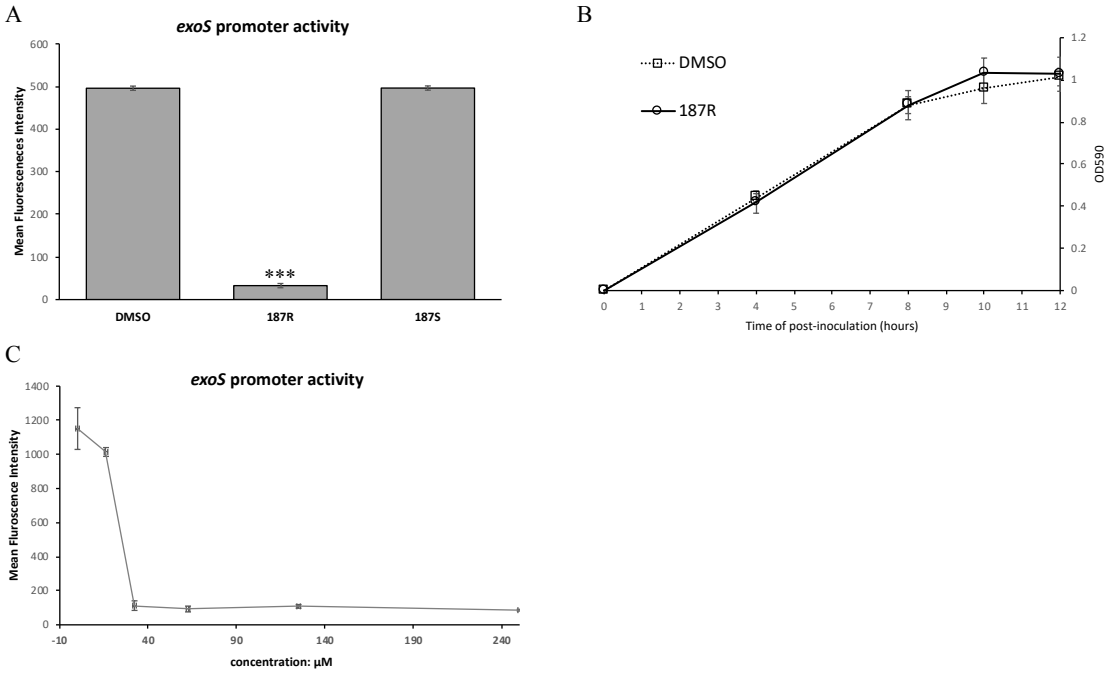

Figure 2.1 Effect of 187 on *P. aeruginosa exoS* promoter activity and growth. *P. aeruginosa* was cultured in T3SS inducing media supplemented with 250µM of compound 187R or DMSO. (A) *exoS* promoter activities supplemented with 187R and DMSO respectively. (B) Growth of *P. aeruginosa* with 250µM of 187R compared with DMSO. (C) Evaluation of the dose-dependent effect of 187R on *exoS* expression.

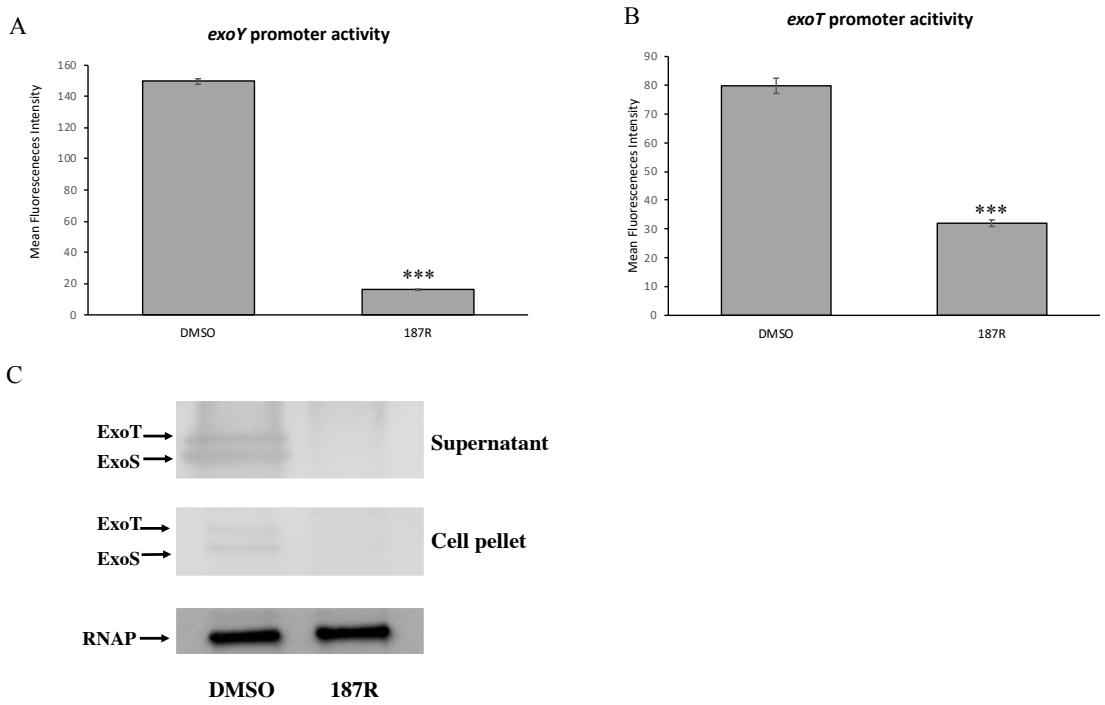

Figure 2.2 187R impairs T3SS expression and reduces the virulence of *P. aeruginosa.* (A) *exoY* promoter activity with 187R or DMSO. (B) *exoT* promoter activity with 187R or DMSO. (C) Western blot of the protein levels of ExoS and ExoT with 187R and DMSO respectively. Statistical significance was determined using an two-tailed student's t-test (***, P<0.001).

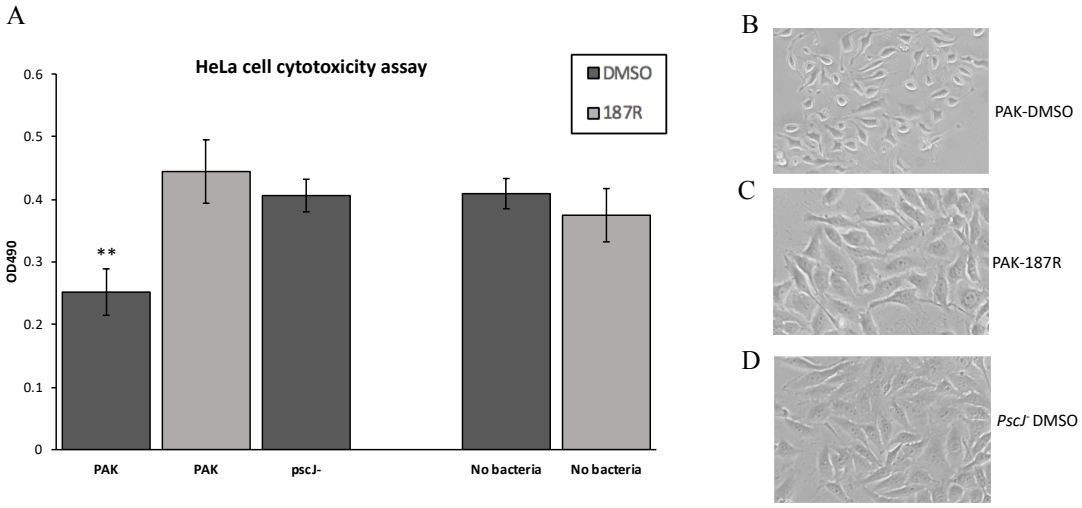

Figure 2.3 HeLa cell cytotoxicity assay. A) Cell lifting assay. Strain names: PAK: wild type PAK, *pscJ*-: PAK *ΔpscJ*, No bacteria: No bacteria but only DMSO or 187R added into the HeLa cell culture. Statistical significance was determined using an ANOVA test with Tukey-HSD analysis. Significant differences were found between DMSO treated PAK infection group and all other groups (**, $P<0.01$). No significant differences found among all other groups. B) HeLa cell morphology post infection with the present of DMSO (B), 187R (C), and D) *pscJ* mutant

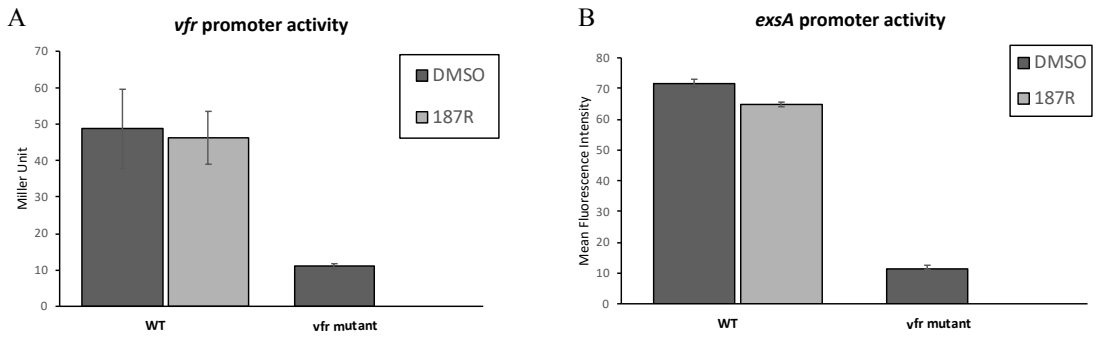

Figure 2.4 *vfr* and *exsA* promoter activity. (A) *exsA* promoter activity in wild type with the presence of 187R and DMSO respectively. *exsA* promoter activity of *vfr* mutant was served as control. (B) *vfr* promoter activity in wild type with the presence of 187R and DMSO, respectively. *vfr* promoter activity of *vfr* mutant was served as control.

