Molecular characterization of a novel pyocin and its immunity protein in pseudomonas aeruginosa

Bahareh Haji Rasouliha

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**MOLECULAR CHARACTERIZATION OF A NOVEL
PYOCIN AND ITS IMMUNITY PROTEIN IN
*PSEUDOMONAS AERUGINOSA***

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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To the dearests

My mother and father, Parvaneh and Hossein

My husband, Ali

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LIST of ABBREVIATIONS

3D	Three-dimensional
ATP	Adenosine Triphosphate
Am	Ampicillin
C	Celsius
Cb	Carbenicillin
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic Acid
FESEM	Field Emission Electron Scanning Microscopy
GFP	Green Fluorescence Protein
HNN	Hierarchical Neural Network
IC50	50% Inhibitory Concentration
LB	Luria–Bertani
mRNA	Messenger RNA
MIC	Minimum Inhibitory Concentration
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sm	Streptomycin
UV	Ultra Violet

SUMMARY

Pseudomonas aeruginosa, a Gram-negative bacterium poses a serious health threat, particularly in immunocompromised patients. Due to its inherent resistance towards many antibiotics, there is a demand for developing an effective strategy to specifically target and treat *P. aeruginosa* infections. *P. aeruginosa* is able to secrete antibacterial bacteriocins, called pyocins. To date, three types of pyocin, namely the F, R, and S types, have been discovered. These pyocins are secreted by *P. aeruginosa* strains, and effective to kill the same or related species. However, pyocin-producing strains are resistant to their own bacteriocins, theoretically due to the presence of probable immunity proteins. Based on the mechanisms of pyocin-killing and pyocin-resistance, pyocins could be applied to attack *P. aeruginosa*. Furthermore, applying pyocins in therapeutic purposes can be advanced due to the specificity of these antibacterial peptides. Pyocins bind to specific receptors on cell surface. In line with above advantages and the urgent demand of therapeutic strategies against *P. aeruginosa*, this thesis first aimed to elucidate the mechanisms of pyocin S5-killing and S5-resistance. To elucidate the killing mechanisms of pyocin S5, pyocin S5 gene from *P. aeruginosa* PAO1 was heterologously expressed in *Escherichia coli*, followed by the assay of killing activity against clinical isolates of *P. aeruginosa* and the study of killing mechanism against the clinical isolate DWW3. It was elucidated that pyocin S5 shows the highest killing activity against DWW3 among a range of *P. aeruginosa* isolates; pyocin S5 is a pore-forming bacteriocin capable of damaging DWW3 cell membrane,

which was evidenced by ATP and nucleic acid leakage, as well as morphological changes including cell shape distortion. To elucidate pyocin S5-resistance mechanism in S5-producing strains of *P. aeruginosa* such as PAO1 (as the second goal of this work), the role of a putative *S5I* gene in conferring resistance to pyocin S5 was identified using fluorescent and FESEM microscopy on DWW3 (a Pyocin S5-sensitive strain) and DWW3-expressing *S5I* strains before and after exposure to pyocin S5. It was shown that following pyocin S5 exposure, morphological changes such as cell shape distortion, occurred in DWW3, while no such cell disturbance appeared in the DWW3 expressing *S5I*. This result indicated that *S5I* conferred pyocin S5 resistance through the prevention of membrane damage by pyocin S5.

Furthermore, this study aimed to explore virulence factor regulation in *P. aeruginosa*, which can help to improve the approaches used to inhibit the pathogenicity, invasion, and antibiotic resistance of this bacterium in patients. Therefore, the regulatory role of *mvaU* gene on a virulence factor (pyoverdine, PVD) of *P. aeruginosa* was illustrated by examining the transcriptome profiling of the *mvaU* mutants vs. wild-type PAO1 strain using DNA microarray. The microarray results showed that in the *mvaU* mutants, three genes involved in PVD biosynthesis—*pvdF*, *pvdJ*, and *pvdG*—have been significantly up-regulated. Hence, It was hypothesized that *mvaU* might be responsible for the regulation of these three genes. This hypothesis was verified by qRT-PCR analysis and PVD measurements in wild type and mutant strains. The PVD measurements demonstrated high concentration of PVD in mutant strain compared to

the wild type. This result beckons further investigations in genome profiling and the possible regulation mechanism of PVD upon *mvaU* in *P. aeruginosa*.

In summary, this thesis verified the antimicrobial mechanism of pyocin S5 against clinical isolates of *P. aeruginosa* from cystic fibrosis patients. Moreover, the efficacy and the mode of action of *S5 immunity* gene were elucidated, which renders the host resistance to pyocin S5. In addition, transcriptome profiling revealed that *mvaU*, a global regulatory gene, is involved in pyoverdine regulation.

The findings in this study, including the relationship between PVD-biosynthesis genes and *mvaU* regulatory gene, and the affirmation of S5 and S5I role, provide valuable insights in understanding virulence gene of *P. aeruginosa* and further expanding therapeutic approaches that can be taken against pathogenicity and antibiotic resistance properties of this bacterium.

CHAPTER 1

INTRODUCTION

1.1 Background

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic Gram-negative pathogen, which is highly resistant to a wide range of antibiotics. This motile bacterium is considered as a common cause of cystic fibrosis in patients with immunodeficiency disorders and the third most common cause of nosocomial infections in the United States (Govan and Deretic 1996, Goldberg, Hancock et al. 2008). It has been revealed that up to 85% of adults with cystic fibrosis can be affected by a *P. aeruginosa* chronic infection (Hart and Winstanley 2002), which leads to therapeutic challenges such as antibiotic resistance or toxicity because of long-term treatments (Fothergill, Winstanley et al. 2012). The ineffective responses to antibiotic therapy caused by several mutations and virulence factors have called for the urgent discovery of new therapeutic approaches to battle this pathogen. Despite a wide range of newly discovered and synthesized antibiotics used against this bacterium, *P. aeruginosa* is still developing its resistance to antibacterial agents, which is critically required to find new therapeutic applications for this bacterium. Toward this aim, first, the extensive knowledge of molecular mechanisms of the resistance and sensitivity in this bacterium

is needed. As the second objective, studying the virulence factors as well as seeking for endogenous and potential antibacterial agents produced by *P. aeruginosa* could be useful in conceptualizing new therapeutic approaches. To date, because of high morbidity and mortality due to antibiotic resistance in *P. aeruginosa*, new therapeutic approaches have been developed to overcome this problem. One of the therapeutic approaches is using bacteriocins produced by bacteria, such as pyocin (Line, Svetoch et al. 2008, Ling, Saeidi et al. 2010, Smith, Martin et al. 2012). Pyocin is a narrow-spectrum antibacterial agent produced by more than 90% of *Pseudomonas* strains (Michel-Briand and Baysse 2002). This specific antibacterial agent is able to kill the same or different species except the producing strain because of the probable immunity protein in the producer bacterium. The killing process begins with the specific binding to the receptor in the cell surface. Different receptors have been detected in pyocins, but in the latest discovered pyocin, pyocin S5, the exact killing mechanism and how this pyocin is not effective in producing strain are still unclear. In this thesis, I demonstrated the pyocin S5 killing mechanism and elucidated pyocin S5 antimicrobial activity against seven clinical *P. aeruginosa* isolates. Furthermore, resistant and sensitive strains of clinical isolates against pyocin S5 were identified. To elucidate how the producing strains could be protected from their own pyocins, I confirmed the identity of a predicted *S5 immunity (S5I)* gene in PAO1. To do so, the *S5I* of PAO1 was constructed and transformed into the pyocin S5-sensitive strain, DWW3. Coherently, the resistance was induced in this strain, and the morphological

changes were studied to suggest the possible reasons of this resistance. Thereby, I demonstrated that the expression of *S5I* in *P. aeruginosa* DWW3 conferred pyocin S5 resistance through the prevention of membrane damages caused by pyocin S5, evidenced by cell viability and membrane morphological assays.

As mentioned, the second objective of this work targets the regulatory study of genes involved in virulence. Among the virulence factors in *P. aeruginosa* PAO1, I studied pyoverdine (PVD) to determine whether this nonribosomal peptide is regulated by the regulatory gene of our interest, *mvaU*. Toward this purpose, the *mvaU* gene was deleted in *P. aeruginosa*, and transcriptome profiling was achieved using a DNA microarray experiment to investigate statistically significant changes in the gene expression of *mvaU* mutants compared to the wild type in the whole genome of *P. aeruginosa* PAO1. Following the transcriptome profiling analysis, derived results showed that three genes involved in PVD biosynthesis (in adaptation, protection cluster) were significantly up regulated. Thus, it was hypothesized that the global regulator, *mvaU*, might regulate these three genes. To validate this hypothesis, the concentration of pyoverdine in both wild type and *mvaU* mutants were measured. Although further study, in particular on molecular basis is required to evidence our hypothesis, this finding will be leading us to find out the regulation mechanism of the virulence factor PVD in *P. aeruginosa* and a promising way to investigate new approaches in silencing PVD-related genes to reduce the pathogenicity of this bacterium. Furthermore, this research will be expanded by DNA microarray on cells

upon pyocin treatment to identify the potential transcription regulators in pyocin S5 and S5I and to detect pyocin S5 receptors. PVDs are fluorescent siderophores with the ability of iron up-take from the environment and transportation of iron molecules into the bacterial cells (Boukhalfa and Crumbliss 2002, Braun and Hantke 2011, Hider 2011). Despite virulence role, this molecule, as a signaling molecule, is able to induce other virulence factors in bacteria (Visca, Imperi et al. 2007).

1.2 Objectives

The purpose of this research is firstly to investigate killing mechanism of the new predicted pyocin, pyocin S5, and S5-resistant mechanism of an immunity gene in *Pseudomonas aeruginosa*. In order to achieve this aim, pyocin S5 and immunity proteins in *P. aeruginosa* and *Escherichia coli* were studied by protein structure analysis softwares such as DNAMAN and HNN. Moreover, membrane permeability and cell morphology studies were performed on pyocin S5-resistant and pyocin S5-sensitive strains of *P. aeruginosa* (PAO1 and DWW3, respectively) upon exposure to pyocin S5. To determine the potential immunity role of the *pyocin S5 immunity (S5I)* gene in *P. aeruginosa*, *S5I* gene was transformed to the sensitive strain DWW3, and morphological responses as well as membrane permeability were studied upon pyocin S5 treatment in wild type and *S5I*-transformed DWW3 strains.

Furthermore, this study endeavored to identify virulence genes in *P. aeruginosa*, which is a common cause of nosocomial infection in cystic fibrosis and immune-deficient

patients. It was shown that virulence factors cause antibiotic resistance that results in high mortality and morbidity in patients. Towards this goal, DNA microarray was conducted to examine genome-wide expression profiles in wild-type and deleted *mvaU* mutants of *P. aeruginosa* PAO1 to investigate any association between *mvaU* gene (a global regulator) and virulence factors in *P. aeruginosa*.

As an overall view, the achievement of this project is an effort that improves the study of virulence genes in *P. aeruginosa* PAO1 and helps screening of *S5I* inhibitors to develop an S5 combinatorial therapeutic strategy to target S5-resistant strains of *P. aeruginosa*.

1.3 Thesis outline

This thesis consists of six chapters. In chapter 1, the background, scientific aims, and objectives of this research are described. In Chapter 2, *P. aeruginosa* and its pyocins are discussed. This chapter is a review of literatures. In chapter 3, the bactericidal activity of the predicted pyocin S5 is described. Here, the pyocin S5 killing mechanism and the morphological changes of *P. aeruginosa* upon pyocin S5 exposure are explained. In chapter 4, the identity of a predicted *S5I* gene of *P. aeruginosa* PAO1 is confirmed, and its immunizing role is discussed. Chapter 5 talks about transcriptome profiling in deleted *mvaU* mutant (*PAO1ΔmvaU*) versus wild-type strain of *P. aeruginosa* PAO1 and the potential role of the *mvaU* gene in the regulation of PVD

biosynthesis genes are explained. In chapter 6 the conclusions and recommendations for future works are presented.

CHAPTER 2

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram negative, motile, and opportunistic bacterium from the genus *Pseudomonas* (Anzai, Kim et al. 2000, Ryan 2004). This rod-shaped aerobic pathogen is about 0.5 to 0.8 μm wide by 1.5 to 3.0 μm long in diameter. The ability of *P. aeruginosa* in catabolizing a high variety of organic molecules makes it adaptable to different environments such as human, animals and plants cells, as well as water, soil, and sewage (Botzenhart and Döring 1993). *P. aeruginosa* causes chronic and acute lung infections. This bacterium is the common cause of cystic fibrosis (CF) in immunocompromised patients (Govan and Deretic 1996). Moreover, *P. aeruginosa* is considered as the third most common nosocomial pathogen in the United States (Goldberg, Hancock et al. 2008). The other infections caused by *P. aeruginosa* are septicemia, urinary tract infection, endocarditis following cardiac surgeries, meningitides and diarrhea. Moreover, *P. aeruginosa* is a major source of antibiotic-resistant infection in hospitalized patients and a significant death cause in patients with cystic fibrosis (Govan and Deretic 1996). The clinical importance of this motile pathogen is related to ineffective response to antibiotic treatment which is due

to a variety of mutations in this microorganism and secretion of various virulence factors. This is a big challenge, particularly to patients with immune deficiency diseases including cancer, acquired immune deficiency syndrome (AIDS) or those who are taking immunosuppressive medications. *P. aeruginosa* can survive in various conditions including up to 42°C temperature, anaerobic conditions as well as minimum nutrient resources. The adaptability of this microorganism is due to different factors such as its ability to use various carbon sources for using energy, recruiting nitrogen as a terminal receptor and biofilm formation (Abd, Wretlind et al. 2008, Matz, Moreno et al. 2008). *P. aeruginosa* degrades host cells through introduction of some virulence factors that can disrupt RNA translation (Woods, Cryz et al. 1982) or production of other factors that entail flagella (Feldman, Bryan et al. 1998) and adhesion (Comolli, Hauser et al. 1999). *P. aeruginosa* can live in two phenotypic modes of growth, planktonic and biofilm, which are reversible to each other mediated by regulatory genes (Goodman, Kulasekara et al. 2004). In planktonic mode, *P. aeruginosa* lives as a single, independent bacterium and causes acute infections. The biofilm mode is resistant to antibiotic treatment, in which causes chronic infection in cystic fibrosis patients (Bjarnsholt 2013). Biofilms are up to 1000-fold antibiotic resistance compared to planktonic cells that is one of the most and well-known traits of biofilm-mode properties (Hoyle and Costerton 1991) in this bacterium. Despite wide range of new antibiotics used against this bacterium, *P. aeruginosa* is still considered as a highly

resistant microorganism to the antibacterial agents, which makes the therapeutic approaches so critical in this bacterium.

2.1.1 *Pseudomonas aeruginosa* genome

The genome of *P. aeruginosa* PAO1 strain was first sequenced by Stover's lab in 2000 revealed that *P. aeruginosa* PAO1 has the largest bacterial genome with high complexity. When PAO1 as a clinical strain was isolated from wound, it was shown that the genome size of 6.3 Mbp contains 5570 predicted open reading frames (ORFs) in this strain (Holloway 1955). A large number of PAO1 genes are associated with outer membrane proteins mediating in motility, efflux pumps, quorum sensing, adhesion, which are considered as virulence factors. The other genes involved in the majority of the localized genes in *P. aeruginosa* PAO1 are related to metabolism and nutrient uptake including enzymes and transport-associated genes (Kung, Ozer et al. 2010). Stover showed that the outer membrane proteins (OMPs) of *P. aeruginosa* PAO1 are encoded by around 150 genes. These proteins are determined as three major families including: the group of outer membrane proteins related to secretion or efflux pumps named OprM (encoded by 18 genes), the OprD family of particular porins (controlled by 19 genes) and the family of gated porins mediates in up taking iron-siderophore (34 genes) (Stover, Pham et al. 2000). More studies in the genome sequence on clinical isolates of *P. aeruginosa* strains implied that the mosaic genome contains conservative sequences and is combined of two parts; the core (90% of total

genome) and the accessory. The genes involved in metabolic and pathogenesis of nearly all strains of *P. aeruginosa*, regardless of their source of isolation (clinical, environmental or in vitro isolates) are categorized in core genome. The core encompasses highly conservative genes. In contrast, the genes that are limited to the specific strains are located in the accessory part, such as the adaptation-related genes (Kung, Ozer et al. 2010). Indeed, the huge size and the variety of virulence genes together with several mutations in *P. aeruginosa* genome force this bacterium to develop antibiotic resistance properties through different approaches such as mobile genetic materials (plasmids) (Lister, Wolter et al. 2009).

2.2 Bacteriocins

Bacteriocins are antimicrobial proteins with mostly low molecular weight produced by gram negative and gram positive bacteria (Farkas-Himsley 1980). Bacteriocin was discovered and introduced by André Gratia, in 1925 (Gratia 1925, Gratia 2000). It has been elucidated that bacteriocins are ribosomally synthesized peptides and are able to kill or inhibit the growth of the other strains but it will not be harmful or toxic against their own producer (Cotter, Ross et al. 2013). These narrow spectrum antibacterial peptides are termed based on their source of production for example, colicin, warnericin, lantibiotics and pyocin that are secreted by *E. coli*, *Staphylococcus warneri*, lactic acid-fermenting bacteria and *Pseudomonas sp.*, respectively. Bacteriocins have shown some benefits over antibiotics such as low toxicity, both narrow- and

broad-spectrum property as well as *in vivo* and *in vitro* applicability, which make them important for therapeutic purposes (Cotter, Ross et al. 2013). Based on these characteristics and the benefits of bacteriocins mentioned above, in recent years, these antimicrobial agents have become more applicable in broad range of Gram-positive and Gram-negative bacteria. A study reported the inhibitory role of entomocin 9 (purified from *Bacillus* sp.) that is able to kill some fungi as well as *P. aeruginosa* and *Listeria monocytogenes* (Cherif, Chehimi et al. 2003). Further studies reported more bacteriocins with killing activity against other strains for example, pyocin S5 from *P. aeruginosa* PAO1 against different clinical isolates of *P. aeruginosa* strains (Ling, Saeidi et al. 2010), enterocin E760 (produced by NRRL B-30745 strain of *Enterococcus durans*, *Enterococcus* sp) with killing ability against a wide range of Gram-positive and Gram-negative bacteria including *Salmonella enterica*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Campylobacter* sp. and *P. aeruginosa* (Line, Svetoch et al. 2008). This thesis mostly focuses on a newly discovered pyocin in *P. aeruginosa* PAO1, named pyocin S5.