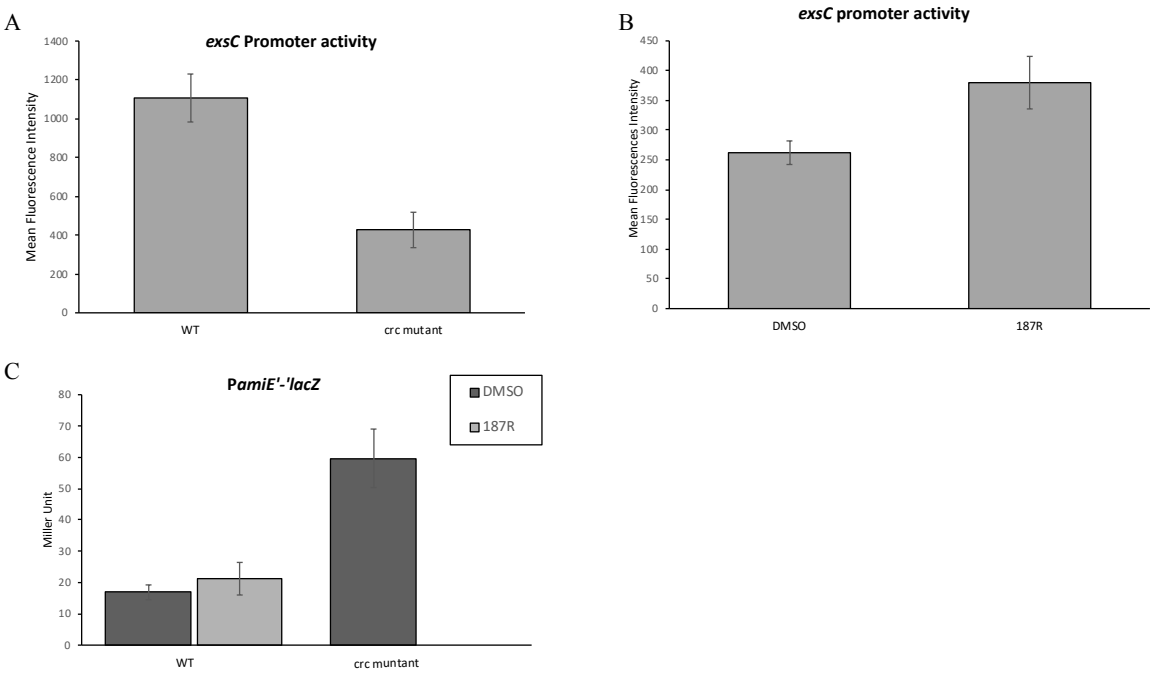

Figure 2.5 *exsC* promoter activity and *amiE'-'lacZ* translational fusion expression (A) *exsC* promoter activity of the wild-type *P. aeruginosa* in the presence of DMSO or187R. (B) *exsC* promoter activity in wild type and *crc* mutant. (C) *amiE'-'lacZ* expression level in the presence of DMSO or 187R, *crc* mutant was served as control.

Expression ratio of *rsmY* and *rsmZ* in 187R/DMSO

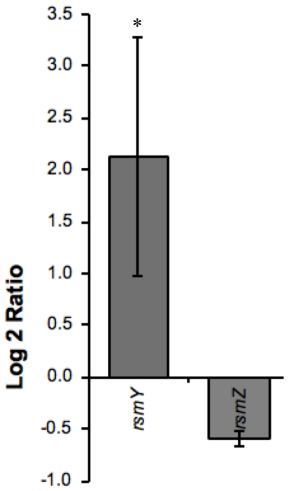

Figure 2.6 Expression ratio of *rsmY* and *rsmZ* mRNA level with the presence of DMSO and 187R. *rpsL* was served as internal control. The fold change was log2 transformed (*, $P<0.05$).

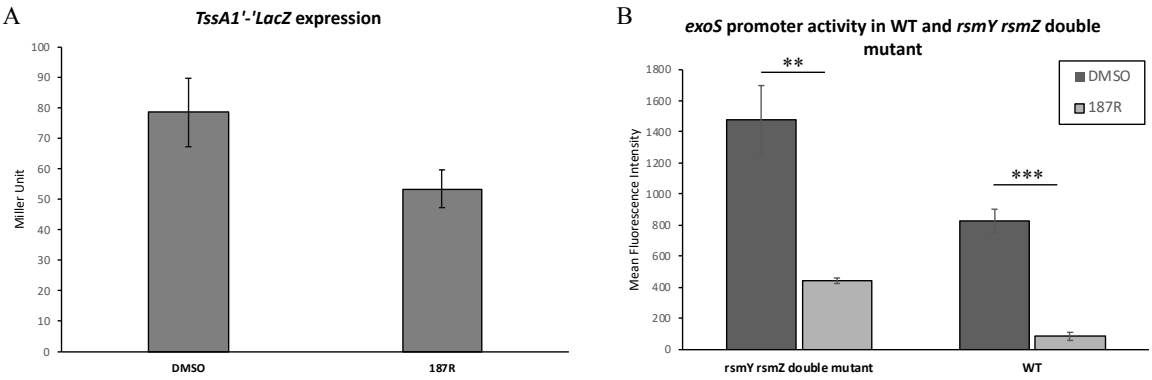

Figure 2.7 Effect of 187R on *rsmY/Z/A* regulatory pathway. (A) expression of *tssA1'-'lacZ* translational fusion reporter with 187R and DMSO respectively. (B) *exoS* promoter activity in in the *rsmYZ* double mutant with the present of 187R and DMSO respectively. Statistical significance was determined using an two-tailed student's t-test (**, P<0.01; ***, P<0.001).

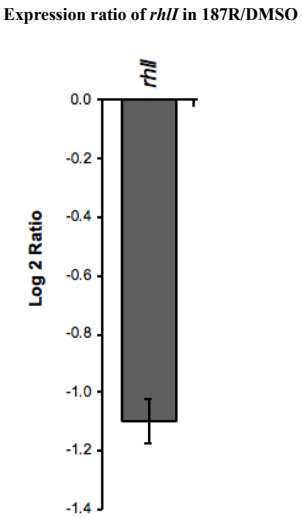

Figure 2.8 Expression ratio of *rhlI* in 187R/DMSO. The ratio waslog2 transformed.

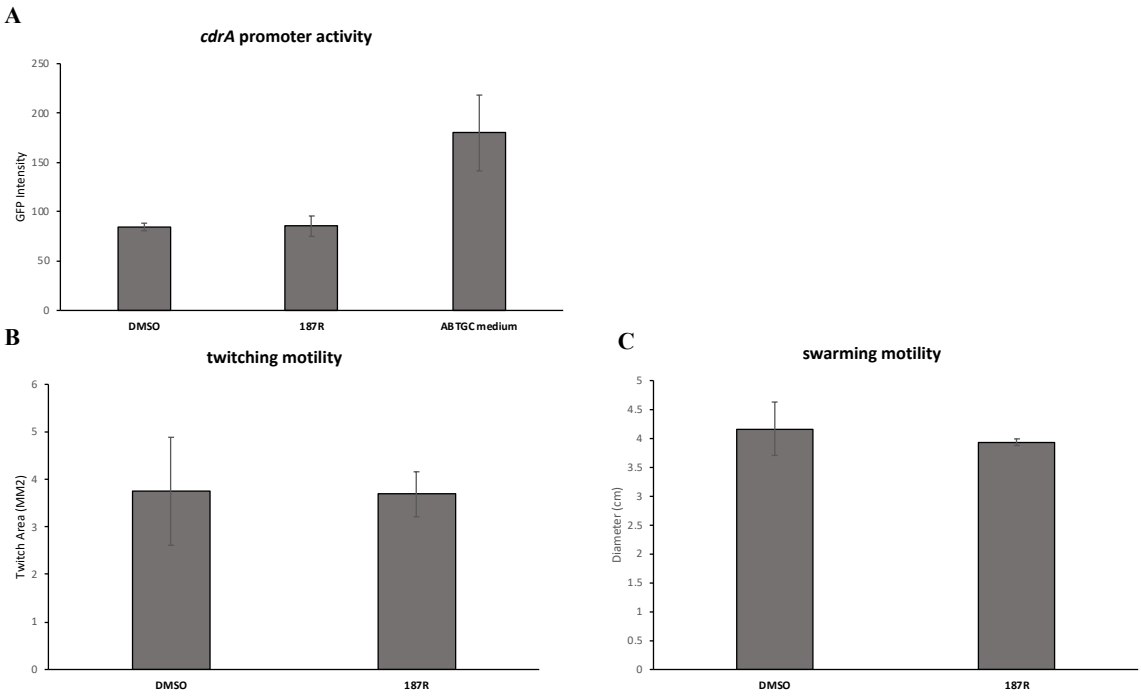

Figure 2.9 Effect of 187R on c-di-GMP level. (A) *cdrA* promoter activity level with the presence of DMSO or 187R. *cdrA* promoter activity in ABTGC medium was used as a control. (B) Twitching and (C) swarming motility of wild-type strain in the presence of DMSO or 187R.

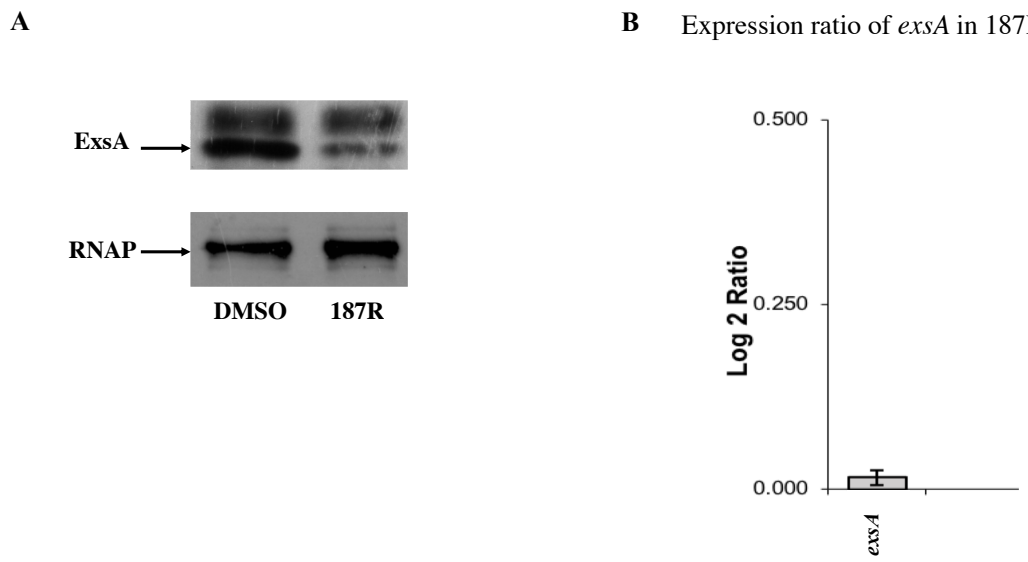

Figure 2.10 Effect of 187R on master regulator *exsA*. A) ExsA protein levels with DMSO and 187R respectively. RNA polymerase (RNAP) was used as the loading control. B) qRT-PCR of *exsA*. Expression ratio of *exsA* in 187R/DMSO. The ratio was log2 transformed. *rpsL* was served as internal control.

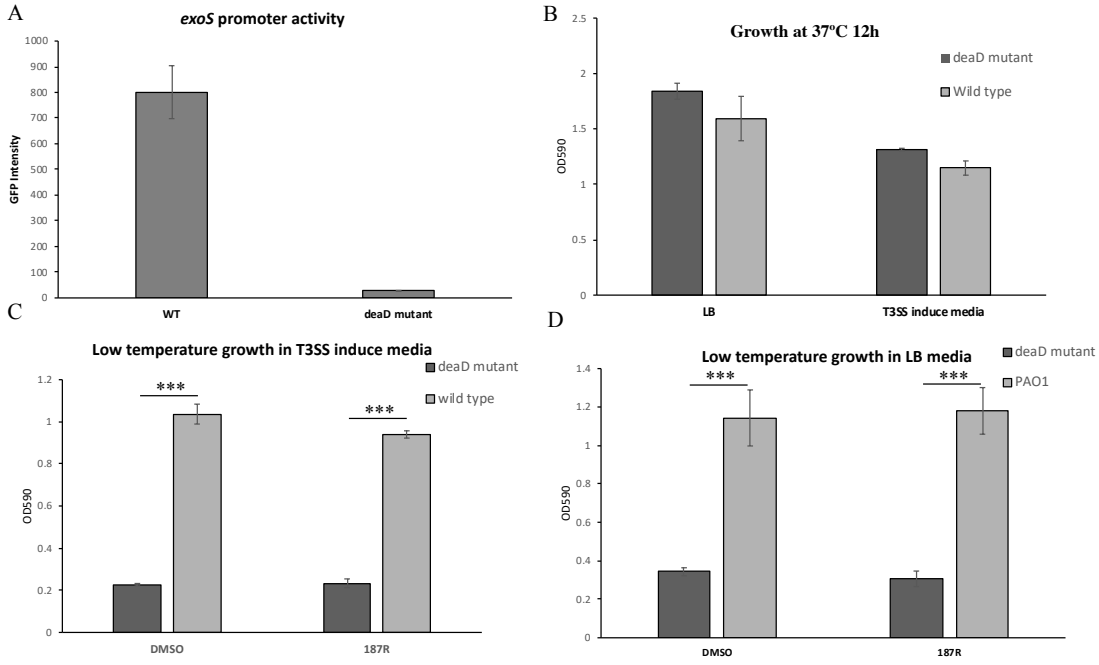

Figure 2.11 *deaD* regulates T3SS expression and low temperature growth. A) *exoS* promoter activity dramatically reduced in *deaD* mutant, adding 187R did not further reduced the *exoS* promoter activity. B) *deaD* did not affect the growth of PAO1 at 37°C. The low temperature (16 °C) growth of *deaD* mutant was measured in T3SS inducing medium (C) and LB (D) medium. *deaD* showed growth defect under the low temperature condition. Adding 187R does not affect the growth of PAO1 at 16 °C. Statistical significance was determined using an two-tailed student's t-test (***, P<0.001).

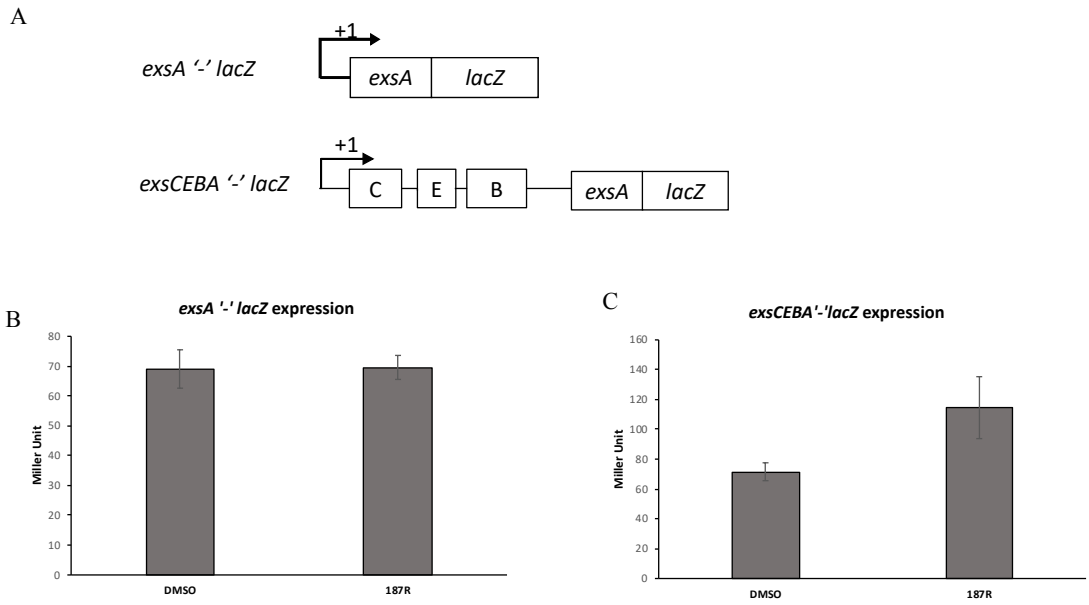

Figure 2.12 Translation of *exsA* with the presence of 187R or DMSO. (A) *exsA'-'lacZ* and *exsCEBA'-'lacZ* translational fusion reporter construction. (B) *exsA'-'lacZ* and (C) *exsCEBA'-'lacZ* expression with the presence of 187R or DMSO