2.3 Pyocins

Pyocins are bacteriocins produced from *Pseudomonads*. More than 90% of *P. aeruginosa* strains are able to produce pyocins (Michel-Briand and Baysse 2002). Pyocin was initially identified by François Jacob when he found out that the released compound from lysed *P. aeruginosa* 10 is able to bind to the same species and kill

them. He reported that this toxic compound is not sensitive to protease enzymes and classified it as R-type pyocins (Jacob, Lwoff et al. 1954, Michel-Briand and Baysse 2002). The production of pyocin in *P. aeruginosa* is in a very low level and the bacteria need to be provoked under stress conditions in order to induce their pyocin production. It has been shown that production of pyocin is induced in *P. aeruginosa* by some DNA damaging agents such as UV radiation, mitomycin C (a mutagenesis component) and hydrogen peroxide (Jacob, Lwoff et al. 1954, Kageyama 1964, Chang, Small et al. 2005). Unlike the colicins in *E. coli* strains that are carried on plasmids, the genetic materials of pyocins are located on the chromosome (Shinomiya, Shiga et al. 1983). All pyocins begin their killing activity through binding to the specific receptor, which is located in the outer membrane of sensitive cells (Ling, Saeidi et al. 2010). It has been confirmed that pyocin producing strains are insensitive to their own pyocins due to expression of an immunity gene (Goodwin, Levin et al. 1972).

The classification of pyocins is based on different parameters such as water solubility, killing mechanism, structure of molecule, and sensitivity to the enzymes. Regarding their killing mechanism, structure and solubility pyocins have been classified into three classes; phage-like pyocins (R type), flexible pyocins (F type), and soluble pyocins (S type).

2.3.1 R-Type Pyocin

The first group of identified pyocins, termed R type, is produced by more than 90% of *P. aeruginosa* strains (Smith, Hirst et al. 1992). This group includes the rod-shaped bacteriocins with phage-like tail and protease-nuclease resistance (Kageyama 1964, Ishii, Nishi et al. 1965). When the R-type pyocin is absorbed by specific receptor on the host cell surface, it penetrates to the cell and releases some intracellular components. After 20 minutes cell death occurs due to depolarization associated with pore formation in cytoplasmic membrane (Kageyama 1964, Ohsumi, Shinomiya et al. 1980, Uratani and Hoshino 1984). R-type pyocin is very efficient such that one molecule of R-type pyocin is able to kill one bacterium (Michel-Briand and Baysse 2002). R-pyocin producing strains are resistant to their pyocin. Although no immunity genes are associated with cognate R-type pyocins, it was hypothesized that the existence of lipopolysaccharide can behaves as a receptor for R-pyocin producing strains (Kohler, Donner et al. 2010).

The structure of R-type pyocins was studied by different scientific groups that confirmed the existence of around 200 protein subunits with contraction ability (Ishii, Nishi et al. 1965, Yui-Furihata 1972, Takeda and Kageyama 1975). Since 1964, when the first pyocin R (R1) was classified by Japanese scientist, several R-type pyocins have been reported and named; R2, R3, R4, and R5 (Ito 1970, Takeda and Kageyama 1975), C9, 430c, and 21 (Higerd, Baechler et al. 1967, Govan 1974, Govan 1974). It was reported that R-type pyocins are not only lethal for *P. aeruginosa*, but for the

other gram negative bacteria such as *Neisseria* sp., and *Haemophilus* sp. because of the common receptor with *P. aeruginosa* (Levin and Stein 1996, Filiatrault, Munson et al. 2001).

R-type pyocin is encoded on the bacterial genome (Shinomiya, Shiga et al. 1983). In 2000, Nakayama and his colleagues showed that 12 out of 16 ORF of *P. aeruginosa* are similar to phage P2. They confirmed the similar gene organization in these two microorganisms (Nakayama, Takashima et al. 2000). Furthermore, it was revealed that there is a high similarity between pyocin R1 and the T-phage tail structure (Figure 2.1) (Michel-Briand and Baysse 2002). Specificity of R-type pyocins related to the tail fiber structure has been used in a novel R-pyocins construction with a therapeutic application (Williams, Gebhart et al. 2008). This property of new engineered R pyocin appeared in a mouse model of *P. aeruginosa* peritonitis and *E. coli* contaminated food for *in vivo* and *in vitro* experiments, respectively (Scholl and Martin 2008, Scholl, Cooley et al. 2009). Meadow and Wells identified the presence of some R-pyocin-specific recognition sites in lipopolysaccharide (LPS) core of *P. aeruginosa*. Moreover, these LPS molecules have the ability of pyocin binding. By the inspiration of these data, two principal roles were proposed for LPS including protection as a shield and a receptor for R-pyocin (Kohler, Donner et al. 2010). A study worked on re-targeting R2 pyocins from *Pseudomonas* strains to *E. coli*. This engineered pyocin has the killing activity for *E. coli* C and K-12. In this method, C-terminal part of the closely related bacteriophage P2 tail fiber was combined to the N-terminal fragment of

the R2 tail fiber in *P. aeruginosa*. This constructed pyocin was named AVR2-P2 and it cannot kill O157:H7 strains (Williams, Gebhart et al. 2008). The similar study also was hypothesized that the adsorption of pyocin by the cell surface will be blocked by proportionately thick O157 antigen in *E. coli* (Ho and Waldor 2007). Recently, the success in engineering R-type pyocins opens the new era in using pyocin as therapeutic approaches (Scholl and Martin 2008, Fothergill, Winstanley et al. 2012).

Figure 2.1 Schematic structure of pyocin R (Yui-Furihata 1972, Michel-Briand and Baysse 2002).

2.3.2 F-Type Pyocin

The second discovered pyocin was named F-type pyocin with flexible but non contractile structure, in contrast with the first discovered pyocin (R-type) (Takeya, Minamishima et al. 1967). Similar to R-type pyocins, pyocins in class F show

protease-nuclease resistance (Govan 1974). These pyocins represent more killing activity against other Gram-negative bacteria compared to R-type pyocins (Filiatrault, Munson et al. 2001). Similarly, this pyocin is also released by more than 90% of *P. aeruginosa* strains (Smith, Hirst et al. 1992). Some similarities have been identified between F-type pyocin and λ phage considered as evidence that this bacterium is derived from λ phage family (Nakayama, Takashima et al. 2000). In comparison with R-type pyocins, more F-type pyocin molecules are required to kill one *P. aeruginosa* (Michel-Briand and Baysse 2002). Later on more F-type pyocins were characterized in different strains of *P. aeruginosa* such as 430f, F1, F2, and F3 (Govan 1974, Kuroda and Kageyama 1979, Kuroda and Kageyama 1981). The structure of F-type pyocin was observed by electron microscopy and showed the phage-like tail with some fiber filaments, which specified this molecule to the receptors of the host cell surface (Figure 2.2) (Kuroda and Kageyama 1981, Michel-Briand and Baysse 2002). Although R- and F-type pyocins are phage-like components, the molecule lacks nucleic acids, which are found in phage and, results in reproduction incapability of R-and F-type pyocins.

Figure 2.2 Schematic structure of pyocin F (Kuroda and Kageyama 1981, Michel-Briand and Baysse 2002).

2.3.3 S-Type Pyocin

Colicin-like bacteriocin, as the last identified bacteriocin of *P. aeruginosa*, is categorized among soluble pyocins (S-type). In contrast with two other R- and F-type pyocins, S-type pyocins are sensitive to protease enzymes. More than 70% of *P. aeruginosa* are able to produce S-type pyocins (Smith, Hirst et al. 1992). To date, 7 types of S-type pyocins have been identified and termed as S1 (Ito 1970), S2 (Ohkawa 1973), Sa (Govan 1986), S3 (Duport, Baysse et al. 1995), S4, S5, and AP41 (Holloway, Rossiter et al. 1973, Parret and De Mot 2000) (Table 2.1).

Table 2.1 The identified S-type pyocins in *P. aeruginosa* strains

S Type Pyocin	Year	Mode of Killing	Discovered by
S1	1970	DNase	Ito, S <i>et al.</i>
S2	1973	DNase	Ohkawa, I <i>et al.</i>
AP41	1973	DNase	Holloway, B. W <i>et al.</i>
Sa	1986	DNase	Govan, J.R.
S3	1995	DNase	Duport, S <i>et al.</i>
S4	2000	tRNase	Parret, A <i>et al.</i>
S5	2000	Pore forming	Parret, A <i>et al.</i>

Purification of S-type pyocins illustrated that these proteins are made of two big and small components that interact with each other (Sano and Kageyama 1981, Sano 1990) (see Figure 2.3). The big part has a killing activity by adopting diverse mechanism in different S-type pyocins. As mentioned in table 1, different S-type pyocins propose various killing mechanisms. Most of the pyocins recruit endonucleases activity in killing cells which results in DNA breakdown and cell death (Michel-Briand and Baysse 2002). Some examples of this class of pyocins are S1-S3 and AP41 with DNase activity (Ito 1970, Holloway, Rossiter *et al.* 1973, Ohkawa 1973, Duport, Baysse *et al.* 1995). However, S4 pyocin was determined with tRNase activity, whilst it was shown that pyocin S5 molecules kill host cells through creating a pore in the membrane of target cells (Parret and De Mot 2000). Big part of S-type pyocins contains 4 domains (Figure 2.4) that are located in the N-terminus. These domains are termed domain I, which is a receptor binding domain, domain II whose role has not been determined, and domain III or translocation domain that is made up of about 240 amino acids and is responsible in translocation across the outer membrane of the target

cells. Domain IV (located in C-terminus) is involved in the killing activity of pyocin (DNase) and is in interaction with a small component (Michel-Briand and Baysse 2002).

Figure 2.3 Schematic structure of pyocin S5 protein (Michel-Briand and Baysse 2002).

The small protein of S-type pyocin (made of 85-153 amino acids) confers immunity to *P. aeruginosa* producing this pyocin (Sano and Kageyama 1993, Sano, Matsui et al. 1993). The similarities between this small compound and its homologues immunity proteins in colicins of *E. coli* strains proposed this small protein as an immunity protein with ability of immunizing *P. aeruginosa* strains against their own produced pyocin (Sano 1990). Discovery of pyocin S5 in *P. aeruginosa* PAO1 by Parret.*et al.* illustrated that this protein (with 498 amino acids) is associated with another small component containing 108 amino acids, which was predicted as an immunity protein. The predicted immunity protein of PAO1 strain is located in the downstream part of pyocin S5 with remarkable similarity to colicin Ia and Ib immunity proteins of *E. coli*.

Pyocin S5 showed high homology with pyocin S2, while the killing domain of S5 pyocin in C-terminus was similar to *E. coli* colicins (Ia and Ib) with pore forming activity (Parret and De Mot 2000) (Figure 2.4).

Figure 2.4 Three known domains of big compound of Pyocin S5 (Ling, Saeidi et al. 2010).

The killing mechanism starts with bacterial exposure upon DNA damaging conditions. Then through its binding domain of the large compound, pyocin binds to the specific receptor in the host cell. The host cell could be different strain of the same species of pyocin-producing bacteria. Some studies reported the inhibitory role of pyocins on the other species (Morse, Vaughan et al. 1976, Sidberry and Sadoff 1977, Scholl, Cooley et al. 2009), as discussed in section 2.2. Various receptors have been identified for S-type pyocins including siderophore pyoverdine receptors. These receptors are expressed within iron starvation conditions. For example, FpvAI (ferripyoverdine A) is used by pyocin S2 and S4 to bind to the host cell (Baysse, Meyer et al. 1999, de Chial, Ghysels et al. 2003). Furthermore, S3 binds to FpvAII on the surface of target cell, while no receptor has been identified for pyocin S5 yet (Elfarash, Wei et al. 2012).

Similar to two other types of pyocins, genomic materials of S-type pyocins of *P. aeruginosa* are located on the chromosome. Their operon contains two open reading frames (ORF). The first ORF encodes large protein, while the small component is

encoded by the second ORF (Michel-Briand and Baysse 2002). Table 2.2 shows the origin of each S-type pyocin in *P. aeruginosa* strains (Ito 1970, Sano and Kageyama 1984, Sano, Matsui et al. 1993, Duport, Baysse et al. 1995, Parret and De Mot 2000). Based on PAO1 genome sequence, the amino acid sequences of pyocin S4 (PA3866) was predicted at 4329990–4327696 of PAO1 genome, while its immunity protein has not been predicted yet. Similarly, the most currently discovered S-type pyocin, pyocin S5 (PA0985) and its immunity protein (PA0984) were predicted at 1067817–1066321 and 1065970–1066296 of *P. aeruginosa* PAO1 strain, respectively (Parret and De Mot 2000).

Table 2.2 Genetic determinants location of S-type pyocins

Name of S-type pyocin	Source of production (<i>P. aeruginosa</i>)
S1	NIH-H, PA03012
S2	NIH-H, PA03012
S3	P12
AP41	PAF41
S4	PAO1
S5	PAO1

2.4 Pyocin Regulation

In *P. aeruginosa*, pyocin production is induced upon exposure to DNA damaging factors such as mitomycin C (Michel-Briand and Baysse 2002). Pyocin regulation studies in *P. aeruginosa* PAO1 implied that pyocins R2, S2, and F2 are controlled by two major genes, *priN* and *priR* (Shinomiya, Shiga et al. 1983, Matsui, Sano et al.

1993). Moreover, a conserved regulatory sequence was identified in the promoter of S-type pyocins including S1-S4 and AP41 termed P box, while such regulatory box has not been characterized in pyocin S5 (Matsui, Sano et al. 1993, Sano and Kageyama 1993, Duport, Baysse et al. 1995, Parret and De Mot 2000). Another regulatory gene of pyocin production was found by Sano and called *recA* (Sano and Kageyama 1987). *prrR* is identified as a negative regulator located on the upstream part of R2-F2 pyocin genes (Nakayama, Takashima et al. 2000). The other regulatory gene *prrR* was determined as a repressor for *prrN* promoter activity (Matsui, Sano et al. 1993). There is a high homology in PrtN protein and cI repressor in Φ 80 phage (Michel-Briand and Baysse 2002).

The regulation mechanism of S-type pyocin synthesis is shown in Figure 2.5. In the absence of mutagenesis factors, S-type pyocin was synthesized by *P. aeruginosa* PAO1 in very low levels because of the repression of *prrN* gene by PrtR proteins (with 256 amino acids). Following exposure of S-type pyocin-producing PAO1 to some inducers such as DNA damaging agents, *recA* gene expression will be induced and results in production of DNA repair proteins, RecA. The RecA molecules then cleave the repressor protein (PrtR) that cause enhancement of *prrN* expression, followed by releasing of PrtN proteins (with 104 amino acids). PrtN proteins activate pyocin synthesis in *P. aeruginosa* PAO1 (Matsui, Sano et al. 1993).

Figure 2.5 Regulation of pyocin producing genes (Michel-Briand and Baysse 2002).

2.5 Histone-Like Nucleoid Structuring (H-NS) Proteins

Chromosome is known as a big component in bacterial cell. To overcome the difficulty of compacting chromosome into the limited size of the bacterial cells, these tiny microorganisms recruit some mechanisms such as macromolecular crowding and DNA supercoiling. Furthermore, small nucleoid-associated proteins are identified as other key molecules in chromosome condensation mechanism (Luijsterburg, Noom et al. 2006, de Vries 2010). Histone-like nucleoid structuring (H-NS) proteins are one of the DNA compactors (Dorman 2004). They are also known as important global regulators

with approximately ~15 kDa in size and are found in various Gram-negative bacteria (Castang, McManus et al. 2008), while to date no archaea and Gram-positive bacteria producing these proteins were found (Tendeng, Soutourina et al. 2003). It was shown that H-NS proteins have an important role in silencing a broad range of genes in gram-negative bacteria. They perform their regulatory role through oligomerization of AT-rich regions in DNA by binding to the specific sites on this molecule (Dorman 2004, Dame, Luijsterburg et al. 2005, Bouffartigues, Buckle et al. 2007, Navarre, McClelland et al. 2007). Initially H-NS proteins were isolated from *E. coli* (Jacquet, Cukier-Kahn et al. 1971). Following nucleotide sequence homology, more H-NS proteins were identified in other enteric-related bacteria such as *Serratia marcescens*, *Salmonella typhimurium*, *Proteus mirabilis* (Ussery, Hinton et al. 1994), and *Haemophilus influenza* (Fleischmann, Adams et al. 1995). The first isolated H-NS-like protein in non-*Enterobacteriaceae* sp. (Goyard and Bertin 1997), implied that despite previous theories, these proteins are not limited to the *Enterobacteriaceae* family (Atlung and Ingmer 1997). Later on, for the first time, Tendeng *et al.* reported the isolation and characterization of H-NS-related gene from a psychrotrophic strain of *Pseudomonas*, Y1000 (Tendeng, Soutourina et al. 2003). Moreover, genome sequence study in different strains of *Pseudomonas* revealed the existence of five putative H-NS proteins in *Pseudomonas putida*, four in *Pseudomonas syringae*, three in *Pseudomonas fluorescens*, and two in *Pseudomonas aeruginosa*, with at least one homologous of H-NS family protein isolated from each of these strains (Tendeng, Soutourina et al.