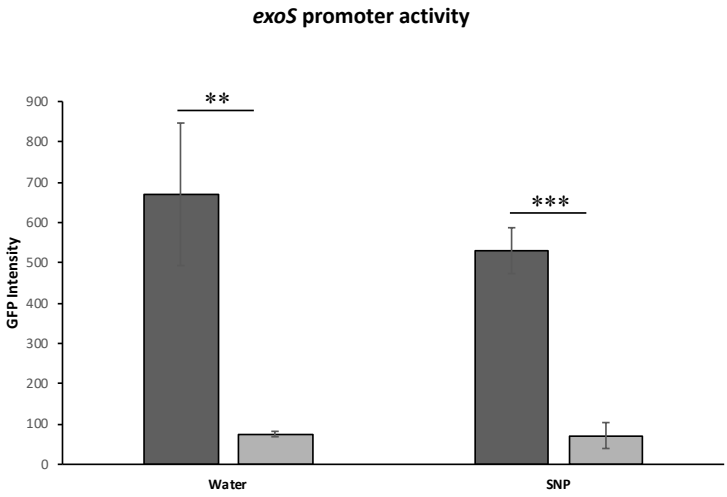

Figure 2.13 187R inhibits T3SS in the presence of SNP (NO donor). *P. aeruginosa* was cultured under T3SS inducing condition. SNP was supplemented in the medium at the concentration of 3mM. Water was supplemented as control. Statistical significance was determined using an two-tailed student's t-test (**, $P<0.01$; ***, $P<0.001$).

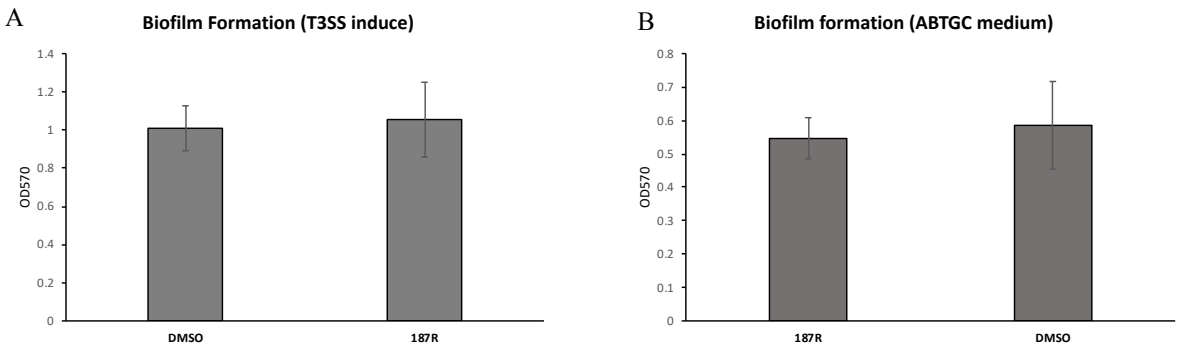

Figure 2.14 Biofilm formation of wild type strain with the presence of 187R and DMSO in T3SS inducing medium (A) and ABTGC medium (B)

Table 2.1 Bacteria strains and plasmids used in this study.

Strain/plasmid	Description	Reference or source
Pseudomonas aeruginosa		
PAO1	Wild-type strain	(109)
PAO1Δ*crc*	*crc* deletion mutant	(74)
PAO1Δ*deaD*	*deaD* deletion mutant	**This study**
PAK	Wild-type strain	(26)
PAKΔ*pscJ*	The *pscJ* mutant	(26)
PAKΔ*vfr*-P*vfr*	*vfr* deletion mutant with *vfr-lacZ* transcriptional fusion integrated into chromosome	(35)
PAK-P*vfr*	PAK wild type **with *vfr-lacZ*** transcriptional fusion integrated into chromosome	(35)
E. coli		
DH5α	Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	(110)
Plasmids		
pPROBE-AT	Cloning vector for transcriptional *gfp* fusions; Cb^r	(83)
exoS-gfp	pPROBE-AT containing the *exoS* promoter; Cb^r	(59)
exoY-gfp	pPROBE-AT containing the *exoY* promoter; Cb^r	**This study**
exoT-gfp	pPROBE-AT containing the *exoT* promoter; Cb^r	**This study**
exsC-gfp	pPROBE-AT containing the *exsC* promoter; Cb^r	**This study**

exsA-gfp	pPROBE-AT containing the *exsA* promoter; Cb^r	**This study**
pME6013	Cloning vector for translational *lacZ* fusions; Tc^r	(111)
pME9655	pME6013 with *amiE'-'lacZ*; Tc^r	(74)
pSW205	*lacZ* translational fusion vector carrying the *P. aeruginosa* 1.8 kb stability fragment; Cb^r	(84)
exsA '-' lacZ	pSW205 carry *exsA* transcriptional start site to +52 codon of *exsA,* driven by LacUV5 promoter	**This study**
exsCEBA '-' lacZ	pSW205 carry *exsC* transcriptional start site to 52 codons of *exsA,* driven by LacUV5 promoter	**This study**
tssA1 '-' lacZ	pSW205 carry +1 to +188 of *tssA1*, driven by LacUV5 promoter	**This study**
pEX18GM	*P. aeruginosa* gene replacement vector; *sacB*, Gm^r	(112)
pEX18GM-deaD	pEX18GM carrying *deaD* deletion allele	**This study**

Table 2.2 Oligonucleotide primers utilized in this study,

underlines indicates restriction enzyme sites

Primer	Sequence (5′→3′)
exoY-gfp	
P*exoY*-F	ATGGATCCGACGGTACGTACTTCGCGAC
P*exoY*-R	ATGAATTCTGCATATCCGCAGGGCGCAG
exoT-gfp	
P*exoT*-F	TAGGATCCCACCAAGAGCCCGTCGCTGC
P*exoT*-R	ATGAATTCCCAGGCGCCCGGCCACGGC
exsC-gfp	
P*exsC*-F	ATTGTCGACGCAGAAGGTCGAGGACCAGATG
P*exsC*-R	ATTGAATTCGATACGGCCTGCGAACTCGGC
exsA-gfp	
P*exsA*-F	ATTGTCGACTACATTGCCTGCTGTTTCGG
P*exsA*-R	ATTGAATTCGGCCAAGAGATTTGGCTCC
cdrA-gfp	
P*cdrA*-F	ATTGTCGACGCAGTTGCAGCTCGTCGAA
P*cdrA*-R	ATTGAATTCCGGACGGACCATGAAAATCT
exsA '-' lacZ translational fusion	
Post *exsA* -F	AAAGAATTCAGGCTTTACACTTTATGCTTCCGGCTCGTAT AATGTGTGG CGTGCTCATGGCTTTGAAAATC
Post *exsA* -R	AAAGGATCC CGCCAGGCAAAAAGTGGAAT
tssA1 '-' lacZ translational fusion	
Post *tssA1* F	AAAGAATTCAGGCTTTACACTTTATGCTTCCGGCTCGTAT
	AATGTGTGGAACCTTTCGAGTCATCCAATAT

Post *tssA1* R	AAGGATCCGCGTCGTACTCCAGATCG
deaD deletion mutant	
deaD Upstream-F	AAA AAGCTTCGAGACGATAGGCTTCGTGG
deaD Upstream-R	GGACCTCAGTCCTCGCGAGTCATGGGTTTGCCTCGTAT
deaD downstream-F	ATACGAGGCAAACCCATGACTCGCGAGGACTGAGGTCC
deaD downstream-R	AAAGGATCC ACGACTTCTACGGCTTTCCG
qRT-PCR primer	
rpsL - F	TGAAGGTCACAACCTGCAAGAGCA
rpsL - R	AACGACCCTGCTTACGGTCTTTGA
exsA - F	CAAGGGAAAGGACAGCCGAA
exsA - R	ACGCTCGACTTCACTCAACA
rsmY-F	TCAGGACATTGCGCAGGAA
rsmY-R	TTTGCAGACCTCTATCCTGACATC
rsmZ-F	GGAACACGCAACCCCGAAGG
rsmZ-R	CCGCCCACTCTTCAGTCCCT
rhlI-F	ATCCGCAAACCCGCTACATC
rhlI-R	TAGGCGAAGACGTCCTTGAG

Table 2.3 Ciprofloxacin susceptibility of *P. aeruginosa* PAO1 grow with DMSO or 187R

Condition	DMSO	187R
MIC[a]	1-2mg/L	1-2mg/L

[a] MIC of ciprofloxacin was measured with DMSO or 187R (250 μM) supplemented in the growth medium. MIC was determined by the minimal concentration of ciprofloxacin without growth.