2003). It was shown that H-NS proteins are able to repress the expression of hundreds of genes (Castang, McManus et al. 2008). Different Gram-negative bacteria such as *Photorhabdus luminescens* (Tendeng 2002), *Vibrio cholerae* (Tendeng, Badaut et al. 2000), *Psychrobacter TAD1* (Tendeng, Krin et al. 2003), and *Yersinia enterocolitica* (Bertin, Hommais et al. 2001) can produce more than one H-NS family member (Tendeng and Bertin 2003).

The two H-NS family members in *P. aeruginosa* are named *mvaU* and *mvaT*. Genome analysis of *P. aeruginosa* PAO1 confirmed the presence of two highly homologous putative genes with P16 subunit of *mvaT* in the soil bacterium *Pseudomonas mevalonii*. This study determined 82% similarity in the first putative gene (PA4315) with *mvaT* subunit in *P. mevalonii* (Rosenthal and Rodwell 1998). The other homologue, PA2667, was characterized with 64% homology with the second subunit of *mvaT* in *P. mevalonii*. Further protein analysis showed that PA2667 was 68% similar to PA4315. Based on these results designated PA4315 and PA2667 were named as *mvaT* and *mvaU*, respectively (Vallet, Diggle et al. 2004). Furthermore, by using tandem affinity purification coupled with mass spectrometry it was revealed that MvaT is in contact with its homologous protein through a direct protein-protein interaction, which occurs in N-terminal regions of these two proteins (Vallet-Gely, Donovan et al. 2005). Based on structural and functional similarities in MvaT and MvaU of *P. aeruginosa* PAO1 these two proteins were categorized in H-NS family in this strain (Vallet, Diggle et al. 2004, Vallet-Gely, Donovan et al. 2005). Structurally, both MvaT and MvaU are

composed of two major domains; the DNA-binding domain in the C-terminus and the oligomerization domain located in N-terminus contains a coiled-coil forms H-NS dimer (Vallet-Gely, Donovan et al. 2005). Later on, genomic study using ChIP-on-chip method illustrated the same AT-rich regions that were occupied by MvaT and MvaU, suggesting that they may regulate common genes coordinately (Castang, McManus et al. 2008).

2.5.1 MvaT protein

mvaT was initially identified as a global regulator of virulence genes in *P. aeruginosa* PAO1 (Diggle, Winzer et al. 2002). Subsequently, the data from microarray analyses of *P. aeruginosa* PAO1 unveiled MvaT protein as the major regulator for more than 150 genes in particular, those are involved in virulence and biofilm formation (Diggle, Winzer et al. 2002, Vallet, Diggle et al. 2004, Westfall, Carty et al. 2006). Consistent with microarray analysis, *mvaT* deletion in *P. aeruginosa* PAO1 induced pyocyanin (a virulence factor) production in this bacterium (Diggle, Winzer et al. 2002).

2.5.1.1 *mvaT* regulatory role

The pathogenicity of *P. aeruginosa* is caused by production of molecules named virulence factors. To date, various virulence factors including elastase, lipase, and some small iron chelating molecules such as siderophores, including pyocyanin and pyoverdine have been identified in *P. aeruginosa* (Meyer, Neely et al. 1996, Lamont, Beare et al. 2002, Potvin, Lehoux et al. 2003). These virulence factors not only have an

effect on growth rate and metabolism of the bacterium, but also able to interrupt the phenotypic characteristics such as biofilm formation and bacterial motility including swarming, twitching, and twisting of *P. aeruginosa* (Potvin, Lehoux et al. 2003). As mentioned above the pathogenicity of *P. aeruginosa* depends on its virulence factors that are controlled by a cascade of regulatory genes. One of the most significant mechanisms recruited by *P. aeruginosa* in order to regulate a diversity of physiological processes is quorum sensing system (Fuqua, Winans et al. 1994). A work was done by Diggle *et al.* showed that deletion of *mvaT* gene enhanced the expression of growth-phase dependent *lecA* and quorum sensing repression. In spite of *lecA* overexpression and pyocyanin increment in *mvaT* mutant of *P. aeruginosa* PAO1 in comparison with the wild type, this mutant strain showed a significant reduction in production of the other virulence factors such as LasA protease, elastase and swarming motility (Diggle, Winzer et al. 2002). Consistent with Diggle's work, another study revealed the role of *mvaT* in biofilm regulation due to repression of *cupA* gene (Vallet-Gely, Donovan et al. 2005), which has been recognized as a prominent gene among biofilm regulatory genes. The regulatory role of *cupA* is due to production of components mediating in fimbrial structures, which facilitates surface attachment in biofilm formation process (Vallet, Olson et al. 2001, Vallet, Diggle et al. 2004).

It has been revealed that the mortality and morbidity of *P. aeruginosa* result in resistance of this bacterium to a wide range of antibiotics. The drug resistance problem raised the need for more studies on resistance-mediated factors such as

energy-dependent multidrug resistance (MDR) efflux pumps in *P. aeruginosa*. In the most of the resistant cases, antibiotic resistance has resulted in the activation of these pumps. To date, seven MDR efflux pumps have been identified and the effect of *mvaT* on repression of some of these efflux pumps has been discussed (Westfall, Carty et al. 2006).

2.5.2 MvaU protein

The second and less abundant member of H-NS family in *P. aeruginosa* PAO1 is named MvaU with limited regulatory role in gene expression of this bacterium (Vallet, Diggle et al. 2004, Vallet-Gely, Donovan et al. 2005). The data from *mvaU* gene deletion showed that in contrast with the close interaction between MvaU and its homologous protein, MvaT (Vallet-Gely, Donovan et al. 2005), deletion of *mvaU* did not show any significant effect on MvaT-regulated genes (Vallet, Diggle et al. 2004, Vallet-Gely, Donovan et al. 2005).

2.5.2.1 *mvaU* regulatory role

To date, fewer studies have been done on the role of *mvaU* in regulation of *P. aeruginosa* virulence genes, while the number of *mvaT* researches is considerably high. It was shown that *mvaU* regulatory role is not as prominent as the *mvaT*. Consequently, Some of the regulatory effects of *mvaT* were not observed in *mvaU* for example, despite of *mvaT*'s role in *cupA* gene regulation, *mvaU* is not involved in such regulatory process (Vallet, Diggle et al. 2004) but it has been demonstrated that the

overexpression of *mvaU* helps deleted *mvaT* strains to retrieve their ability in cupA gene repression (Vallet-Gely, Donovan et al. 2005). Some reports revealed that depletion of both *mvaU* and *mvaT* in *P. aeruginosa* strains cannot be tolerated and the cell growth rate will be dramatically reduced in double mutant strains (Castang, McManus et al. 2008) while, depletion of one of these H-NS members (*mvaT* or *mvaU*) induces the production of another one (Vallet-Gely, Donovan et al. 2005). Later on, the double mutants of *mvaU* and *mvaT* were constructed and it was found that while the growth rate of double mutant strains is significantly lower than wild type or single mutant, some other abilities such as Pf4 expression and consequently, phage activation have been increased in these double-knocked out strains (Li, Wally et al. 2009, Castang and Dove 2012). In contrast with overexpression of pf4 genes, pyocyanin production was inhibited in the *mvaT* and *mvaU* double mutants (Li, Wally et al. 2009).

2.6 Pyoverdine

Like other organisms, pathogenic bacteria need to uptake iron in order to better growth and pathogenesis (Neilands 1987). Due to limitation of iron in the host cells and the environment, *P. aeruginosa* produces iron-chelating components with high Fe (III) binding affinity to overcome this iron poor condition and acquires sufficient iron (Lamont, Beare et al. 2002). One of the most important iron-chelating compounds is termed siderophore (Guerinot 1994). In addition to its role in hunting and transporting

the iron molecules in iron starvation states, this non-ribosomal protein is considered as a signaling molecule and regulates gene expression in bacteria (Lamont, Beare et al. 2002, Visca, Leoni et al. 2002, Visca, Imperi et al. 2007). Till now, two types of siderophores, psychelin (low affinity) and pyoverdine (high affinity), have been discovered in *P. aeruginosa* (Cox 1980, Cox and Adams 1985). Pyoverdines or pseudobactins are yellow-greenish fluorescent pigments that uptake iron from the environment and transport it to the bacterial cells in iron-depleted conditions (Boukhalfa and Crumbliss 2002, Braun and Hantke 2011, Hider 2011). Pyoverdines are produced by fluorescent *Pseudomonas* species including *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens* (Meyer JM 1978), *P. putida* and *P. syringae* (Palleroni 1984) as well as *P. jessenii* and *P. mandelii* (Verhille, Baida et al. 1999), *P. monteilii* (Elomari, Coroler et al. 1997), *P. rhodesiae* (Coroler L 1996) and *P. veronii* (Elomari, Coroler et al. 1996) and secreted in the cytoplasm. In addition to iron acquisition of pyoverdine, this fluorescent pigment is able to regulate some other bacterial functions such as biofilm formation, and cell-to-cell communication (Visca, Imperi et al. 2007). Furthermore, pyoverdine as a virulence factor is able to modulate the production of some other virulence factors including exotoxin A, PrpL, and its own regulation (Lamont, Beare et al. 2002). Since the first discovery of pyoverdine in 1892 (MC 1892), more than 50 pyoverdine molecules have now been detected from different species of *Pseudomonas* (Budzikiewicz 2004). Despite the high diversity in pyoverdine structure in different strains, three major parts are represented in all strains

and species of *Pseudomonas*: (1) dihydroxyquinolone chromophore as a conserved fluorescent domain; (2) an acyl side chain (either dicarboxylic acid or amide) that binds to the amino group of the chromophore; and (3) a variable peptide chain linked by an amide group binding to the commonly C1 carboxyl group of the chromophore (Visca, Imperi et al. 2007). In *P. aeruginosa* strains three types of pyoverdines are produced, while each strain can synthesize just one type of pyoverdine (Jimenez, Koch et al. 2012) (see Figure 2.6).

Figure 2.6 Three types of pyoverdine molecules produced by different *P. aeruginosa* strains (Jimenez, Koch et al. 2012).

2.6.1 Pyoverdine regulation

As described in section 2.6, pyoverdine is an important signaling molecule with the ability of iron binding and iron transportation in iron starvation conditions. This molecule makes the bacterial cells more invasive and pathogenic to the host cells through production of some other virulence factors in addition to itself, which is proposed as a virulence compound. Therefore, function of pyoverdines is directly environmental dependent and is based on cell-to-cell communication processes. Consequently, following iron-depletion conditions pyoverdine production will be increased and diversely, due to high iron concentration in the environment it will be

repressed (Meyer JM 1978). In this process, an alternative sigma factor termed PvdS plays a major and essential positive regulatory role in pyoverdine biosynthesis (Cunliffe, Merriman et al. 1995, Miyazaki, Kato et al. 1995, Leoni, Orsi et al. 2000, Wilson and Lamont 2000). Another regulatory gene was named *ferric uptake regulatory* gene, *fur* was identified as a repressor in iron-rich conditions in PAO1 strain that inhibits pyoverdine biosynthesis through suppression of *pvdS* transcription (Prince, Storey et al. 1991, Leoni, Ciervo et al. 1996). It has been implied that *pvdS* expression is regulated by an inducer gene called *cysB*, which binds to the *pvdS* promoter and results in expression of PvdS protein (Imperi, Tiburzi et al. 2010). Coherently, due to PvdS production, the responsible genes in pyoverdine synthesis are expressed causing pyoverdine production. There are two sets of genes responsible in regulation of pyoverdine including *pvd* and *pvc* as peptide and chromophore biosynthesis regulators, respectively (Stintzi, Cornelis et al. 1996, Stintzi, Johnson et al. 1999). In the category of *pvd* cluster, four major pyoverdine synthesis genes (*pvdD*, *pvdI*, *pvdJ*, and *pvdL*) have been identified in *P. aeruginosa* PAO1 (Mossialos, Ochsner et al. 2002, Ackerley, Caradoc-Davies et al. 2003, Ravel and Cornelis 2003). Moreover, ferric pyoverdine receptor gene (*fvpA*) is also classified in *pvd* cluster (Poole, Neshat et al. 1993). It has been revealed that in the presence of pyoverdine and iron starvation conditions, pyoverdine binds to the FpvA receptor on the cell surface. Furthermore this siderophore bounds to the Fe (III), and this triplet complex activates FpvR. As an antagonist of PvdS, FpvR activates PvdS production that results in

producing more virulence factors including *tox*A, PrpL, and pyoverdine itself (Beare, For et al. 2003). In addition to these mentioned-above genes and proteins, another protein named TonB1 was found with a key role in pyoverdine transportation and signaling pathway (Shirley and Lamont 2009). A list of genes controlling pyoverdine biosynthesis and transportation is shown in Table 2.3 (the data were collected from literatures) Regarding all the studies about pyoverdine regulation, I did not find any research focusing on finding the relation between H-NS family proteins (*mvaT* and *mvaU*) and any related-pyoverdine regulatory genes except one study discussed about *mvaT* role in biosynthesis of pyoverdine through *pvdS* regulation (Imperi, Tiburzi et al. 2010). In this study no correlation between *mvaT* and *pvdS* was found. In line with their experiments, microarray results confirmed their conclusion (Vallet, Diggle et al. 2004). Consistent with these findings and all the studies that are yet to be done yet, I was eager to work on regulatory role of *mvaU* and if this gene can regulate any pyoverdine biosynthesis genes.

Some studies working on pyoverdine biosynthesis and mediating factors such as initiators, enzymes, cofactors and transporters elucidated *pvdJ* as one of four most important pyoverdine synthesis genes in PAO1 strain of *P. aeruginosa*, which are capable to produce synthetase enzymes in this process (Mossialos, Ochsner et al. 2002, Ackerley, Caradoc-Davies et al. 2003, Ravel and Cornelis 2003). Moreover, *pvdF* together with *pvdA* were identified as catalyzing enzymes of

N⁵-formyl-N⁵-hydroxyornithine (fOHOrn) synthesis that is a pyoverdine component (Visca, Serino et al. 1992, McMorran, Shanta Kumara et al. 2001).

Table 2.3 List of genes mediating in pyoverdine synthesis and transportation

Gene ID	Name	PseudoCAP Function Class	Reference
PA1754	cysB	Transcriptional regulators	Imperi <i>et al</i> , 2010
PA2398	fpvA	Transport of small molecules	Poole <i>et al</i> , 1993
PA2387	fpvI	Transcriptional regulators, Transport of small molecules	Beare <i>et al</i> , 2003
PA2388	fpvR	Transcriptional regulators, Transport of small molecules, Membrane proteins	Lamont <i>et al</i> , 2002, Beare <i>et al</i> , 2003
PA2254	pvcA	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintzi <i>et al</i> , 1996
PA2255	pvcB	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintzi <i>et al</i> , 1996, 1999
PA2256	pvcC	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintzi <i>et al</i> , 1996, 1999
PA2257	pvcD	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintzi <i>et al</i> , 1996, 1999
PA2386	pvdA	Adaptation, Protection	Visca <i>et al</i> , 1994
PA2399	pvdD	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)	Merriman <i>et al</i> , 1995
PA2397	pvdE	Membrane proteins, Transport of small molecules, Adaptation, Protection	McMorran <i>et al</i> , 1996
PA2396	pvdF	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)	McMorran <i>et al</i> , 2001
PA2425	pvdG	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2413	pvdH	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2402	pvdI	Putative enzymes	Lehoux <i>et al</i> , 2000
PA2400	pvdJ	Adaptation, Protection	Lehoux <i>et al</i> , 2000
PA2424	pvdL	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2393	pvdM	Central intermediary metabolism	Lamont <i>et al</i> , 2003
PA2394	pvdN	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2395	pvdO	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2392	pvdP	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2385	pvdQ	Adaptation, Protection	Lamont <i>et al</i> , 2003
PA2389	pvdR	Transport of small molecules	Imperi <i>et al</i> , 2009
PA2426	pvdS	Transcriptional regulators	Cunliffe <i>et al</i> , Miyazaki <i>et al</i> 1995
PA2390	pvdT	Membrane proteins, Transport of small molecules	Imperi <i>et al</i> , 2009
PA5531	tonB1	Transport of small molecules	Shirley <i>et al</i> , 2009

CHAPTER 3

A Predicted S-type Pyocin Shows a Bactericidal Activity against Clinical *Pseudomonas aeruginosa* Isolates through Membrane Damage

3.1 Abstract

Based on the genome sequence, the nucleic acid sequence at the positions 1067817–1066321 of *Pseudomonas aeruginosa* PAO1 genome was predicted to encode a novel S-type pyocin, designated S5. However, its antimicrobial spectrum, activity and mechanism have not been investigated. Herein, I report that pyocin S5 has an antimicrobial activity against seven clinical isolates of *P. aeruginosa* (DWW3, InA, InB, In3, In4, In7, and In8). Among them, DWW3 is the most sensitive strain with a minimum inhibitory concentration of $12.6 \mu\text{g mL}^{-1}$ and a killing percentage of 95.7 at $225 \mu\text{g mL}^{-1}$ pyocin S5. Further, I demonstrated that the antimicrobial mechanism of pyocin S5 is membrane damage, evidenced by the leakage of intracellular materials, the increase of membrane permeability, and cell surface disruption.