Chapter 3 : Anti-virulence compound preserve the microbial community better than the traditional antibiotics

Abstract

Antibiotic therapies are known to disrupt the microbiota and have long-term negative influence on the hosts' health status. Anti-virulence compounds target the virulence factors but do not affect cell components that are essential for cell survival. Therefore, anti-virulence therapies are considered as alternative antimicrobial therapies that can better preserve the host-associated microbial community. Although several studies evaluating the efficacy of anti-virulence compounds on infectious diseases have been conducted, the impact of anti-virulence compounds on host microbial community has been seldom investigated. In this study, we used 16S Illumumia sequencing to test the impact of the anti-virulence compound, designated as 187R, on the composition of a *P. aeruginosa* hosts microbial community (*Arabidopsis* phyllosphere) in comparison to the treatment of streptomycin. Although the culture independent sequencing method is a powerful tool for studying the microbial community, it cannot assess the bioactivity and viability of the microbial community. To address this limitation, we also used the Biolog EcoPlate assay to compare the viability and metabolic function of the 187R-and streptomycin-treated microbial community. After examining the Illumina sequencing results and the carbon profile of 187R-, DMSO- and streptomycin-treated phyllosphere. We found that microbial community composition and metabolic function of the 187R-treated phyllosphere were more similar to the DMSO-treated phyllosphere (negative control) than the streptomycin-treated group. The results suggest that anti-virulence compounds can preserve the microbial community better than antibiotics.

Introduction

Microorganisms inhabit almost every imaginable environment in the biosphere. These microbes play crucial roles in the ecosystems all around the world for cycling elements such as carbon, oxygen, nitrogen and metal (113). For example, the soil microbial community is

essential for decomposing the organic matters in forest soil, further influencing the carbon cycle and nutrient composition in the soil (114). In addition, in many ecosystems such as the tropical forest ecology system, the microbial community is considered as a key factor for maintaining the bio-diversity through negative feedback mechanisms (i.e., accumulation of pathogens inhibits the growth of plant seedlings) (115). The microbial communities in different areas, or symbiotic microbes in different hosts, feature their unique compositions and diversities (116). These microbial communities also have special functions affecting the hosts' health status. For example, the gut microbial community provides nutrients to the host, represses pathogens and parasites, and stimulates the development of the host's immune system (63, 64). Studies have suggested that phyllosphere microbial communities (i.e., microbial community on the leaf surface) limit the number of invasive pathogenic bacteria on the leaf surface via competition, as a result alleviating diseases (117).

Antibiotic therapy has been widely applied for treating the bacterial and fungal infections in human and animals since Alexander Fleming discovered penicillin in 1928. However, the current antibiotic therapy targets the cellular components which are essential for cell survival. The strong selective pressure leads to the development of antibiotic resistance and causes the accumulation of antibiotic resistant genes in the host gut microbiota. As a result, the host-associated microbiota becomes a potential pool of resistant genes and poses public health risk (118). Moreover, the cellular components serving as the targets of antibiotics, especially the broad-spectrum antibiotics, are conserved in many different bacteria species including both pathogenic and nonpathogenic microbes. Therefore, the antibiotic treatment also affects the nonpathogenic microbes and disturbs the hosts' microbial communities (58, 119). The disruption of hosts' microbes sometimes causes severe diseases. For example, gut microbial disorder correlates with *Clostridium difficile* infection (68) and inflammatory bowel disease (69). The recovery of microbiota after treatment with antibiotics can take up to years (70),

which could potentially shorten the life span of the host (58, 120).

The anti-virulence therapies target the virulence factors instead of the essential components for cell survival. Therefore, it has been expected that the anti-virulence therapies can minimize the damage to the hosts' microbiota (72). However, few studies have been done to prove the theory. This study aimed to evaluate the anti-virulence compound impacts on host (i.e., *Arabidopsis* phyllosphere) from the perspective of the microbial community. Three treatments, including the virulence compound (i.e., 187R), control group (i.e., DMSO) and antibiotics (i.e., streptomycin) were applied to observe the corresponding impacts on phyllosphere microbes. Our results showed that 187R treatment group had more similar *Arabidopsis* phyllosphere to the control group compared with streptomycin, indicating less disturbance to host microbiota from this anti-virulence compound. This observation suggests such alternate antimicrobial therapy may be able to preserve non-pathogen host microbiota, causing less negative impacts on the host health condition compared to the widely-applied antibiotics.

Anti-virulence compound is considered as narrow-spectrum or even species-specific since the regulatory of virulence factors such as T3SS are very different in different pathogens. In our study, we also tested the spectrum of our anti-virulence compound 187R by testing the impact of 187R on a Hrp (Hypersensitive responses and pathogenicity) T3SS. Hrp T3SS is conserved among plant pathogens such as *D. dadantii*, *E. amylovora* and *Psedomonas syringae* (121). The Hrp T3SS in these three bacteria is activated by alternative sigma factor HrpL. In our experiment, we tested if the expression of *D. dadantii* and *E. amylovora hrpA* gene, which encodes a major component of T3SS pilus, altered by 187R (122, 123). We found that the *hrpA* expression has not changed in both *D. dadantii* and *E. amylovora*. These results showed that 187R is not a broad-spectrum anti-virulence compound.

Materials and Methods

Phyllosphere collection

For testing the effect of 187R on the phyllosphere, eight weeks *Arabidopsis thaliana* Col-1 were moved from the pot to 2mL microcentrifuge tubes with the root immersed in water. The leaves were sprayed twice (8 hours between each spray) with 187R (35 μM), DMSO (negative control) or streptomycin (200 ppm), respectively. DMSO was also added into the streptomycin solution before spraying. The leaves were collected after the plants were kept at room temperature for overnight. The collected leaves were gently washed in PBS for cleaning the remaining compound and antibiotics. The washed leaves were placed in sterile tubes containing 30 mL PBS. The phyllosphere were collected by sonicating the test tubes in a water bath sonicator for 15 minutes. Samples were collected in triplicate for each treatment. A total of 22-24 plants were used in each treatment.

16S rRNA gene illumina sequencing and data analysis

PBS containing phyllosphere microbes collected from above were centrifuged and the cell pellets were used for microbial DNA extraction with the Qiagen Dneasy Powersoil kit (Qiagen, Germany) following manufacturer's instructions. The quantities of the genomic DNA were measured by Nanodrop spectrophotometer. Approximately 10ng of DNA were added into the PCR reaction as template. The V4 region of 16s rRNA gene was amplified using the 16S_515F and 16S_806R primers with Illumina sequencing adaptors (16S_515F: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG CCA GCM GCC GCG GTA A; 16S_806R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACH VGG GTW TCT AAT) (124). PCR amplification consisted of 95°C for 45 s, followed by 38 cycles of 95°C for 15 s, 78°C for 10 s, 60°C for 30 s, and 72°C for 30 s (125). PNA (peptide nucleic acid) clamps

mPNA and pPNA clamps (mPNA: GGC AAG TGT TCT TCG GA and pPNA: GGC TCA ACC CTG GAC AG) (126, 127), which binds to and blocks the amplification of mitochondria and chloroplast DNA, respectively, were added to the PCR reaction at a final concentration of 0.75 μM. The PCR products was purified by QIAquick Gel Extraction Kit (QIAGEN).

Microbial community Illumina sequencing data was analyzed by Qiime version 1.9.1 (128). Open-reference OTU (Operational Taxonomy Unit) picking method using GreenGenes reference database version 13.8 (129) was performed for OTU clustering and taxonomy assignments. Principle coordinates analysis (PcoA) of microbial communities was performed based on the Unifrac distance matrix (130).

Testing the impact of 187R on phyllosphere with culture dependent Biolog EcoPlate.

The different members of a microbial community have different growth requirements and utilize different carbon sources, therefore, the microbial community can decompose different organic carbon and involved in the nutrient cycling. Alternating the microbial community may change its carbon utilization. In this assay, the ability of the phyllosphere microbial communities treated by 187R, DMSO or streptomycin, utilizing the carbon sources were assessed by BioLog EcoPlate. Each EcoPlate has 96 wells containing 31 carbon sources and one blank control in three replicas. Tetrazolium violet redox dye was used to evaluate the substrate (carbon source) metabolization. For the EcoPlate assay, 2mL of PBS containing the suspended phyllosphere was diluted 1:20 (vol:vol) in sterile PBS. PBS contains diluted phyllosphere suspension was aliquoted into each well (120μL) of the Biolog EcoPlate. The carbon utilization was determined by Telcon plate reader using OD590nm after 48 hours incubation. The results were analyzed by Principle Component Analysis (PCA) using R (90) package FactoMineR (131). The heatmap with clustering analysis was generated based on

average OD590 value of each carbon source within each treatment using R package gplots (132).

Testing the expression of T3SS in *Erwinia amylovora* and *Dickeya dadantii*

Erwinia amylovora 273 and *Dickeya dadantii* 3937 are plant pathogens processing T3SS. We tested the T3SS gene expression by measuring the T3SS regulon gene *hrpA* expression in these two strains. *E. amylovora* 273 and *D. didantii* 3937 carrying a phrpA reporter plasmid were used in this assay. The reporter plasmid phrpA for *E. amylovora* 273 was constructed by cloning *E. amylovora hrpA* promoter region into pPROBE-NT (123). The reporter plasmid phrpA for *D. dadantii* 3937 was constructed by cloning *D. dadantii* 3937 *hrpA* promoter region into pPROBE-AT (133). The strains carrying phrpA reporter plasmid at 1:1000 were inoculated into Minimal Medium (133) with 250 μM of 187R. *E. amylovora* or *D. didantii* 3937 harboring the T3SS *hrpA -gfp* transcriptional fusion reporter treated with DMSO (solvent for screening compound) were used as negative control. Bacteria were cultured overnight at 28°C before harvesting. The harvested bacterial cells were 1:10 diluted in PBS and the GFP intensity was measured using fluorescence-activated cell sorter flow cytometry (FACS) (BD Biosciences, CA).

Results

Comparison of the impact of 187R and streptomycin on phyllosphere microbe compositions

There were a total of 19 phyla were observed for each treatment group, with *Proteobacteria* and *Bacteroidetes* as the two most abundant phyla. In the control group, *Proteobacteria* and *Bacteroidetes* composed on average 67% and 25% of the whole community, respectively. Phylum *Verrucomicrobia* and phylum *Firmicutes* each occupied about 1% of the whole

community (Fig. 3.1). Compare to the control group, the 187R-treated group, in which *Proteobacteria* and *Bacteroidetes* composed on average 62% and 30% of the whole community, showed similar relative abundances of different phyla (Fig. 3.1 & Fig. 3.2). Compared to the DMSO treated group, the antibiotic treated-group showed reduced relative abundance of *Proteobacteria* and increased relative abundance of *Bacteroidetes* and *Verrucomicrobia*. In particular, the average abundance of *Proteobacteria* reduced to 37%; *Bacteroidetes* and *Verrucomicrobia* increased to 51% and 6%, respectively (Fig. 3.1 & Fig. 3.2).

To further evaluate similarities of the microbial communities of different treatments, principle coordinates analysis (PcoA) was performed based on the Unifrac distance of different phyllosphere samples (Fig. 3.3). The antibiotic-treated phyllosphere was well separated from the 187R- and DMSO-treated phyllosphere along PC1, indicating antibiotic altered phyllosphere to a larger extent compared to 187R. Unifrac monte carlo significance test were conducted checking the phylogenetical differences between each pair of samples in our experiment. The test confirmed that all the streptomycin-treated phyllosphere are significantly different from the DMSO-treated phyllosphere ($p < 0.05$); however, no significant difference was observed when compared 187R-and DMSO-treated phylospheres. Overall, these results suggested that anti-virulence compound 187R preserved the microbial community composition better than the antibiotics.

187R preserved the metabolic functions of the phyllosphere better than antibiotics.

The change of microbial metabolic function such as carbon metabolism may reflect the viability and metabolism of the microbial community. In order to test whether compositions of phyllosphere in 187R and antibiotics treatment groups can comprehensively represent the corresponding functional profiles, we used the EcoPlate assay to test the carbon metabolism (an important function related to microbe survival and nutrient cycling) of 187R-, DMSO- or

streptomycin-treated phyllosphere. The heatmap with clustering analysis was generated based on average OD590 of each carbon source within each treatment. The results demonstrated that 187R-and DMSO-treated phyllospheres shared similar pattern in terms of carbon metabolism profile (Fig. 3.4). However, the carbon metabolism profile of streptomycin-treated phyllosphere was different from the DMSO-and 187R-treated phyllospheres. Besides 2-Hydroxy Benzoic Acid and L-Threonine, which cannot be used by phyllosphere microbes as carbon sources, 13 out of 29 carbon sources showed more than two-fold reduction of OD590 (carbon metabolism) compared to those in the DMSO-treated phyllospheres. Moreover, the antibiotic-treated phyllospheres even lost the ability of using phenylethylamine as carbon source (indicated by arrow in Fig. 3.4). However, 187R-treated phyllosphere, has not shown such dramatic change in terms of carbon utilization (Fig. 3.4). The PCA analysis for carbon profiles of all 12 samples suggested that the carbon profiles of all three streptomycin-treated phyllospheres were greatly differed from the carbon profiles of DMSO-treated (control) phyllosphere. The samples were well separated along dimension 1 (Dim1), which explained 49.69% of the variation. Compared to carbon profiles of streptomycin-treated phyllosphere, the carbon profiles of three 187R-treated phyllospheres showed higher similarity to the carbon profiles of DMSO-treated (control) phyllospheres (Fig. 3.5). The results confirmed that there were drastic changes in antibiotic-treated phyllospheres but not in 187R-treated phyllospheres. Moreover, the results also suggested that compared to the antibiotic streptomycin, anti-virulence compound 187R showed less impact on the microbial community carbon metabolism.

187R does not inhibits the T3SS of two other bacterial pathogens.

To test the specificity of 187R on other Gram negative bacteria possessing T3SS, we treated the *E. amylovora* and *D. dadantii* 3937 with 187R under T3SS inducing conditions. The expression of *hrpA* gene, which encodes the major subunit of the Hrp pilus, is under the

regulation of sigma factor HrpL (123, 133). We found that the expression of *hrpA* was not affected by adding 187R (Fig. 3.6), indicating that the anti-virulence compound 187R may be specific to the T3SS of *P. aeruginosa.*

Discussion

We found that by targeting the virulence factors of pathogens can reduce their ability to cause infections while preserving the host-associated microbiota. In this research, culture-independent and culture-dependent method were applied for testing the impact of an anti-virulence compound on *P. aeruginosa* host microbial community (*Arabidopsis* phyllosphere). Our results suggested that compared to antibiotics, 187R, and likely other anti-virulence compounds preserved the microbial community in terms of composition and function better than the traditional antibiotics. In addition, our anti-virulence compound did not affect the T3SS of *E. amylovora* and *D. dadantii* 3937, which indicates 187R-mediated T3SS inhibition is specific to *P. aeruginosa.*

In this experiment, we observed that the addition of PNAs greatly reduced the amplification of hosts' DNA including the mitochondria and chloroplast DNA (125). In our sequencing dataset, two sequences of chloroplast were found and no mitochondria sequences were observed, suggesting that supplementation of PNAs decreased the bias of microbial community analysis caused by host DNA amplification.

The EcoPlate assay highlighted the effect of anti-virulence compound on preserving the microbial community composition and metabolic functions, suggesting that the anti-virulence compound greatly reduced the damage of hosts' microbial community compared to the traditional antibiotics. A major limitation of the using culture-independent method examining the microbial community is that it cannot differentiate the viable and dead microbes in the ecosystem; therefore, the effects of treatments on the phyllosphere may be underestimated

(134). In addition, microbial communities are not only characterized by their species composition, they are also featured with different functional genes (for example, genes related to carbon and nitrogen metabolism) that are responsible for the microbes' bioactivities such as nutrient cycling and immunomodulation (135, 136). It has always been discussed that changing the microbial community composition would not necessarily result in the alternation of microbial community function due to its high functional redundancy (137, 138). Therefore, it is questionable that whether alternating the species composition would affect the hosts and ecosystems. Our culture-dependent EcoPlate method addressed these limitations of how antibiotics and anti-virulence compounds affect the viability and metabolic functions microbial communities. In this study, the EcoPlate assay testing the carbon metabolism profile of microbial communities showed that while 187R-treated samples showed similar carbon utilization profile to the DMSO control, the antibiotic-treated phyllosphere showed reduced capability of carbon utilization.

While checking the carbon metabolism profile of different phyllosphere, we observed that the carbon utilization profile was greatly altered in the microbial community treated with antibiotics. (Fig 3.4 & 5). Besides the altered composition of bacteria in the antibiotic-treated samples, the inhibitory mechanism of streptomycin may also contribute to the altered carbon metabolism. Streptomycin is a bacteriostatic agent. The research conducted in Gram negative bacteria *E. coli* showed that streptomycin interferes with protein synthesis by disturbing the stability of mRNA-ribosomal complex and inducing misreading of genetic code (139). As a result, although the bacteria may still survive in the community, the accumulation of mutated protein hampered their bioactivity and caused a decreased carbon metabolic rate. Further research for elucidating the impact of bacteriostatic agents, such as streptomycin, on the metabolic functions need to be done.