3.2 Introduction

Pseudomonas aeruginosa is a widely-spread environmental Gram-negative bacterium with high diversity in environments such as soil, sewage, and living organisms. This

bacterium is an opportunistic pathogen causing acute and chronic infection in immunocompromised individuals, such as cystic fibrosis (CF) patients (Govan and Deretic 1996). In human body, *P. aeruginosa* disrupts the transduction system through secretion of effector proteins (type III) (Lee, Smith et al. 2005). Moreover this bacterium is able to release a variety of virulence factors including flagella, exotoxin A, siderophores, and adhesion factors, which result in high morbidity and mortality in individuals with defective immune system (Woods, Cryz et al. 1982, Tang, Kays et al. 1995, Feldman, Bryan et al. 1998, Comolli, Hauser et al. 1999). More than 90% of *P. aeruginosa* strains produce bacteriocins, called pyocins. Bacteriocins are antimicrobial peptides produced by variety of bacterial species (Galvez, Lopez et al. 2008). Pyocins in *P. aeruginosa* are classified into three types: R-, F- and S-type. Ecologically, pyocin production may ensure the predominance of a producing strain in a bacterial niche upon exposure to mutagenic agents that provoke DNA damage, such as mitomycin C, UV, and H₂O₂ (Michel-Briand and Baysse 2002, Chang, Small et al. 2005, Waite and Curtis 2009). The killing activity of pyocins is initiated by the binding of pyocins to the specific outer membrane receptors of the susceptible cells. Most pyocinogenic strains are insensitive to their own pyocins mainly because of immunity proteins, which help preserve the initial predominance of pyocinogenic bacteria against pyocin-sensitive cells (Goodwin, Levin et al. 1972). The R-type (rod-like) pyocins, related to P2 phage and required for competitive growth advantage (Nakayama, Takashima et al. 2000), bind their tail fibers to targeted bacterial surface receptors and

then kill them through forming pores in the target cell membrane (Scholl and Martin 2008). The F-type (flexible and non-contractible) pyocins are related to λ phage with similar structural and serological properties but different receptor specificities (Nakayama, Takashima et al. 2000). The S-type (soluble) pyocins, including S1, S2, S3, and AP41, have similar structure and mode of action to the secreted bacteriocins by *Escherichia coli*, colicins (Michel-Briand and Baysse 2002, Barreteau, Bouhss et al. 2009). The S-type pyocins' structure is combined of an effector with DNase killing activity and an immunity component, (Seo and Galloway 1990, Sano, Matsui et al. 1993, Duport, Baysse et al. 1995). In general, an effector component of the S-type pyocins, which has a range of 498–776 amino acids, consists of a receptor binding domain for binding to the cell surface receptor, a translocation domain for translocation across the membrane, and a killing domain with killing property. The immunity component, which has 87–153 amino acids, immunizes pyocinogenic strains to their own pyocins. Recently, a novel pyocin from clinical isolates of *P. aeruginosa* JJ692 and 6077 exoU was shown to exhibit colicin M (ColM)-like hydrolase activity towards peptidoglycan precursors (Barreteau, Bouhss et al. 2009). The antimicrobial activity and mechanisms of pyocins S1, S2, S3, and AF41 have been studied. The availability of *P. aeruginosa* genome sequences helps to predict more pyocin-related genes. Recently a new pyocin (PA0985) at positions 1067817–1066321 of nucleic acid sequences has been predicted in *P. aeruginosa* PAO1 genome. This predicted pyocin, designated S5, contains translocation-receptor, receptor-binding (Figure 3.1), and the

killing domains. The killing domain shares 46% homology with that of colicin Ia, a pore-forming bacteriocin (Parret and De Mot 2000). In this study, for the first time, the antimicrobial spectrum, activity and mechanism of pyocin S5 has been experimentally demonstrated.

Figure 3.1 A schematic diagram of pyocin S5 domains.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

Escherichia coli BL21 (DE3) and TOP10 were grown in Luria–Bertani (LB) broth at 37°C with shaking at 250 rpm. Fifteen *Pseudomonas aeruginosa* strains, In1–8 (Fyfe, Harris et al. 1984), InA–E (Fyfe, Harris et al. 1984), DWW1, DWW3 (Waite and Curtis 2009) (clinical isolates) were kindly provided by Dr. R.D. Waite at Queen Mary University of London. *P. aeruginosa* strains were grown in Mueller–Hinton broth (MHB) or Tryptone soya broth (TSB) medium at 37°C. Solid media were prepared by adding 1.5% (wt/vol) agar to the broth. Tryptone soya agar (TSA) plates and soft agar (1.0% peptone and 0.5% agar) were used for the overlay assay (Waite and Curtis 2009). For the selection of *E. coli* BL21 transformants (containing pET30b plasmid, Invitrogen, USA), kanamycin (50 µg mL⁻¹) was added to LB agar plates. All the

strains and primers used in this study are listed in this chapter (see Table 3.1 and Table 3.2).

Table 3.1 Bacterial strains and plasmid used in this study

Strain/Plasmid	Characteristics	Reference or Source
<i>P. aeruginosa</i>		
PAO1	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
DWW1	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
DWW3	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In1	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In2	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In3	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In4	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In5	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In6	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In7	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
In8	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
InA	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
InB	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
InC	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
InD	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
InE	Wild type (clinical isolate)	Dr. R.D. Waite at Queen Mary University of London
<i>Escherichia coli</i>		
BL21	Wild type	Merck (Germany)
BL21	Containing PyoS5- pET30b	This study
W31010	Wild type	Martinez-Morales, F. <i>et al.</i> , 1999
ATCC15597	Wild type	
TOP10	Competent cell	Invitrogen (USA)
<i>S. aureus</i>		
ATCC6538	Wild type	
<i>Plasmids</i>		
pET30b (K ⁿ ^r)	Pyocin S5 gene-containing plasmid	Invitrogen (USA)

Table 3.2 List of primers used in this study

Primers	Sequence	Reference/Source
S5F2	5' CAGTCCAACGCCAGAGCA3'	This study
S5R2	5' CTGCGTCGTGTCGTTTCG3'	This study
S5PR	5' TTAGACTTCTTCATTGGTGAGTGT3'	This study
S5PF	5' ATCTCTTCGGTAAAACGC3'	This study

3.3.2 Competent cell preparation

3.3.2.1 *E. coli* competent cells

First, a single colony of *E. coli* TOP10, on LB medium without antibiotic, was inoculated into LB broth (5 mL) and incubated overnight at 37°C and 250 rpm. The bacterial culture was diluted 100 times in fresh LB broth (100 mL) and incubated at 37°C with 250 rpm shaking for 2-3 h to reach OD₆₀₀ of 0.5. Then the cell culture was aliquot to pre-chilled 50-mL centrifuge tubes and incubated on ice for 10 min. The tubes were centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was discard from each tube and the pellet cells were resuspended in ice-cold MgCl₂ (0.1 M, 30 mL). Both tubes were centrifuged for 5 min at 4°C, 5000 rpm and supernatant was discarded. The pelleted cells were resuspended with ice-cold CaCl₂ (0.1 M, 20 mL) and incubated on ice for 30 min. after that, both tubes were centrifuged for 5 min at 4°C, 5000 rpm and supernatant was discard from each tube followed by re-suspending the pelleted cells in the mixture of ice-cold CaCl₂ (0.1 M) and 15% glycerol (1.5 mL of mixture for each tube). Finally the cell suspension was aliquot into 1.5 mL tubes and kept at -80°C.

3.3.2.2 *P. aeruginosa* competent cells

A single colony of *P. aeruginosa* (isolated from TSA medium without antibiotic) was inoculated in LB broth (6 mL) overnight at 37°C and 250 rpm shaking. After overnight incubation, the bacterial culture was diluted (100x) into fresh LB broth and incubated for 1.5 h at 37°C and 250 rpm shaking to reach the OD₆₀₀ of 0.8-1. Then the cell culture was aliquot into microcentrifuge tubes and centrifuged for 2 min at room temperature (RT) and 16000 g. The supernatant was discarded and bacterial cells were washed two times with sucrose (1 mL, 300 mM sucrose for each 1.5 mL of cell culture). Cells from each tube were mixed together and were kept on ice for maximum 1 h.

3.3.3 Secondary structure of pyocin S5

The Hierarchical Neural Network (HNN) (Combet, Blanchet et al. 2000) and the SOSUI method (Hirokawa, Boon-Chieng et al. 1998) reportedly provide a high accuracy of protein structure and trans-membrane protein prediction. The HNN and SOSUI programs were employed to predict the secondary structure and trans-membrane region of pyocin S5 (PA0985), respectively. The secondary structure of colicin Ia (GenBank Acc. No. ZP_02783400.1) was analyzed by the HNN method for comparison with pyocin S5.

3.3.4 Overexpression and purification of pyocin S5 in *E. coli* BL21 (DE3)

Pyocin S5 gene (pyoS5, PA0985, 1497 bp, encoding 498 aa) was synthesized (GeneArt, Germany) and ligated into pET30b (+) (Merck, Germany) followed by *EcoRI* / *HindIII* (New England BioLabs, USA) double digestion, and then was transformed into *E. coli* BL21 (DE3). Overexpression of pyocin S5 was induced for 3 h by adding IPTG (1.0 mM) into the cell culture (OD₆₀₀ of 0.5–1.0) of a single colony; followed by 3 h incubation at 37°C and 250 rpm shaking. Then, the recombinant plasmid (pET30b-pyoS5) was harbored. The total protein was analyzed by SDS–PAGE.

To purify pyocin S5, after induction, the cells were centrifuged for 20 min at 4°C and 6000 g. Then supernatant was discarded and the pelleted cells were resuspended in 1x PBS containing imidazole (10 mM, pH 7.4), and lysed three times by digital Sonifier ultrasonic cell disrupter S-450 (Branson, USA), while the cells were kept on ice-water bath to avoid dissipating heat from sonicator. Sonicator controller was set at 37%, 5 sec on and 10 sec off for 5 min. The cell debris was separated by centrifugation at 4°C, 10000 rpm for 10 min followed by filtration of supernatant using 0.45 µm filters. The cell-free lysate was used for purification by His Trap™ HP columns (GE Healthcare, USA) through Amersham Akta fast protein liquid chromatography (FPLC) (GE Healthcare). Five-milliliters of HisTrap column was used to desalt. Finally, the protein was eluted into the protein sample buffer (10 mM PO₄³⁻, 20% glycerol, and pH 6.0).

The purity and concentration were measured by SDS–PAGE and Bradford Assay Kit (Bio-Rad, CA, USA), respectively.

To purify pyocin S5, the required buffers such as washing, elution, and desalting buffers were prepared based on Table 3.3.

Table 3.3 List of buffers and solutions were used for pyocin S5 purification

	Elution buffer	Wash buffer	Desalting buffer	Binding buffer
pH	8	8	6	7.4
HK₂PO₄, 3H₂O (PO₄⁻³)	20 mM	20 mM	10 mM	-
NaCl	250 mM	250 mM	-	-
Imidazole	500 mM	10 mM	-	10 mM
Glycerol	-	-	20 % (v/v)	-
PBS (1X)	-	-	-	Variable

3.3.5 Antimicrobial spectrum and bactericidal activity

The antimicrobial spectrum of pyocin S5 was determined based on the overlay assay as previously described (Iwatani, Zendo et al. 2007, Waite and Curtis 2009). Briefly, the overlay assay employs bacteria-seeded soft agar as a top layer covering a bottom layer of agar, with antimicrobial compounds spotted onto the bottom layer, followed by a subsequent determination of inhibition zones. In our study, TSA plates were covered by soft agar (2.5 mL) containing 100 µL cell cultures at a log phase (approximately 10^7 cells mL⁻¹) of each of 16 *P. aeruginosa* strains (PAO1, In1–8, InA–E, DWW1, and DWW3), three *E. coli* strains (BL21, W3110, and ATCC15597) and *Staphylococcus aureus* ATCC6538. Ten microliters of pyocin S5 protein with two fold serial dilution was spotted onto the strain lawns. After overnight incubation at

30°C, inhibition zones on the bacterial lawns were examined. Minimum inhibition concentrations (MICs) were determined as the lowest concentration of purified pyocin S5 that induced a clear inhibition zone on the bacterial lawns (Fujita, Ichimasa et al. 2007, Iwatani, Zendo et al. 2007).

Colony forming unit (CFU) counting

To elucidate the effects of pyocin S5 on the viability of *P. aeruginosa* DWW3, the cells at a middle-log phase were washed three times and adjusted to an OD₆₀₀ of 1.0. An aliquot of the cells resuspended in sterile NaCl (0.9%) was mixed with pyocin S5 at a range of concentrations (3.5, 14, 56, 225 µg mL⁻¹). The mixtures were incubated at 37°C with a shaking at 250 rpm for 20 min, 40 min, or 60 min. The cell aliquots from each time point were collected and used for the colony forming unit (CFU) counting.

3.3.6 Antimicrobial mechanisms

To detect the release of the nucleic acids from the DWW3 cells, the absorbance at 260 nm (A₂₆₀) in the cell-free supernatant was measured after treatment with pyocin S5 (225 µg mL⁻¹) by Nano Drop (Thermo, USA). ATP release from the cells in the supernatant was analyzed by BacTiter-Glo Microbial Cell Viability Assay Kit and GloMax 20/20 Luminometer (Promega, USA). The control cells were prepared in the same way but without pyocin S5 treatment. Furthermore, the cells after the treatment were stained by using LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, CA,

USA), and finally observed under AxioA1 fluorescence microscope (Carl Zeiss, Germany).

The field emission scanning electron microscopy (FESEM) produces clearer and less electrostatically distorted images compared to the conventional SEM and has widely been used to observe details of the cell surface. The cells were washed with sodium cacodylate (0.1 M, pH 7.4) after 1 h incubation with pyocin S5 (225 $\mu\text{g mL}^{-1}$). The first fixation was performed with glutaraldehyde (GTA) (2.5%) at 4°C for overnight, followed by washing and the second fixation was done with osmium tetroxide (1.0%) at RT for 1.5 h. The cells were dehydrated by ethanol (37%, 67%, 95%, and 100%) and loaded onto silicon slides. After air dry, the cells were observed using JOEL FESEM JSM-6700F (JOEL, Japan).

3.4 Results and discussion

3.4.1 Overexpression and purification of pyocin S5

Based on *Pseudomonas aeruginosa* PAO1 genome sequence (<http://www.pseudomonas.com/>), the nucleic acid sequences at positions 1067817–1066321 (the gene number: PA0985) were predicted to encode a novel pyocin, designated S5 (Parret and De Mot 2000), with 498 amino acids, the killing domain which shares 46% homology with that of colicin Ia. In this study, 23 alpha-helices were detected in the secondary structure of pyocin S5 using the Hierarchical Neural Network (HNN) method (Combet, Blanchet et al. 2000), whereas 24 alpha-helices

(Figure 3.2) were identified in colicin Ia. Furthermore, the trans-membrane region prediction was performed by using the SOSUI program (Hirokawa, Boon-Chieng et al. 1998), indicates that pyocin S5 has two trans-membrane helices close to C-terminus. These trans-membrane regions were determined as PFFVKIETLAAGAAASWLVGIAF and ATATPIGILGFALVMAVTGAMID (Table 3.4A and B). As mentioned above, similarly, colicin Ia has 24 alpha helices, which include two trans-membrane helices near C-terminus (Figure 3.2). This similar secondary structure and trans-membrane region, together with the 46% homology of the killing domains, suggest pyocin S5 as a pore-forming bacteriocin.

Table 3.4 A Trans-membrane regions of pyocin S5

No	Trans-membrane region	N-terminal	C-terminal	Length	Type
1	PFFVKIETLAAGAAASWLVGIAF	437	459	23	Secondary
2	ATATPIGILGFALVMAVTGAMID	462	484	23	Primary

Table 3.4 B Trans-membrane helices of colicin Ia

No	Trans-membrane region	N-terminal	C-terminal	Length	Type
1	VKTEAIIAGNAATALVALVFSIL	569	591	23	Primary
2	LGIIGYGLLMAVTGALIDESLVE	596	618	23	Primary

Figure 3.2 The secondary structure of pyocin S5 and colicin Ia. The secondary structures of pyocin S5 (498 amino acids) from *P. aeruginosa* and colicin Ia from *E. coli* (626 amino acids, GenBank ID: ZP_02783400.1), were analyzed using the Hierarchical Neural Network (HNN) program. The numbers (i.e. 0, 50, 100, and 150) stand for amino acids (aa) position. The long, middle and short bars represent the amino acids that form helix, sheet and coil, respectively.

Pyocin S5 is reportedly produced under stress conditions caused by H₂O₂ (Chang, Small et al. 2005) and ceftazidime (Blazquez, Gomez-Gomez et al. 2006). In biofilm state, *P. aeruginosa* is able to secrete pyocin upon both aerobic and anaerobic condition (Waite and Curtis 2009). In this study, to express and purify pyocin S5, pyocin S5 gene was chemically synthesized, introduced into the constitutive vector, pET30b (+), and the recombinant construct was subsequently transformed into the host cell *E. coli* BL21 (DE3). As shown in Figure 3.3, pyocin S5 was efficiently expressed by IPTG induction. To facilitate the purification, His tag-S tag (around 4.8 kDa) was fused onto the N-terminus of pyocin S5 (MW 57.6 kDa). Purified pyocin S5 protein was obtained using FPLC (with 2.16 mg mL⁻¹ final concentration). SDS-page running

illustrated a single band at 62.4 kDa of MW indicating successfully purified pyocin S5 (Figure 3.3, lane 4).

Figure 3.3 SDS–PAGE analysis of overexpressed and purified pyocin S5. Lanes 1–4: protein standard (Invitrogen), *E. coli* BL21 (DE3) (host cell), harboring pET30b-S5 without and with IPTG induction, and pyocin S5 after purification.

3.4.2 Antimicrobial spectrum and kinetics of bactericidal activity against *P. aeruginosa*

To determine the antimicrobial spectrum of pyocin S5 and to identify sensitive *P. aeruginosa* strains, I examined the susceptibility of sixteen *P. aeruginosa* strains (PAO1, In1–8, InA–E, DWW1, and DWW3 [5]), three *E. coli* strains (BL21 (DE3), W3110, and ATCC15597) and *S. aureus* ATCC6538, a Gram-negative bacterium, against pyocin S5. Table 3.5 shows that seven *P. aeruginosa* strains (DWW3, In3, In4,

In7, In8, InA, and InB) displayed sensitivity to pyocin S5, which showed clear inhibition zones with diffused boundaries. *P. aeruginosa* PAO1, *E. coli* (BL21 (DE3), W3110, and ATCC15597) and *S. aureus* ATCC6538 were insensitive to pyocin S5. This insensitivity against pyocin S5 might stem from that: (i) those strains have no specific receptor on the cell surface and/or no translocation system for pyocin S5, and/or (ii) the immunity protein expressed in the insensitive strains might inactivate pyocin S5 action by binding to the C terminus of pyocin S5. S5 immunity protein (108 amino acids) encoded by PA0984 potentially resides in the inner membrane and enables specific intra-membrane helix–helix interactions with pyocin S5 to perform immunity. The antimicrobial activity is initiated by binding of pyocin S5 to its receptor on the sensitive cell membrane. Although several receptors for pore-forming colicins and other pyocins have been characterized (e.g. Cir, BtuB, FpvA, and lipopolysaccharide) (Lazdunski, Bouveret et al. 1998, Cascales, Buchanan et al. 2007, Barreteau, Bouhss et al. 2009, Kohler, Donner et al. 2010), the receptor for pyocin S5 remains unknown. To evaluate the antimicrobial activity of pyocin S5 against different sensitive strains, the MICs were measured. In regard with data in table 3.5, it was revealed that among the seven sensitive strains (DWW3, In3, In4, In7, In8, InA, and InB), DWW3 that is a clinical isolate from cystic fibrosis patients (Waite and Curtis 2009), showed the highest sensitivity to pyocin S5, with a MIC of $12.6 \mu\text{g mL}^{-1}$ (0.2 μM). This MIC is more than 60 times lower than that against In8. At the MICs of

pyocin S5 against these sensitive strains, clear inhibition zones with 10–15 mm diameters and diffused boundaries were observed.

Table 3.5 MIC (Minimum Inhibitory Concentration) of pyocin S5 against *P. aeruginosa* strains

Strains	DWW3	In7	InA	InB	In4	In3	In8
MIC ($\mu\text{g mL}^{-1}$)	12.6	50.5	50.5	151.6	202.2	404.4	808.8

To further study, the killing rate of pyocin S5 against DWW3 was studied through colony forming units (CFUs) during time course (i.e. 20, 40, and 60 min) at different concentrations of 3.5, 14, 56, and 225 $\mu\text{g mL}^{-1}$. Figure 3.4 shows that the killing percentage of pyocin S5 reached $58.9 \pm 1.76\%$ after 20 min treatment at 14 $\mu\text{g mL}^{-1}$ and was increased to $95.7 \pm 0.87\%$ after 60 min treatment with pyocin S5 (225 $\mu\text{g mL}^{-1}$). In addition, pyocin S5 at 3.5 $\mu\text{g mL}^{-1}$ displays at least three times lower killing efficacy than at 225 $\mu\text{g mL}^{-1}$ against DWW3. During the time course, the killing activity showed a general trend of increase except for that at 3.5 $\mu\text{g mL}^{-1}$ of pyocin S5.

Figure 3.4 The kinetics of killing activity of pyocin S5 against *P. aeruginosa* DWW3.

3.4.3 Membrane leakage and permeability

Tokuda *et al.* proposed that colicin Ia binds to a specific outer membrane receptor and is subsequently translocated to the cytoplasmic membrane (Tokuda and Konisky 1978). Since pyocin S5 shows 46% homology to colicin Ia in the killing domain (Parret and De Mot 2000), I hypothesized that the mode of action of pyocin S5 might be cytoplasmic membrane damage, which causes the leakage of intracellular materials, enhancement of membrane permeability, and membrane disruption.

To investigate the leakage of intracellular materials such as nucleic acids and ATP, absorbance at 260 nm and ATP release in cell-free supernatant were measured in DWW3 cells upon exposure to 225 µg mL⁻¹ of pyocin S5. The control cells were prepared in the same way but without pyocin S5 treatment. As shown in Figure 3.5A,

after exposure to pyocin S5, absorbance at 260 nm is more than three times higher than that of the control. Since a purine or pyrimidine base shows characteristic absorbance at 260 nm, this increase in absorbance at 260 nm indicates the leakage of nucleic acids from DWW3 cells into the supernatant after exposure to pyocin S5. Similarly, ATP assay shows that the relative luminescence unit (RLU) in the supernatant after 1 h incubation with pyocin S5 is more than two times higher than that of the control (Figure 3.5B). The luminescence signal represents luminescent light intensity after ATP-luciferin reaction catalyzed by luciferase in the presence of Mg^{2+} , and molecular oxygen. Thus, the increased RLU in the supernatant represents ATP release from the cells through membrane damage after exposure to pyocin S5.

Figure 3.5 Assay used to examine membrane damage of *P. aeruginosa* DWW3 upon exposure to pyocin S5. (A) The leakage of intracellular components in the supernatant by the measurement of absorbance at 260 nm; (B) ATP release from the cells (a relative luminescence unit).

3.4.4 LIVE/DEAD viability staining assay

Besides the leakage of intracellular materials, a change in membrane permeability is another sign of membrane damage. Figure 3.6 illustrates that most DWW3 cells, which were used as a control are stained with SYTO 9 (green) (Figure 3.6A), whereas pyocin S5 addition caused the majority of the cells to be stained with PI (red) (Figure 3.6B). Fluorescent nucleic acid dye SYTO 9 penetrates and labels the bacteria with intact and damaged membrane; however, PI can only penetrate damaged membrane. Therefore, the DWW3 cells stained with PI (red) represent the cells with damaged membrane.

Figure 3.6 Cell viability of *P. aeruginosa* DWW3 cells using SYTO 9 (green) and PI (red) staining. (A) *P. aeruginosa* DWW3 cells without pyocin S5 exposure. *P. aeruginosa* DWW3 cells were shown in green remained intact and healthy. (B) *P. aeruginosa* DWW3 cells upon pyocin S5 exposure. The cells were red, which implied dead and damaged cells due to their sensitivity to pyocin S5.

3.4.5 Membrane damage observed under FESEM

To further characterize membrane damage caused by pyocin S5, the cell morphology of DWW3 was examined upon exposure to pyocin S5 by FESEM. Figure 3.7A and B show that: (i) more than 60% of the cells shrunk to a smaller size after the exposure, likely due to the leakage of intracellular materials; (ii) the shape of the majority of the cells became irregular or distorted; and (iii) the cell surface turned very rough with observable protuberances and concaves. Moreover, the majority of the DWW3 cells exhibited shrinkage, rough surface and/or distortion after 1 h exposure. This difference in the cell morphology demonstrates that pyocin S5 caused membrane damage to DWW3 cells.

Figure 3.7 Cell morphology changes of DWW3 with and without exposure to pyocin S5 under FESEM microscopy. (A) *P. aeruginosa* DWW3 cells without pyocin S5 treatment; the cells looked healthy with intact membrane (B) *P. aeruginosa* DWW3 cells upon pyocin S5 exposure indicates damaged membrane in the cells.

3.5 Conclusion

To summarize, this study has provided the first experimental evidence that the nucleic acid sequences at positions 1067817–1066321 (PA0985) encode a bacteriocin protein. I have shown that this bacteriocin, previously designated pyocin S5, is effective against seven clinical *P. aeruginosa* strains including DWW3, a clinical isolate from cystic fibrosis patients. The MIC of pyocin S5 against DWW3 was 12.6 $\mu\text{g mL}^{-1}$ (0.2 μM) and the killing percentage was 95.7% at 225 $\mu\text{g mL}^{-1}$ concentration and 60 min exposure. Furthermore, this research demonstrated that pyocin S5 causes membrane damage in DWW3, which was evidenced by (i) the increased leakage of intracellular materials, nucleic acids and ATP; (ii) the enhancement of PI penetration through membrane; and (iii) the shrinkage and distortion of the cell shape, and the rough cell surface. *P. aeruginosa* poses a serious health threat to the immunocompromised patients and also to the public because of its resistance to many synthetic antibiotics. Given the significance of the discovery of antimicrobial molecules effective against clinical isolates of *P. aeruginosa*, this study may suggest the potential use of pyocin S5 for *P. aeruginosa* treatment. Furthermore, a specific binding capability of pyocins to their receptors and the killing mechanism of pyocins can be exploited for developing a novel treatment regimen that has a low propensity to cause antimicrobial resistance and specifically targets *P. aeruginosa*. Alternatively, this narrow antimicrobial spectrum can be tailored for broader applications by developing chimeric pyocins.

CHAPTER 4

A Predicted Immunity Protein Confers Resistance to Pyocin S5 in a Sensitive Strain of *Pseudomonas aeruginosa*

4.1 Abstract

Many strains of *Pseudomonas aeruginosa* produce bacteriocins named pyocins. I previously verified the identity and antimicrobial mechanisms of a predicted S-type pyocin designated S5. Theoretically, the immunity proteins in *P. aeruginosa* would render pyocin-producing strains insensitive to their own pyocins. Based on the genome sequence of *P. aeruginosa* PAO1, an immunity protein was predicted at the positions 1065970-1066296 but the role of this protein has yet to be experimentally ascertained. In this study, the identity of a predicted *S5 immunity (S5I)* gene of *P. aeruginosa* PAO1 was confirmed. Moreover, the immunizing role of this gene was elucidated in resistant strain against pyocin S5. To evaluate the immunizing role of the predicted *S5I* gene, this gene was expressed in a pyocin S5-sensitive strain of *P. aeruginosa*, DWW3, which is devoid of *S5I*. Furthermore, this project demonstrates that the expression of S5 immunity in *P. aeruginosa* DWW3 conferred pyocin S5 resistance through prevention of membrane damages by pyocin S5, evidenced by cell viability and membrane morphological assays. This study provides novel insights into exploiting pyocins as a specific treatment against *Pseudomonas* strains.

4.2 Introduction

Pseudomonas aeruginosa is one of the leading etiological agents, which causes nosocomial infections among cystic fibrosis and immunocompromised patients (Govan and Deretic 1996). Unlike other infectious Gram-negative bacteria, *P. aeruginosa* is equipped with highly robust and adaptable metabolic pathways, which enable the pathogen (i) to utilize diverse carbon substrates ranging from glucose to diesel for growth and metabolism and (ii) to utilize nitrate or arginine as respiratory electron acceptors for proliferation in aerobic or anaerobic environments (Abd, Wretlind et al. 2008, Matz, Moreno et al. 2008). The infection process begins with an adhesive attachment of *P. aeruginosa* to host cells, followed by the release of virulence factors such as effector proteins, toxins and pigments (Woods, Cryz et al. 1982, Feldman, Bryan et al. 1998, Comolli, Hauser et al. 1999). Despite the array of antibiotics currently available, *P. aeruginosa* is still among the leading causes of nosocomial infections due to its inherent resistance towards many antibiotics and antimicrobials. This is likely due to the presence of effective multi-drug efflux systems coupled with the ability to rapidly acquire drug tolerance. Therefore, there is an urgent demand for developing an effective strategy to specifically target and treat *P. aeruginosa* infections.

In a competitive environment, *P. aeruginosa* secretes bacteriocins to limit the growth of other related-species. Pyocins are narrow-spectrum bacteriocins produced by *P. aeruginosa* and were termed after the species' former name *Pseudomonas pyocyanea*

(Jacob, Lwoff et al. 1954). Unlike plasmid-bound colicins, pyocins are located in the chromosomes of *P. aeruginosa*, but are similarly expressed under stress conditions such as nutrient starvation and UV exposure (Kageyama 1964, Michel-Briand and Baysse 2002, Chang, Small et al. 2005, Waite and Curtis 2009). Based on the killing mechanisms, structures and solubility, three types of pyocins (F, R, and S) are secreted by *P. aeruginosa* (Michel-Briand and Baysse 2002). S-type pyocins are soluble and sensitive to protease and heat treatments. Pyocin S1–S3 and AP41 exhibit DNase activity (Seo and Galloway 1990, Sano, Matsui et al. 1993, Duport, Baysse et al. 1995), while in silico analysis of *P. aeruginosa* genome predicted two more S-type pyocins S4 and S5 (Parret and De Mot 2000). The predicted S5 shows high degree of sequence homology to S2, and comparable homology exists between the C-terminal domain of S5 and the active domain of the pore-forming colicins Ia and Ib (Wiener, Freymann et al. 1997, Parret and De Mot 2000). Likewise, a putative pyocin S5 immunity protein (S5I) shows considerable homology to the colicin Ia and Ib immunity proteins, but the role of this protein has yet to be experimentally described (Parret and De Mot 2000, Iwatani, Zendo et al. 2007). In theory, the immunity proteins of *P. aeruginosa* render pyocin-producing strains insensitive to their own pyocins (Sano and Kageyama 1993, Sano, Matsui et al. 1993). In our previous study, the bactericidal activity of pyocin S5 through membrane damage was demonstrated against clinical isolates of *P. aeruginosa* (Ling, Saeidi et al. 2010). The study also revealed DWW3 as the most sensitive strain from the antimicrobial activity assay of pyocin S5 against clinical isolates. As the

presence of an immunity protein is supposed to provide protection against pyocins, I hypothesized that the reason for the sensitivity and resistance against pyocin S5 might be related to the absence and presence of S5 immunity gene (*S5I*), respectively. Towards this aim, the previously predicted *S5I* gene was characterized, and changes in sensitivity towards pyocin S5 were examined in a pyocin S5-sensitive strain of *P. aeruginosa*. In this study, we confirmed the identity of the predicted pyocin S5 immunity gene (*S5I*) in *P. aeruginosa* PAO1 (ATCC 15692), and elucidated its protective role in conferring resistance to pyocin S5 in the S5-sensitive strain *P. aeruginosa* DWW3. Furthermore, we demonstrated that expression of *S5I* in S5-sensitive strain conferred pyocin S5 resistance through prevention of membrane damage. This study provides potentially useful insights for exploiting pyocins as specific treatment against *Pseudomonas* Strains (Figure 4.1).

Figure 4.1 The schematic figure of predicted S5 immunity role in immunizing pyocin S5-producing strains. The putative S5 immunity (S5I) protein confers resistance to pyocin S5 in *P. aeruginosa*. We show that S5I prevents cellular lysis in cells expressing S5I protein (Rasouliha et al. 2013).

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used in this study, including *E. coli* TOP10 (Invitrogen, USA), were cultured in Luria-Bertani broth (LB) medium at 37°C and 250 rpm shaking. *P. aeruginosa* strains, PAO1 (ATCC 15692) and DWW3 [18], were cultured in LB medium at 37°C and 250 rpm shaking. For the selection of *P. aeruginosa* transformants, carbenicillin (100 $\mu\text{g mL}^{-1}$) was added to LB agar plates. Soft agar (1.0% peptone and 0.5% agar) was used for the overlay assay (Fyfe, Harris et

al. 1984, Waite and Curtis 2009). The strains and plasmid used in this study are listed in Table 4.1.

Table 4.1 List of bacterial strains and plasmids were used in this study

Strain or Plasmid	Characteristics	Source
<i>P. aeruginosa</i>		
PAO1	Wild type pyocin S5-resistant strain Competent cell	Dr. R.D. Waite at Queen Mary University of London
DWW3	Wild type pyocin S5-sensitive strain	Dr. R.D. Waite at Queen Mary University of London
DWW3- <i>S5I</i>	Pyocin S5-sensitive strain containing S5 immunity (<i>S5I</i>)	This study
<i>E. coli</i>		
TOP10	Containing pUCP18 ^{Cbr} - <i>S5I</i>	Invitrogen
BL21	Containing PyoS5-pET30b	Merck (Germany)
<i>Plasmids</i>		
pUCP18 (Cb ^r)	Pyocin S5 immunity gene-containing plasmid	Prof. Pete Greenberg at University of Washington
pET30b (Kn ^r)	Pyocin S5 gene-containing plasmid	Merck (Germany)

4.3.2 Ligation, purification and transformation

The synthesized *S5I* gene (GeneArt, Invitrogen, USA) and pUCP18 (Davies, Parsek et al. 1998) were double digested by restriction enzymes *EcoRI* and *SacI*. Subsequently, DNA fragments were purified and ligated using T4 ligase (New England BioLabs, USA). The ligated product (pUCP18-*S5I*) was transformed into *E. coli* TOP10

competent cells. Positive transformants were screened on LB agar with carbenicillin ($100 \mu\text{g mL}^{-1}$), and single colonies were characterized by colony PCR and sequencing.

The pUCP18-*S5I* was purified using miniprep plasmid purification kit (Qiagen, USA) according to the manufactures protocol. The purified pUCP18-*S5I* (500 ng) was transformed into *P. aeruginosa* DWW3 competent cells (100 μL) by electroporation using Gene Pulser (BioRad, USA).