By testing the T3SS of *E. amylovora* and *D. dadantii* 3937, we found that 187R does not inhibit the *hrp* T3SS of these two bacteria, thus indicating that 187R may be a narrow-spectrum T3SS inhibitor specific for *P. aeruginosa*. The narrow-spectrum of 187R may also contributed to the preservation of the host microbial community since T3SS may also involve in shaping the bacterial habitat via interacting with the fungi and change its morphology (121).

In conclusion, using high-throughput sequencing and culture dependent EcoPlate assay, we studied the impact of anti-virulence compound on host microbial communities and the host-associated microbial community composition and metabolic function better than the traditional antibiotics. This discovery may allow us to target virulence factors of the pathogen while not altering the host microbiota. Our study indicates that anti-virulence compounds are good alternative strategy for new antimicrobial therapy development.

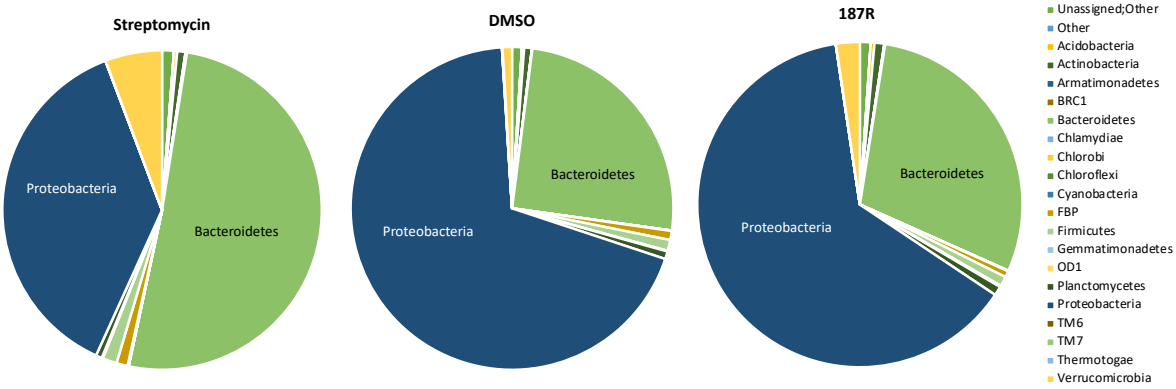

Figure 3.1 Average relative abundance of microbial communities at phylum level. Legend listed on the right side of the figure. 187R, phyllosphere treated by T3SS inhibitor 187R; DMSO, phyllosphere treated by DMSO; Streptomycin, phyllosphere treated by antibiotic streptomycin. Each pie graph demonstrates the mean abundance of three individual samples.

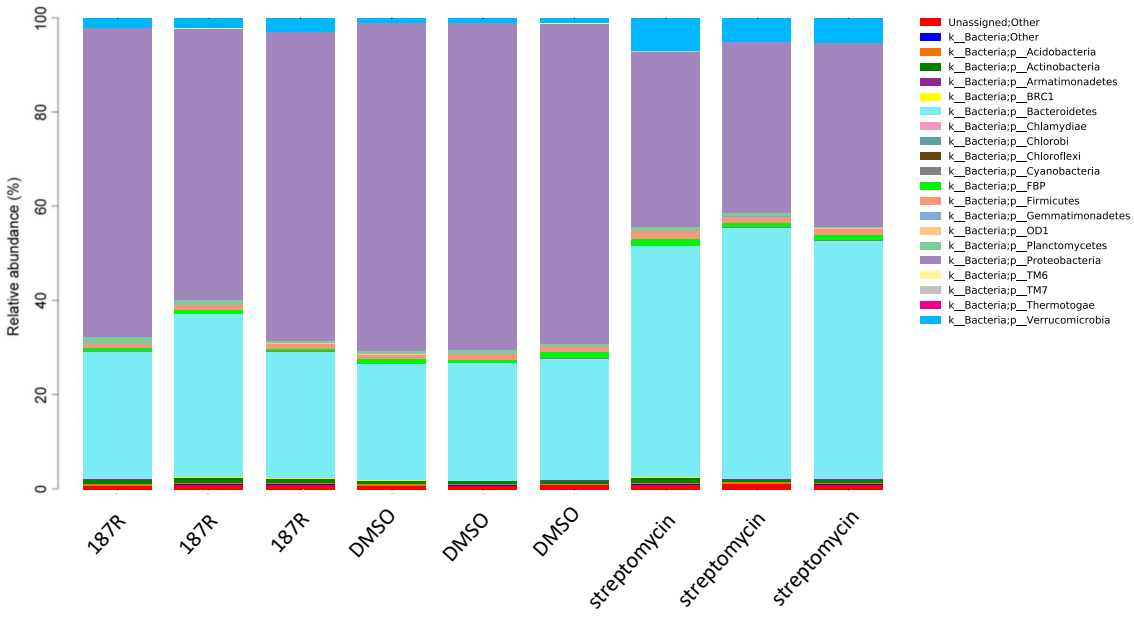

Figure 3.2 Relative abundance of microbial communities at phylum level. Legend listed on the right side of the figure. 187R, phyllosphere treated by T3SS inhibitor 187R; DMSO, phyllosphere treated by DMSO; Streptomycin, phyllosphere treated by antibiotic streptomycin.

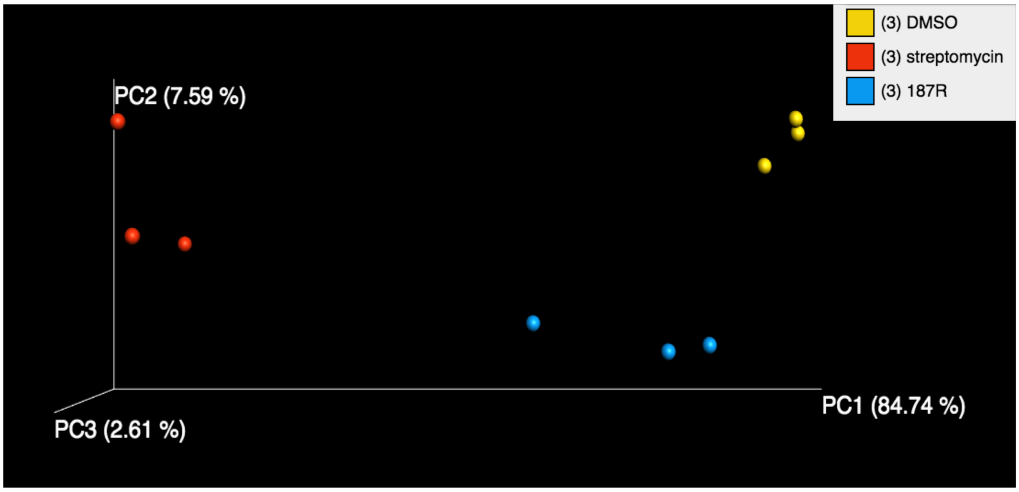

Figure 3.3 Principal Coordinate Analysis (PcoA) of microbial communities based on weighted Unifrac distance. Legend listed on the right side of the figure. 187R, phyllosphere treated by T3SS inhibitor 187R; DMSO, phyllosphere treated by DMSO; Streptomycin, phyllosphere treated by antibiotic streptomycin.