4.3.3 Secondary structure and trans-membrane prediction study

The secondary structure and trans-membrane regions of the predicted pyocin S5 immunity protein (GenBank Acc. No.NP_249675.1) were analyzed and compared to the well-characterized colicins Ia (GenBank Acc. No. YP_003502719.1) and E1 (GenBank Acc. No.ZP_12077771.1) immunity proteins by using DNAMAN software, Hierarchical Neural Network (HNN) (Hirokawa, Boon-Chieng et al. 1998) and the SOSUI (Combet, Blanchet et al. 2000) programs, respectively.

4.3.4 RNA extraction

Extraction of RNA was performed by RNeasy Protect Bacteria Mini Kit (Qiagen, USA). According to provider's protocol, around 5×10^9 bacterial cells in exponential phase (OD_{600} of 1.0) were harvested (1 mL) for each RNA extraction. Subsequently, RNA was treated with RQ1 RNase-Free DNase (Promega, USA) to remove any leftover DNA. To check the quality of extracted RNA, gel electrophoresis was performed on 1% agarose gel and two clear bands (16S and 23S) were observed.

Moreover, the absorbance 260/280 was measured by Nano-Drop and checked to be between 1.6-1.8. Extracted RNA concentration was determined by OD₂₆₀ using Nano-Drop.

4.3.5 Reverse transcription PCR (RT-PCR)

Equal quantities of purified RNA were converted into cDNA using iScript cDNA Synthesis Kit (Biorad, USA). The concentration of cDNA was determined by spectrophotometry, Nano-Drop. PCR was carried out using Taq polymerase and specific primers (Table 4.2) with the settings as follows:

Stage	Temperature (°C)	Time (min)		
Initial denaturation	95	5		
Denaturation	94	1	}	25 cycles
Annealing	60	1		
Extension	72	1		
Final extension	72	10		

In the final step, equal amounts of PCR samples were run on 1.5% agarose gel and visualized with 6X DNA Loading Dye (Thermo Scientific, USA) and observed using Molecular Imager Gel Doc XR (Bio-Rad, USA).

4.3.6 Real time PCR (qRT-PCR)

Real time PCR (qRT-PCR) was performed using SsoFast EvaGreen (Bio-Rad, USA), where two biological and four technical replicates were analyzed to maximize the statistical power.

The equal quantities of prepared cDNA (100 ng mL^{-1}) from each sample were added to the super mix contains dNTPs, Sso 7d-fusion polymerase, MgCl_2 , EvaGreen dye and stabilizers in 96-well plate. The specific and reference primers were added separately. In order to remove any bubbles formed due to pipetting, the plate is centrifuged at 3000 g for 30 sec at 4°C , and then loaded into the real time PCR machine with the following setting:

Stage	Temperature ($^\circ\text{C}$)	Time	
Enzyme activation	95	5 min	
Denaturation	94	1 min	} 40 cycles
Annealing/extention	59	1 min	
Melting curve	55	30 sec	60 cycles

Real time PCR is considered as a quantitative and powerful method to quantify gene expression. In real time PCR, threshold cycle (C_T) is the quantitative end point, which is number of cycles when the fluorescent signal of reporter dye reaches a fluorescent

intensity. The higher C_T is due to the lower amount of expressed gene (Schmittgen and Livak 2008). There are two major quantification methods, absolute and relative, in real time PCR analysis. In this study, I calculated gene expression based on relative quantification. In this method, comparative C_T was measured and the expression of target gene was calculated relative to an internal control (a housekeeping/reference gene) (Livak and Schmittgen 2001). I used the formula below (Schmittgen and Livak 2008) to calculate normalized gene expression (*S5I* gene) in DWW3 (wild type) and DWW3-*S5I*.

$$2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ reference gene}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ reference gene}) \text{ sample B}]$$

In our study, *S5I* gene expression was normalized to the reference gene (housekeeping gene):

$$2^{-\Delta\Delta C_T} = [(C_T \text{ of } S5I - C_T \text{ of } rpoD) \text{ DWW3-}S5I - (C_T \text{ of } S5I - C_T \text{ of } rpoD) \text{ DWW3}]$$

iQ5 real-time PCR detection system (Bio-Rad, USA) was employed for qRT-PCR experiments and sample analysis was performed by using iQ5 Optical System Software version 2.0.

For both RT- and qRT-PCR methods, *rpoD* was used as reference gene. The housekeeping and specific primers used in this step are listed in Table 4.2.

Table 4.2 List of primer sequences were used in this study

Primers	Sequence	Source
PAO1 <i>S5I</i> -R (RT-PCR)	5'-ATAAGAATGCGGCGGCAATGTCCTTTAAATATTATTGGGC-3'	This study
PAO1 <i>S5I</i> -F (RT-PCR)	5'-CCGCTCGAGGCTGGCTTTGCCATATTATA-3'	This study
PAO1 <i>S5I</i> -R (q-PCR)	5'-TAGCAGCTCAGATAAAAAACGGCATAACAGAC-3'	This study
PAO1 <i>S5I</i> -F(q-PCR)	5'-GCACCATCTGTTTCCGTTTAGCGTTAAAC-3'	This study
rpoD-R	5'-GTTCTCCACCTACGCCACC-3'	This study
rpoD-F	5'-TCAGTACCTTGC GGATCTTG-3'	This study
PyoS5I-R	5'-CCGCTCGAGGGTGGGTTTGCCATATTATA-3'	This study

4.3.7 Overlay assay

A single colony of each strain with and without *S5I* gene was inoculated overnight in LB (5 mL) containing carbenicillin ($100 \mu\text{g mL}^{-1}$) at 37°C and 250 rpm shaking. The cell culture was diluted to OD_{600} of 0.02 and incubated until OD_{600} reached 1.0. An overlay of soft agar (2.4 mL, Difco peptone 1% and agar 0.5%) with DWW3 and DWW3-*S5I* cultured cells (0.1 mL) at the exponential phase were prepared separately in LB containing carbenicillin ($100 \mu\text{g mL}^{-1}$), and poured over the LB agar plate. After the agar was solidified, serially diluted pyocin S5 (10 μL) at various concentrations (0.036, 0.072, 0.143, 0.287, 0.575, 1.15, 2.3, and 4.6 mg mL^{-1}) was spotted on top of the dried agar plate and incubated overnight at 30°C to observe the inhibition halos (Iwatani, Zendo et al. 2007, Waite and Curtis 2009).

4.3.8 IC₅₀ (half maximal inhibitory concentration) measurment

The half maximal inhibitory concentration (IC₅₀) of pyocin S5 against DWW3 and DWW3-S5I was determined by measuring the growth curve of OD₆₀₀ of cells treated with different pyocin S5 concentrations. The cultured of DWW3 and DWW3-S5I (100 µL) were grown to OD₆₀₀ of 0.1 and incubated for 6 h with varying pyocin S5 concentrations (0.1125, 0.2246, 0.4492, 0.8984, 1.7969, 3.5938, 7.1875, 14.375, 28.75, 57.5, 115, 230, and 460 µg mL⁻¹). The OD₆₀₀ was measured hourly and the exponential growth slope was determined. The slope values (initial velocity, V₀) were plotted against log₁₀ [Pyocin S5] and fitted with 1:1 inhibition model to yield the IC₅₀ values (Sigmaplot).

4.3.9 Colony forming unit (CFU) counting

Single colony was inoculated from each strain as described above. Cultured cells (1 mL) with OD₆₀₀ of 1.0 were washed with NaCl (0.85%, 1 mL) and centrifuged at 10,000 rpm for 1 min. The pelleted cells were washed twice with NaCl (0.85%, 1 mL) and subsequently were treated with different concentrations of pyocin S5 (3.1, 12.4, 49.6, and 198.4 µg mL⁻¹) for 1 h at 37°C and 250 rpm shaking. The untreated samples of each strain were prepared as control. After 1 h incubation, colony forming unit (CFU) of each strain was counted on LB agar plates with two biological replicates.

4.3.10 Live/Dead viability staining assay

The LIVE/DEAD BacLight Bacterial Viability Kit was used for cell staining, which consists of two separate nucleic acid stains, SYTO9 and propidium iodide (PI). To determine the protective role of *S5I* gene against pyocin S5, single colonies of DWW3 and DWW3 -*S5I* were inoculated as described in the overlay assay section. Cultured cells (1 mL) were washed and treated with pyocin S5 as explained in CFU part but the treatment was conducted with the highest pyocin concentration ($198 \mu\text{g mL}^{-1}$) for 1 h. The suspension of each sample (1 mL) was mixed with dye (1.5 μL of propidium iodide and 1.5 μL of SYTO 9) and incubated in the dark for 15 min at RT. Finally, stained cells (1–2 μL) were observed using Axio A1 fluorescent microscope.

4.3.11 Field emission scanning electron microscopy (FESEM)

The Scanning Electron Microscopy was used to observe the morphological changes upon pyocin S5 treatment on both DWW3-*S5I* and the control. FESEM can provide a clear image with less electrostatic disturbance and more details on cell surface. Silicon slide used for FESEM was cleaned using ethanol (70%) with Polyethyleneimine (PEI) (Qiu, Pei et al. 2005) for 30 min. After the cells were treated with pyocin S5, previously described in CFU section, they were washed with sodium cacodylate (pH 7.4, 0.1 M) (100 μL) and centrifuged at 2500 rpm for 15 min. Cells were washed twice and centrifuged. The supernatant was discarded. Subsequently, the pelleted cells were fixed with glutaraldehyde (2.5%, 150 μL) in sodium cacodylate (0.1 M, pH 7.4) and

kept overnight at 4°C. After the first overnight fixation, the cells were collected by centrifugation at 2500 rpm for 15 min at RT, and were washed three times with sodium cacodylate (0.1 M, pH 7.4). The cell pellet was re-suspended in sodium cacodylate (0.1 M, 30 µL). Then suspended cells (2 µL) were loaded onto the PEI coated silicon slide, followed by a 30 min incubation at RT. The second fixation was done with osmium tetroxide (1%) in sodium cacodylate (0.1 M, pH 7.4) at RT for 90 min. Afterward, the cells were washed three times with sodium cacodylate (0.1 M), and were dehydrated by submerging the slides in serial concentrations of absolute ethanol (37%, 67%, and 95%) and three times in ethanol (100%) for 15 min. The slides were dried in vacuum drier at RT. Finally, the prepared samples were coated with platinum and examined using JOEL FESEM JSM-6700F microscope.

4.4 Results and discussion

4.4.1 Secondary structure and trans-membrane region study

Among the S-type proteins, pyocin S5 is an important bacteriocin with a pore-forming activity similar to colicin Ia. It has been reported that there is high homology of protein sequences and the secondary structures between pyocin S5 and colicin Ia (Ling, Saeidi et al. 2010). This high homology likely suggests similarities of S5 immunity protein (S5I) and colicin immunity proteins, which has not been reported yet. BlastP (BlastP, <http://blast.ncbi.nlm.nih.gov>) was used to identify similarities between S5I (PA0984, 108 aa) encoded by *P. aeruginosa* (<http://www.pseudomonas.com/>) and colicin

immunity proteins (E1 and Ia), and DNAMAN software was used for the alignment (Lynnon Corp., Quebec, Canada). The results indicated multiple regions with high degrees of amino acid sequence conservation between S5I and colicin Ia and E1 immunity proteins (Figure 4.2A). Furthermore, the secondary structure and transmembrane domains of S5I, Ia and E1 immunity proteins were analyzed by the Hierarchical Neural Network (HNN) tool (Combet, Blanchet et al. 2000) and the SOSUI method (Hirokawa, Boon-Chieng et al. 1998), respectively. The HNN analysis predicted nine random coils in S5I, and nine and seven random coils in colicin Ia and E1 immunity proteins, respectively (Figure 4.2B). By the SOSUI analysis, putative trans-membrane domains (VAWKGSVFPSLASVNPLVVAGLS, and GLYAVFYLSICYLFSIPLGMVFLF) were found at C- and N-termini of S5I. Colicin Ia and E1 immunity proteins have similar C- and N-terminal transmembrane domains; thus, I hypothesized that S5I is a transmembrane protein with an immunizing role against pyocin S5.

4.4.2 Semi quantitative and quantitative real-time PCR (RT and qRT-PCR)

The results of semi-quantitative and quantitative PCR assays confirmed the lack of *S5I* in DWW3, pyocin S5-sensitive strain (Figure 4.3A) and the expression of *S5I* gene in DWW3-*S5I* (Figure 4.3B).

Figure 4.3 *S5I* gene expression analysis using (A) RT-PCR and (B) qRT-PCR. In RT-PCR gel image, lane 1 indicates absence of *S5I* expression in DWW3 (wild type), and lane 2 confirms *S5I* expression in DWW3 transformant cells with *S5I*. In this experiment *rpoD* was used as the reference gene (lane 3). The qRT-PCR analysis shows high expression level of *S5I* mRNA in DWW3 with *S5I* (DWW3-*S5I*), which confirms *S5I* expression in this cell following transformation.

4.4.3 Overlay assay

To validate the functional expression of *S5I* at the translational level and the changes in sensitivity of DWW3 strain to pyocin S5, an overlay assay was conducted with purified pyocin S5. Consistent with the reported sensitivity of DWW3 to pyocin S5 (Ling, Saeidi et al. 2010), growth of wild type cells (DWW3) was inhibited after exposure to a range of concentrations of purified pyocin followed by an overnight incubation at 30°C (Figure 4.4A). In contrast, *S5I* expressing DWW3 (DWW3-*S5I*) cells showed unimpeded growth after pyocin S5 treatment (Figure 4.4B), Thus demonstrating that expression of *S5I* in DWW3 provides resistance towards pyocin S5, relieving the inhibition of cell growth.

The results of overlay assay were further supported by quantitative cell viability tests, such as half-maximal inhibitory concentration (IC_{50}) and quantitative method of cell viability by colony forming unit (CFU) count.

Figure 4.4 Overlay assays of S5-treated cells. Pyocin S5 sensitivity tested by overnight incubation of (A) DWW3 and (B) DWW3-S5I cells with various concentrations of pyocin S5 (overlay assay). Inhibitory zones represent the sensitivity of DWW3 cells against pyocin S5.

4.4.4 IC_{50} measurement

To determine IC_{50} , the OD_{600} of DWW3 and DWW3-S5I strains of *P. aeruginosa* against pyocin S5 was measured over time. The cultured cells of each strain (100 μ L) was collected with OD_{600} of 0.1 and incubated for 6 h with various concentrations of pyocin S5 (0.1125-460 μ g mL^{-1}). The OD_{600} was measured every hour and the IC_{50} was calculated by plotting the exponential growth (3-6 h) slope (initial velocity, V_0) against various pyocin concentrations used. This measurement was conducted in duplicates for each strain. DWW3-S5I showed 6.1 fold higher resistance against pyocin S5 (IC_{50} values of 0.5337 and 3.2674 μ g mL^{-1} for DWW3 and DWW3-S5I, respectively, with pyocin S5; Figure 4.5A and 4.5B). The growth rate comparison of wild type, DWW3, (Figure 4.5C) and DWW3-S5I (Figure 4.5D) revealed the

remarkable resistance of DWW3-S5I against various concentrations of pyocin S5 in contrast with wild type.

Figure 4.5 Half maximal inhibitory concentration (IC_{50}) and growth curve of pyocin S5 at exponential growth phase (3–6 h) against (A and C) DWW3 and (B and D) DWW3-S5I strains of *P. aeruginosa* were examined ($IC_{50}=0.5337$ and $3.2674 \mu\text{g mL}^{-1}$ for DWW3 and DWW3-S5I, respectively). V_0 (initial velocity) is cellular division rate with various pyocin concentrations. The growth rate of DWW3 (C) and DWW3-S5I (D) strains vary over different concentration of pyocin S5. In comparison with strain, DWW3 cells, DWW3-S5I strain shows significant cell growth upon pyocin exposure.

4.4.5 Colony forming unit (CFU) counting

To validate the IC_{50} values, we performed CFU counting for DWW3 and DWW3-S5I cells treated with various concentrations of pyocin S5 (3.1, 12.4, 49.6, and $198.4 \mu\text{g mL}^{-1}$). DWW3 strain was not viable after exposure to pyocin S5, even at the lowest

concentration ($3.1 \mu\text{g mL}^{-1}$), whereas DWW3-S5I was viable at the highest concentration ($198.4 \mu\text{g mL}^{-1}$) examined (Figure 4.6).

Figure 4.6 Tabulation of colony forming unit of DWW3-S5I cells with and without treatment with pyocin S5 (1 h incubation at 37°C). As it was shown, in the absence of pyocin S5 both strains grew well, while upon different concentrations of pyocin S5 only DWW3-S5I could survive and the CFU counting of DWW3 cells after 1 h incubation with pyocin S5 (3.1 - $198.40 \mu\text{g mL}^{-1}$) indicated no cell growth.

4.4.6 LIVE/DEAD cell viability staining assay

To investigate the role of the S5I protein in protecting the cellular membrane, DWW3 and DWW3-S5I cells were stained with LIVE/DEAD BacLight Bacterial Viability Kit before and after exposure to pyocin S5. Consistent with our previous study, pyocin S5-mediated cell damage resulted in cellular content leakage, including nucleic acids. We investigated pyocin S5-mediated membrane damage by using the nucleic acid-labeling fluorescent dye, SYTO 9, to assess membrane permeability (Ling, Saeidi et al. 2010). First, DWW3 and DWW3-S5I cells were stained and viewed by using a fluorescent microscope. It was shown that without pyocin S5 treatment, both DWW3

and DWW3-*S5I* cells showed high green fluorescence intensity (Figure 4.7A and B). This high intensity of green illustrates the intact membrane and cellular survival. Then both strains were incubated with pyocin S5 ($198.4 \mu\text{g mL}^{-1}$) for 1 h, stained, and observed under a fluorescent microscope. Most of the pyocin S5-treated DWW3 cells fluoresced red, thus indicating a high proportion of dead cells ($62.23 \pm 1.5\%$; Figure 4.8A). The remaining S5-treated DWW3 cells fluoresced green, but weaker than treated DWW3-*S5I* cells, which is indicating low concentrations of nucleic acid, possibly a result of membrane damage (Figure 4.8A and B). In contrast, most of the S5-treated DWW3-*S5I* cells ($99.2 \pm 1.4\%$) showed high green fluorescence, thus demonstrating that they are viable with intact membrane (Figure 4.8B). On the basis of our previous study, this study postulates that cell death of S5-treated DWW3 is attributable to cell surface damage.

Figure 4.7 Fluorescence cell imaging of DWW3 and DWW3-*S5I* without pyocin S5 treatment. Live/Dead cell viability assay of A) DWW3 and 2) DWW3-*S5I* before treatment with pyocin S5. Both strains were shown in green with high intensity, which indicates they are intact and alive.

Figure 4.8 Fluorescence cell imaging of DWW3 and DWW3-*S5I* treated with pyocin S5. SYTO 9 (green) and PI (red) staining of (A) DWW3 cells and (B) DWW3-*S5I* cells after 1 h pyocin S5 treatment. DWW3 cells have high population of cells that fluoresce red (cell sensitivity towards pyocin S5), whereas most DWW3-*S5I* cells fluoresce green (cellular survival) resulting from pyocin S5 resistance, upon pyocin treatment.

4.4.7 Cell surface morphology using FESEM

To further validate that cell death is attributable to cell surface damage by pyocin S5, field emission scanning electron microscopy (FESEM) was conducted on DWW3 and DWW3-*S5I* cells before and after treatment with pyocin S5. To investigate cell morphology, DWW3 and DWW3-*S5I* cells were treated with pyocin S5 ($198 \mu\text{g mL}^{-1}$), fixed and dried as described in section 4.3.11. Cells were coated with platinum (20 mA, 80 sec), and cell morphology was examined by FESEM at 5.0 kV (Qi, Zhou et al. 2010). There was no damage to the cell envelope of DWW3 without S5 treatment (Figure 4.9A and B). The membrane integrity appeared to be normal for both DWW3 cells with or without *S5I* expression in the absence of pyocin S5 (Figure 4.9A and B), whereas most of the S5-treated DWW3 cells had shrunk and showed visible

protuberances and concave areas on the cell surface (Figure 4.9C), thus suggesting cell surface damage upon exposure to pyocin S5, in support of the aforementioned membrane damage as proved by the LIVE/DEAD viability staining assay. In contrast, S5-treated DWW3-*S5I* showed normal cell morphology indicating the immunizing role of *S5I* in DWW3-*S5I* against pyocin S5 (Figure 4.9D). The differences in cell morphology indicate that expression of *S5I* in DWW3 prevented cell surface damage by pyocin S5, consistent with our observation by fluorescence microscopy. In conclusion, the differences in cell morphology in DWW3 and DWW3 expressing *S5I* (DWW3-*S5I*) indicate that expression of *S5I* in DWW3 prevents cell surface damage by pyocin S5.

Figure 4.9 FESEM microscopy of DWW3 and DWW-S5I with and without pyocin S5 treatment. FESEM cell imaging of cell morphology changes in (A) DWW3 and (B) DWW3-S5I cells, without pyocin S5 treatment, and (C) DWW3 and (D) DWW3-S5I cells after 1 h pyocin S5 treatment ($198.4 \mu\text{g mL}^{-1}$).

4.5 Conclusion

The previous study reported similarities in protein structure between pyocin S5 and colicin Ia (Ling, Saeidi et al. 2010). In this study, I strived to elucidate the similarities between S5 immunity protein of *P. aeruginosa* and colicins Ia and EI immunity proteins of *E. coli*. Moreover, the immunity role of S5I gene in immunizing pyocin S5-producing strains was verified by analyzing the protein structures of pyocin S5I and colicin immunity proteins. The result revealed the similarities of protein sequence,

secondary structure, and trans-membrane domains among these immunity proteins. Based on such similarities, it was predicted that *S5I* is a trans-membrane protein probably with an immunizing role against pyocin S5. Accordingly, I selected DWW3, a pyocin-sensitive strain of *P. aeruginosa* that is devoid of *S5I* gene expression, to examine the extended immunity that can be introduced by an exogenous *S5I* expression.

This project demonstrated that the expression of *S5I* in an S5-sensitive strain resulted in a significant resistance towards pyocin S5 using agar, colony forming unit and LIVE/DEAD cell viability staining assays. The integrity of cell membrane was closely examined using FESEM, which further demonstrated protection against membrane damage induced by pyocin S5 at a 15-fold higher concentration of the reported MIC ($12.6 \mu\text{g mL}^{-1}$) (Ling, Saeidi et al. 2010). Therefore, this study successfully confirmed the protective role of putative *S5I* gene in *P. aeruginosa*, where the sensitivity of the cells to pyocin S5 is determined by the presence or absence of this gene. Furthermore, this study also showed that with the *S5I* gene, a pyocin-S5 sensitive strain could become resistant to pyocin S5.

As structural information on both pyocin S5 and *S5I* is inadequately available, protein interaction mechanisms underlying the immunity conferred by *S5I* gene are not clear. Based on the studies on immunity proteins against colicins, the immunity proteins provide protection towards self-produced colicins by tightly binding to their cognate

colicins, thereby preventing the lethal nuclease activity of colicins. Furthermore, it is also a membrane protein that prevents the opening of lethal channels in the producing cell (Cascales, Buchanan et al. 2007, Jakes and Cramer 2012). As pyocin S5 has structure or activity that is similar to colicin Ia, it can be postulated that S5I may also share similar mechanisms of providing protection against pyocin S5. S5I may mitigate the pore-forming activity of pyocin S5 by preventing the translocation and insertion of the pore-forming domain of pyocin S5 to the outer membrane surface. Although elucidating the mechanisms behind immunity by *S5I* gene certainly warrants further investigation, the findings presented herein substantiates the potential role of *S5I* as a therapeutic target that can sensitize *P. aeruginosa* to pyocin S5 treatment. Pyocin S5 has already shown its therapeutic potential in a proof-of-concept study which utilized synthetic biology to engineer cells that are able to specifically release pyocin S5 upon sensing the presence of *P.aeruginosa* (Saeidi, Wong et al. 2011). Therefore, by combining a strategy to target *S5I* to reverse the immunity of the target cells with selective pyocin S5 release, a more refined and effective treatment against *P. aeruginosa* can be achieved.

In conclusion, this project identified the putative *S5I* gene in *P. aeruginosa* PAO1. Furthermore, I proved that S5I confers pyocin S5 resistance through prevention of membrane damage. Future efforts might include screening for S5I inhibitors, for an S5 combinatorial therapeutic strategy to target S5-resistant strains.

CHAPTER 5

***mvaU* Regulatory Role in *Pseudomonas aeruginosa* Pyoverdine Biosynthesis Genes**

5.1 Abstract

Despite considerable studies on regulatory role of *mvaT* in *Pseudomonas aeruginosa*, its homologous component, *mvaU*, receives less attention in related research fields. Here, a microarray analysis demonstrated that in the *mvaU* mutant of PAO1 strains, the expression of pyoverdine-related genes such as *pvdF*, *pvdJ*, and *pvdG* were significantly increased in comparison with wild-type PAO1 in *P. aeruginosa*. For the first time, this study discussed that *mvaU* deletion enhances pyoverdine (PVD) production in *P. aeruginosa* PAO1. Based on the transcriptome analysis and supporting evidences discussed in this thesis, I propose that *mvaU* might be involved in the regulation of PVD biosynthesis genes.

5.2 Introduction

Histone-like nucleoid-structuring (H-NS) proteins are important global regulators identified in various Gram-negative bacteria including *Pseudomonas aeruginosa* (*P. aeruginosa*) (Castang, McManus *et al.* 2008). The regulatory role of H-NS family

genes is mostly gene silencing (Navarre, Porwollik *et al.* 2006, Dorman 2007). Two putative H-NS proteins, *mvaT* (PA4315) and *mvaU* (PA2667) were determined in *P. aeruginosa* using genome sequence study (Rosenthal and Rodwell 1998). These two proteins showed high homology with each other, which resulted in similar regulatory functions (Vallet-Gely, Donovan *et al.* 2005). Moreover, tandem affinity purification coupled with mass spectrometry demonstrated that the MvaT protein is in contact with a homologous protein through a direct protein-protein interaction, which occurs in the N-terminal regions of these two proteins (Vallet-Gely, Donovan *et al.* 2005). *mvaT* and *mvaU* are assumed as virulence factor regulators in *P. aeruginosa* PAO1 strain. This was confirmed when the deletion of the *mvaT* gene enhanced the expression of growth-phase dependent *lecA* and quorum sensing (Diggle, Winzer *et al.* 2002). In line with Diggle's work, it was shown that *mvaT* represses the expression of the *cupA* gene, a prominent gene among biofilm regulatory genes (Vallet-Gely, Donovan *et al.* 2005). Moreover, Diggle, *et al.* revealed that *mvaT* deletion alters swarming motility in *P. aeruginosa* PAO1. This group demonstrated that *mvaT* regulates pyocyanin production as well as LasA protease and elastase. (Diggle, Winzer *et al.* 2002). Some studies indicated the regulatory role of *mvaT* in antibiotic resistance genes; however, such a regulatory role has not been identified in its homologous *mvaU* (Westfall, Carty *et al.* 2006). Recently, a study was conducted by Imperi *et al.* to elucidate the regulatory role of *mvaT* in PVD synthesis through *pvdS* regulation. PVD is one of two major iron-chelating molecules, known as siderophores, with high-affinity iron binding

property. This fluorescent pigment is secreted by *P. aeruginosa* PAO1 to compensate the iron deficiency in this bacterium by up taking Fe (III) from the environment and transporting it to the cell (Cox 1980, Boukhalfa and Crumbliss 2002). PVD molecules are important in *P. aeruginosa* study because of their role as virulence factors and their efficiency in cell-to-cell communication, which is vital in biofilm formation and *P. aeruginosa* pathogenicity (Visca, Imperi et al. 2007). PVD biosynthesis and transportation are regulated by several genes (shown in Table 5.8). One of the key and well-studied genes in PVD biosynthesis is *pvdS*, which is known as an activator (Cunliffe, Merriman et al. 1995). The signaling pathways of PVD molecules are shown in Figure 5.1. (Jimenez, Koch et al. 2012). As shown in Figure 5.1, in the presence of PVD-Fe, this complex binds to a cell surface receptor, FpvA protein, and then interacts with the anti-sigma factor named FpvR. Following this interaction, the regulators PvdS and FpvI are expressed. Expression of PvdS enhances the production of virulence factors such as ToxA, PrpL, and PVD, while FpvI regulates production of FpvA (Beare, For et al. 2003). From the other side, TonB1 binds to the TonB box in FpvA molecule to provide the required energy for PVD-Fe transportation (Shirley and Lamont 2009). Imperi and his group showed that there is no relation between *pvdS*, a major positive regulator in PVD biosynthesis (Wilson and Lamont 2000), and *mvaT* (Imperi, Tiburzi et al. 2010). Although the secretion mechanism of PVD molecules remains unknown, some molecules such as multidrug efflux pumps or transporters were proposed as potential candidates for this purpose (Poole, Heinrichs et al. 1993,

McMorran, Merriman et al. 1996). Imperi *et al.* identified another molecule known as PvdRT-OpmQ that is responsible in recycling and transportation of PVD (Imperi, Tiburzi et al. 2009). However, despite the regulatory role of *mvaT* in PAO1 genes, very few studies have been done on the regulatory role of *mvaU* and its mechanism. In this thesis, I aimed to find out the linkage between *mvaU*, as a global regulator in PAO1, and PVD biosynthesis genes. Toward this aim, I performed transcriptomic profiling to exploit the responsible genes in PVD regulation. Following microarray data analysis, I demonstrated that *mvaU* deletion in *P. aeruginosa* PAO1 resulted in the overexpression of PVD biosynthesis genes such as *pvdJ*, *pvdF*, and *pvdG*. Accordingly, I carried out quantitative real-time PCR (qRT-PCR) to validate the microarray data. As the next step, PVD measurement experiments were performed in wild-type PAO1 and deleted mutant (PAO1 Δ *mvaU*::Sm^r). The results demonstrated that PVD concentration was significantly higher in deleted mutant compared to the wild type. Therefore, further investigation is required to elucidate the role of *mvaU* in PVD biosynthesis regulation. Subsequently, due to the role of PVD as a virulence factor further study in particular, at molecular and transcriptome levels might result in remarkable findings in *P. aeruginosa* virulence factors and antibiotic resistance.

Figure 5.1 Signaling pathway of PVD in *P. aeruginosa* (Jimenez, Koch et al. 2012).

5.3 Materials and methods

5.3.1 Bacterial strains and growth conditions

The bacterial strains used in this study including *P. aeruginosa* PAO1 (wild type) and PAO1 $\Delta mvaU$ (deleted *mvaU* mutant), *E. coli* BL21 were grown in Luria–Bertani (LB) broth at 37°C, with shaking at 250 rpm. For selection of *P. aeruginosa* and *E. coli* transformants, carbenicillin (Cb), streptomycin (Sm), and kanamycin (Kn) were added to the LB broth and LB agar plates at the final concentrations of 100, 50, and 30 $\mu\text{g mL}^{-1}$, respectively. The following plasmids were used in this section: pET30b and pEX18Ap were received from (Merck, Germany) and Prof. Jürgen Heesemann (Max von Pettenkofer Institut, Germany) (see Table 5.1).

Table 5.1 List of bacterial strains and plasmids were used in this study

Strain or Plasmid	Characteristics	Reference or Source
<i>P. aeruginosa</i>		
PAO1	Wild type pyocin S5-resistant strain Competent cell	Dr. R.D. Waite at Queen Mary University of London
PAO1 $\Delta mvaU::Sm$	Deleted <i>mvaU</i> strain	This study
<i>E. Coli</i>		
DH5 α	Containing pEX18Ap (Amp ^r)	Prof. Jürgen Heesemann, Max von Pettenkofer Institut, Germany
<i>Plasmids</i>		
pEX18Ap (Sm ^r)	<i>mvaU</i> gene-containing plasmid	Prof. Jürgen Heesemann, Max von Pettenkofer Institut, Germany
pET30b (K ⁿ ^r)	Pyocin S5 gene-containing plasmid	Merck (Germany)

5.3.2 *mvaU* knock-out mutant construction

In order to delete the *mvaU* gene in *P. aeruginosa* PAO1, I employed a gene-replacement method using suicide plasmid, pEX18Ap (Cb^r), which carries the Cb resistance gene and the *sacB* as a marker gene that confers sucrose sensitivity (Westfall, Luna et al. 2004). Plasmid pEX18Ap was purified from *E. coli* DH5 α -pEX18Ap (generously provided by Dr. Jürgen Heesemann) using a miniprep plasmid purification kit based on the manufacturer's protocol. The downstream and upstream sequences of the *mvaU* gene (with Sm^r and pLac promoter) were constructed and then were amplified using polymerase chain reaction (PCR). This constructed sequence (*mvaU::Sm*) was digested with *Sma*I, then ligated and cloned into pEX18Ap

using T4 DNA ligase (New England Bio-Labs, USA). The ligated product was transformed into *P. aeruginosa* PAO1 by electroporation using the Gene Pulser. Transformants were screened on LB broth containing Sm (50 $\mu\text{g mL}^{-1}$) and sucrose (5%). Following this two-step homologous recombination, the *mvaU* gene was removed together with the *sacB* marker and Cb resistance gene. Figure 5.2 shows the homologous recombination method used in gene deletion.

Figure 5.2 Overview of gene deletion via suicide vector and homologous recombination method.

5.3.3 Complementary PAO1 Δ *mvaUC*::Cb

E. coli TOP10 carrying plasmid pUCP18-*mvaU* was inoculated in LB broth with Cb (100 $\mu\text{g mL}^{-1}$) to obtain and purify pUCP18-*mvaU*. Plasmid pUCP18 containing the *mvaU* gene (pUCP18-*mvaU*) was purified using the miniprep plasmid purification kit

according to the manufacturer's protocol. About 1 μL of purified plasmid (512 ng mL^{-1}) was added to *P. aeruginosa* PAO1- $\Delta mvaU$ competent cells in a 1.5 mL tube and swirled gently (pipetting up-down mixing were avoided). Then the mixture was transferred to a chilled-out cuvette (Bio-Rad, USA) using a micropipette. To remove any bubbles and excess moisture, the cuvette was tapped gently and wiped off with a soft tissue paper. Then the cuvette was placed into the Eppendorf Multiporator (Eppendorf, Germany) and pulsed-shock. Immediately after shocking, LB broth (1 mL) was added into the cuvette, and the mixture was transferred into a round-bottom 10 mL tube, incubated at 37°C for 1 h, and shaken at 170 rpm. Subsequently, 100 μL of recovery cells was cultured into LB agar with Cb ($100 \text{ } \mu\text{g mL}^{-1}$) and incubated overnight at 37°C . Then colony PCR was performed to confirm the successful transformation by visualizing the corresponding band in 1.3% agarose gel (the *mvaU* target size: 354 bp) (see Figure 5.3).

Figure 5.3 Colony PCR on selected PAO1 $\Delta mvaUC$ (complementary strain). The first lane represents the ladder, lanes 1 and 2 represent the *mvaU* gene with the size of 354 bp in our selected colonies, and lane 3 is the negative control from deleted *mvaU* mutant.