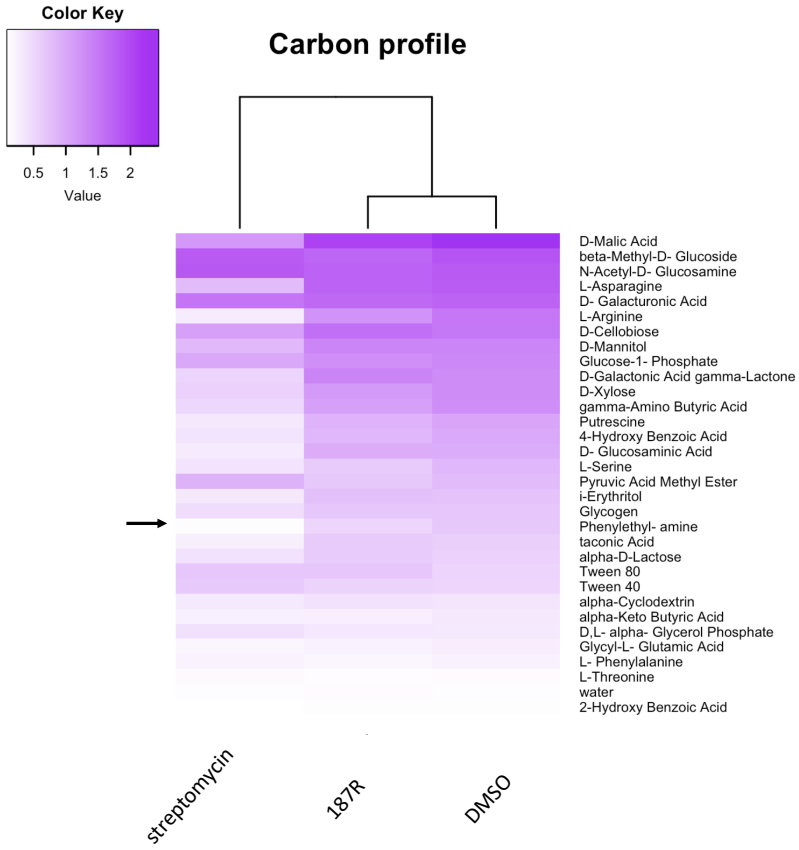

Figure 3.4 Heatmap with cluster analysis of carbon metabolism profile. Color key indicates the OD590 value listed on the left side of the figure. Carbon source listed on the right side of the figure. 187R, phyllosphere treated by T3SS inhibitor 187R; DMSO, phyllosphere treated by DMSO; Streptomycin, phyllosphere treated by antibiotic streptomycin. Arrow indicated the phenylethylamine, the carbon source cannot be utilized by streptomycin treated phyllosphere.

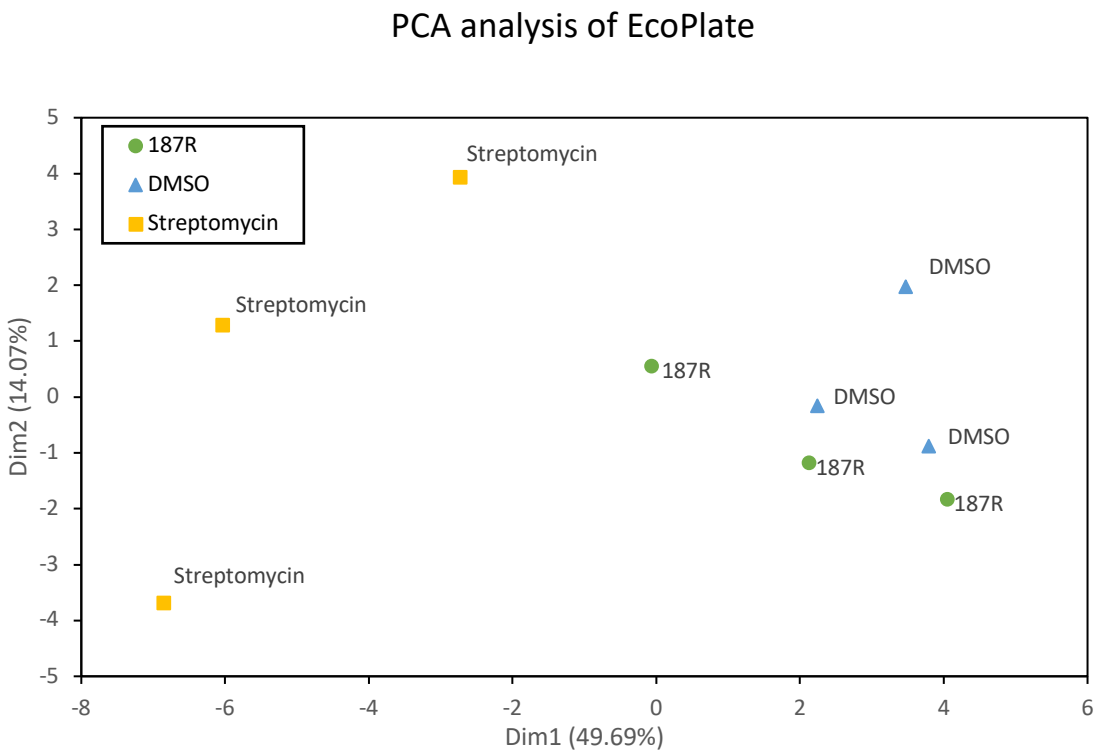

Figure 3.5 Principal Component Analysis (PCA) of microbial communities carbon metabolism profile. Legend listed on the inside of the figure. 187R, phyllosphere treated by T3SS inhibitor 187R; DMSO, phyllosphere treated by DMSO; Streptomycin, phyllosphere treated by antibiotic streptomycin. The percentage under the axis indicate the variation the axis explained.

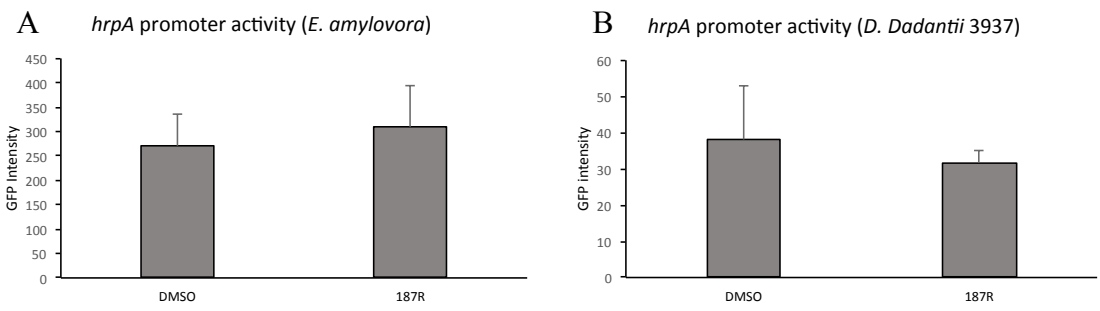

Figure 3.6 *hrpA* promoter activity of *E. amylovora* (A) and *D. dadantii 3937* (B) in the presence of DMSO or 187R.

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Curriculum Vitae

EDUCATION

University of Wisconsin-Milwaukee

Ph.D in Biological Sciences
August, 2019

Dissertation: Mechanism of 187R inhibits *Pseudomonas aeruginosa* Type III Secretion System

Dissertation Advisor: Dr. Ching-Hong Yang, Ph.D

Northeastern University

M.S. in Regulatory Affairs for drugs, biologics and medical devices
May, 2013

Zhejiang Chinese Medical University

B.S. in biotechnology
June, 2009

AWARDS

Chancellor's Graduate Student Fellowship **2013-2018**

Teaching Experience

TEACHING EXPERIENCE: University of Wisconsin-Milwaukee

Teaching Assistant—Anatomy and Physiology I **2013-2015**

Assisted with overall class structure, including weekly lab practicum, and administered all grading for assigned laboratory sections.

Teaching Assistant - General survey of microbiology **2016-2018**

Responsible for instructing students on experimental techniques and research method development. Assisted in the assessment of student performance, and grading of laboratory work.

Teaching Assistant - Experimental Microbiology **2017-2018**

Designed special projects assigned by the course professor. Implementation of enhanced techniques for improving student experimental outcomes.

Teaching Assistant – General microbiology **2017**

Responsible for instructing students on experimental techniques and research method development. Assisted in the assessment of student performance, and grading of laboratory work.

Teaching Assistant-Laboratory Techniques in Molecular biology **2018**

Assisted with student experiments and lab preparation.

Teaching Assistant- Elements of biology **2019**

Responsible for instructing students on experimental techniques and research method development. Assisted in the assessment of student performance, and grading of laboratory work.

ACADEMIC RESEARCH EXPERIENCE

- Discovery of novel compounds inhibit the virulence factor of *Pseudomonas aeruginosa* (T3SS). Found a potent T3SS inhibitor 187R and elucidated the inhibitory mechanism of the compound. Determined inhibitory mechanism of the 187R on *P. aeruginosa* T3SS.
- Analysis the anti-virulence compounds' impact on the microbial community using culture dependent and independent methods. Using illumina sequencing method and bioinformatics tools illustrated that anti-virulence compound cause less impact on host-associated microbial community compared to traditional antibiotics.
- Investigating the role of soil biota in negative-feedback mechanism which maintaining the biodiversity in the tropical rainforest using the culture independent sequencing method and bioinformatic tools.
- Established the animal skin infection model.
- Studied the production of camptothecine (anti-cancer compound) from *Nothapodytes nimmoniana* tissue culture with HPLC analysis

Publication

Fan,S., **Fang, T**., Fang. L., Yang, CH., He., Y.Transcriptional responses of *Xanthomonas oryzae*pv. *oryzae* to type III secretion system inhibitor *ortho*-coumaric acid in *BMC Microbiology,* No.163, July 2019

Fang, L. Study on Rapid Propagation and Effective Anticancer Constituents of *Nothapodytes nimmoniana* In *JOUR. OF ZHEJIANG FOR. SCI. & TECH*, pp. 27-29, Vol.28. No.6, Nov. 2008.