5.3.4 Microarray experiment

Microarray is a method for gene expression profiling that is able to quantify thousands of genes in a single RNA sample, purified mRNA. By using this method, I will be able to get the relative expression levels of genes in a sample as well as the relative expression fold change of a gene between multiple samples. Figure 5.4 shows the schematic image of the mRNA microarray.

Figure 5.4 Schematic figure of DNA microarray.

In this work, I performed microarray expression assay by taking advantage of the Agilent Microarray Scanner G25505B (Agilent Technologies, USA) following the steps below:

1. High-quality RNA extraction and integrity analysis using NanoChip via Bioanalyzer
2. cDNA labeling with fluorescence dyes (two colors: Cy3 and Cy5) using the FairPlay III Microarray Labeling Kit

3. Hybridization using Agilent two-color GE Hybridization Kit
4. Washing and array scanning
5. Feature extraction
6. Data analysis using GeneSpring software

Figure 5.5 Flow chart of microarray sample preparation steps.

5.3.4.1 RNA extraction and integrity analysis

Cell cultures of wild-type PAO1 and PAO1 $\Delta mvaU$ mutant strains were collected at OD₆₀₀ of 1.0 to isolate the RNA. Total RNA was extracted using RNeasy Protect Bacteria Mini Kit as described in chapter 4. In brief, according to the protocol, 1.0 mL of bacterial culture with OD₆₀₀ of 1.0 was lysed by adding lysozyme (egg yolk,

Sigma-Aldrich, USA) in Tris-EDTA buffer. The DNA in each sample was removed using RQ1 RNase-Free DNase, and finally, the extracted RNA was purified using the columns in the kit. The primary qualification and quantification of RNA samples were performed by running agarose gel (1.2%) and using Nano-Drop, respectively. In microarray experiments, the high quality of the extracted RNA is required. To achieve this, the integrity of the extracted RNA was validated using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer (Agilent, USA) (see Figure 5.6).

Figure 5.6 The integrity of RNA samples was verified using Bioanalyzer. “L” represents the ladder used in this experiment and samples from 1-8 represents bacterial samples. As shown in this figure, all the samples were extracted with high integrity.

5.3.4.2 cDNA synthesis and labeling

cDNA was synthesized based on the iScriptc DNA Synthesis Kit (Bio-Rad, USA). To couple the nucleotide samples with fluorescent dyes, the reverse-transcribed cDNA was conjugated with fluorescent dyes Cy3 (cyanine 3) and Cy5 (cyanine 5) (Amersham, UK) and FairPlay III Microarray Labeling Kit (Stratagene, USA). The

experiment was conducted in duplicates for each sample. According to the manufacture's protocol, one of the biological replicates was labeled with "Cy3" and the other biological duplicate was labeled with "Cy5" in order to reduce bias caused by the different conjugation efficiencies of the two dyes. The labeled cDNA was then purified by using a column provided in the Fairplay kit. I mixed equal amounts of Cy3-labeled RNA and Cy5-labeled RNA. Then I performed hybridization using probes that are represented by individual spots on the *P. aeruginosa* gene expression microarray slides (Agilent, USA).

5.3.4.3 Hybridization, washing, and scanning

The labeled samples were hybridized using a microarray hybridization kit (Agilent, USA), according to the manufacturer's protocol, in a hybridization oven for 16 h at 65°C. Then unbound RNA samples were washed away from the microarray slide using two different Gene Expression Wash Buffers (Agilent, 5188-5327, USA with Triton-X). The absolute and relative intensities of the two dyes in a spot were scanned using microarray scanner (Agilent, USA).

5.3.4.4 Feature extraction

Agilent Feature Extraction software v. 9.5.3.1 extracted the raw fluorescent signals. The software calculates and determines data by performing background subtraction, normalization, outlier removal, and statistical analysis. On the other hand, each spot's signal, representing the quantified mRNA, was normalized using a Quantile method to

the numerical mean of all samples. To make the extracted data more significant and accurate, one-way ANOVA was selected in the program and the calculations were performed with *t*-test correction (*p*-value < 0.01).

5.3.4.5 Fold change and data analysis

The ratio of signal intensity in PAO1 $\Delta mvaU$ to the signal intensity of wild-type PAO1 was calculated to derive fold changes. Here, the intensity indicates the quantified mRNA of each gene. On the other word, fold changes of each gene expression level were measured by a ratio of the normalized mRNA quantity from the mutant samples to the normalized mRNA quantity of the wild type (control) sample based on the following equation:

$$\frac{\text{Normalized signal intensity of a gene in mutant strain}}{\text{Normalized signal intensity of a gene in wild type strain}}$$

In this formula, gene-deleted and wild-type strains represent PAO1 $\Delta mvaU$ and PAO1, respectively. In this chapter, common statistically significant expressed genes in duplicates were selected based on a *t*-test *p*-value of 0.01 and fold change > 5.0. For further analysis of extracted data, GeneSpring GX11 was used.

5.3.5 RNA extraction and cDNA synthesis

This step was previously explained in more detail before (in chapter 4). In brief, RNA samples were extracted from *P. aeruginosa* PAO1, PAO1 $\Delta mvaU$, and complementary PAO1 $\Delta mvaU$ cell culture at OD₆₀₀ of 1.0 (exponential phase) using RNeasy Protect Bacteria Mini Kit according to the manufacturer's protocol. For quality control of the

RNA samples, the OD 260/280 ratio was measured and checked to be between 1.6 and 1.8. I used OD₂₆₀ to measure RNA concentration. The equal amount of the extracted RNA was employed for cDNA synthesis using iScriptc DNA Synthesis Kit, as described in chapter 4. The concentration of reverse-transcribed cDNA was measured using Nano-Drop.

5.3.6 RT-PCR and qRT-PCR

Semi quantitative PCR (RT-PCR) was employed to check the expression levels of our target genes; *pvdF*, *pvdG*, *pvdJ*, and *rpoD* (as the reference gene). The RT-PCR procedure was explained in more detail in chapter 4. RT-PCR was carried out with the transcribed cDNA using Taq DNA Polymerase (New England BioLabs, USA) and specific primers (shown in Table 5.2) for the mentioned gene targets with the following settings:

Stage	Temperature (°C)	Time (min)		
Initial denaturation	95	5		
Denaturation	94	1	}	25 cycles
Annealing	60	1		
Extension	72	1		
Final extension	72	10		

In the final step, equal amounts of PCR samples were run on 1.3% agarose gel and visualized with 6X DNA Loading Dye and observed using Molecular Imager Gel Doc XR.

To validate the microarray data, qRT-PCR was conducted. Primers used in this experiment are summarized in Table 5.2. The procedure was completely described in chapter 4. The PCR program used for PCR conditions was specified as follows:

Stage	Temperature (°C)	Time (sec)	
Enzyme activation	95	30	
Denaturation	95	5	} 35 cycles
Annealing/extension	60	10	
Melting curve	60	10	73 cycles

Real-time analysis was carried out with two biological and three technical replicates for each sample to increase the accuracy of data. The excel datasheet calculates the C_T values according to the formula shown in chapter 4 that is briefly,

$$\Delta C_T (\text{sample}) = C_T (\text{sample}) - C_T (\text{control})$$

$$\text{Fold change} = 2^{-\Delta C_T (\text{Sample})}$$

Normalized:

$$\text{Fold change: } 2^{-(\Delta \Delta C_T)} = 2^{\Delta C_T (\text{sample}) - \Delta C_T (\text{control})}$$

For example:

$$\Delta \Delta C_T (\text{PAO1}\Delta mvaU) = \Delta C_T (\text{PAO1}\Delta mvaU) - \Delta C_T (rpoD)$$

$$\text{Fold change} = 2^{-\Delta \Delta C_T (\text{PAO1}\Delta mvaU)}$$

The control here is our wild-type PAO1, which was compared with the threshold cycles (C_T) of our samples (PAO1 $\Delta mvaU$ and PAO1 $\Delta mvaUC$).

All the control and sample C_T were normalized to the housekeeping gene (*rpoD*).

Table 5.2 List of primer sequences used in this study

Primers	Sequence	Source
<i>mvaU</i> and <i>mvaU</i> complementary primers for qRT-PCR		
mvaUR	5'-CCGCTCGAGGCGTTGCAGCCAGGATTCGAC-3'	This study
mvaUF	5'-GGAATTCCATATGTCCAAACTTGCCGAGTTCCG-3'	This study
mvaUCR	5'-TTAGCGTTGCAGCCAGGATTC-3'	This study
mvaUCF	5'-ATGTCCAAACTTGCCGAGTTCC-3'	This study
Reference (internal/housekeeping) gene		
rpoD-R	5'-GTTCTCCACCTACGCCACC-3'	This study
rpoD-F	5'-TCAGTACCTTGCGGATCTTG-3'	This study
Streptomycin primers for <i>mvaU</i> gene deletion		
SmR	5'- <u>GTCGACT</u> CTAGACCCGGGTCACCTACCCACTACCTTAGTGAT-3'	This study
SmF	5'- <u>GATTACGA</u> ATTCGATGCGCGAAGCGGTTATTG-3'	This study
primers for pyoverdine-related genes		
pvdJR	5'-GAGCGCTCCAGGGATTTAGCC-3'	This study
pvdJF	5'-GTCGATGTTCACCGCGGAGACAC-3'	This study
pvdFR	5'-CGGGTTCAGAGCTTCTCGGCG-3'	This study
pvdFF	5'-CGATTCCGG CGAAGTGTTCCATGA-3'	This study
pvdGR	5'-CCTCAACTGGCGGCGCGCAT-3'	This study
pvdGF	5'-TCACCCTCGACCTGCTCGACG-3'	This study

5.3.7 Pyoverdine (PVD) measurements ^(Imperi, Tiburzi et al. 2009)

5.3.7.1 Intracellular PVD measurement

Single colonies of our *P. aeruginosa* strains, wild-type PAO1, PAO1 $\Delta mvaU$ (deleted mutant) and PAO1 $\Delta mvaUC$ (complementary), were each incubated in LB broth (5 mL) with Sm (50 $\mu\text{g mL}^{-1}$) for the deleted mutant and Cb (100 $\mu\text{g mL}^{-1}$) for the complementary strains in a 250 rpm shaking incubator at 37°C, overnight. The cells were diluted in LB broth (5 mL) with the same respective antibiotics mentioned above to get OD₆₀₀ of 0.05 and incubated for 16 h at the same conditions to reach OD₆₀₀ of 3.0 (late exponential phase). About 1 mL of bacterial cells was collected (5×10^9 cells) and washed with Tris-HCl (pH 7, 30 mM) and NaCl (150 mM) and centrifuged at 5000 rpm for 5 min, at 4°C. The pelleted cells were washed and centrifuged twice again at the same condition. The bacterial pellets were collected at the bottom of 2 mL tubes and were resuspended in 1 mL of Tris-HCl (pH 8, 10 mM) and NaCl (100 mM). Then 50 mg acid-washed glass beads ($\leq 106 \mu\text{m}$) (Sigma-Aldrich, USA) were added to the resuspended cells in 2 mL tubes, which were then suspended and lysed using FastPrep-24 Instrument (MP Biomedicals, USA). The lysis procedure was performed two times, each time for 60 s, with a 5 min break in between. In all steps, samples were kept on ice. After lysis, the tubes were collected while carried on the ice and were centrifuged at 7000 g at 4°C for 10 min to remove the cell debris. Supernatants were then used for PVD measurement. 100 μL of supernatant from each sample was added

to each well of a Grenier 96-well plate with a clear bottom (Grenier, Germany). Intracellular PVD was quantified fluorometrically by recording the emission at 455 nm upon excitation at 398 nm in an Infinite 200 PRO multiplate reader equipped with a fluorescent detector (Tecan, Switzerland). Then, a standard curve was generated with known PVD concentrations (0, 1, 2, and 3 M in 100 mM of Tris-HCl [pH 8]) from PVD standards stock (10 mg mL⁻¹) (Sigma-Aldrich, USA).

5.3.7.2 Supernatant PVD measurement

The cell preparation was performed as described in intracellular PVD measurement. Briefly, the single colonies of our three strains were incubated in LB broth (5 mL) with respective antibiotics in a 250 rpm shaking incubator at 37°C with overnight. After 100 times of dilution, they were incubated for 16 h at the same conditions to reach OD₆₀₀ of 3.0 (late exponential phase), and 1 mL of each sample was collected (5×10⁹ cells) and centrifuged at 5000 rpm for 5 min at 4°C. The washing and centrifugation steps were repeated two more times. The bacterial pellets were collected at the bottom of 2 mL tubes and were resuspended in 1 mL of Tris-HCl (pH 8, 100 mM). The supernatant was then collected from individual samples, and 100 µl was added to each well of a TPP 96-well plate (Tissue Culture Test Plates, Switzerland). OD₆₀₀ and OD₄₀₅ were measured for bacterial cell culture and bacterial supernatant, respectively, using Synergy HT Multi-Mode Microplate Reader (BioTek, USA). Finally, PVD from

cultured cells' supernatants was diluted in 100 mM of Tris-HCl (pH 8) and measured as OD₄₀₅, then normalized by the cell density of bacterial cultures (measured at OD₆₀₀).

5.4 Results and discussion

5.4.1 Growth rate

In order to compare the growth rate of PAO1 $\Delta mvaU$ (mutant) and PAO1 (wild type), we first inoculated single colonies of mutant and wild type strains in LB broth with and without antibiotic (100 $\mu\text{g mL}^{-1}$ of Sm), respectively, at 250 rpm shaking and 37°C for overnight. Then cells were diluted to obtain OD₆₀₀ of 0.05 and incubated at the same conditions. The OD₆₀₀ of duplicates of each strain was measured every one hour for four h to generate the growth rate curve. The growth curve indicates nonsignificant differences in the growth rate of wild-type and mutant strains. As shown in Figure 5.8, the minor variation in growth rate is because of the manipulation in the PAO sequence and *mvaU* mutation, as was expected. In line with growth rate measurement, we noticed that the appearance of the cell culture was remarkably different in each strain (see Figure 5.7). The PAO1 cell culture appeared in typical yellow color, while the cell culture of mutant PAO1 $\Delta mvaU$ strain turned to yellow-green, probably due to the overproduction of siderophore molecules such as pyocyanin and pyoverdine. Previously it was reported that the overproduction of pyocyanin makes PAO1 cell culture more greenish (Diggle, Winzer et al. 2002). Although in our mutant strain *mvaT* gene was not knocked out, this experiment might prove the existence of similar

functions between these two homologous H-NS members of *P. aeruginosa* PAO1 (Vallet, Diggle et al. 2004, Vallet-Gely, Donovan et al. 2005).

Figure 5.7 Cell culture appearances of PAO1 and PAO1 $\Delta mvaU$ after overnight incubation at 37°C and 250 rpm shaking. The right image indicates the wild-type PAO1 cell culture with normal color, while the image on the left shows PAO1 $\Delta mvaU$ (deleted mutant), which is greenish probably because of the enhancement of two fluorescent pigments, such as pyocyanin and pyoverdine production.

Figure 5.8 Growth curve of PAO1 and PAO1ΔmvaU strains within four h incubation at 37°C.

5.4.2 Microarray data analysis

Microarray analysis was conducted to examine genome-wide expression profiles of PAO1 and PAO1ΔmvaU mutants, using two biological replicates. This experiment was conducted to see how the deletion of the *mvaU* gene would influence the gene expression in *P. aeruginosa* PAO1 cells. I used PAO1 cells as the control, and PAO1ΔmvaU mutant cells were compared with them. Statistically differentially expressed genes were selected based on *p*-value (≤ 0.01 , *t*-test) and fold change (≥ 2.0). Following selection, the significantly up- and down-regulated genes were analyzed using GeneSpring GX11 and then clustered using Gene Ontology (GO) and *P.*

aeruginosa genome databases. Consequently, this transcriptome profiling analysis demonstrated that totally 247 out of 5570 genes showed statistically significant changes in *mvaU* mutants compared to the wild-type, PAO1 (see Figure 5.9A). Among these statistically significant expressed genes in *mvaU* mutant, mRNA levels of 240 and seven genes were increased and decreased, respectively (Table 5.3 and Table 5.4). Fold change was calculated based on the ratio of the mRNA level of each gene in PAO1 Δ *mvaU* mutants compared to PAO1 (in a log 2 scale).

Table 5.3 List of statistically significant up-regulated genes of PAO1 Δ *mvaU* mutant compared with PAO1. *p*-value ≤ 0.01 and fold change ≥ 2.0

Gene ID	Name	Fold change	<i>P</i> value	PseudoCAP Function Class
PA5262	fimS	8.7	0.000	Two-component regulatory systems, motility & attachment
PA4814	fadH2	7.4	0.003	Fatty acid and phospholipid metabolism
PA2425	pvdG	7.2	0.008	Adaptation, Protection
PA2842		6.8	0.001	Hypothetical, unclassified, unknown
PA2396	pvdF	6.7	0.006	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)
PA3520		6.6	0.004	Hypothetical, unclassified, unknown
PA2400	pvdJ	6.3	0.006	Adaptation, Protection
PA4189		6.1	0.004	Putative enzymes
PA1921		6.1	0.001	Hypothetical, unclassified, unknown
PA0185		6.1	0.007	Membrane proteins, Transport of small molecules
PA0094		6.0	0.001	Hypothetical, unclassified, unknown
PA1907		5.7	0.000	Hypothetical, unclassified, unknown
PA3034		5.7	0.008	Transcriptional regulators
PA0883		5.7	0.001	Putative enzymes
PA4230	pchB	5.6	0.000	Secreted Factors (toxins, enzymes, alginate); Transport of small molecules
PA3580		5.5	0.009	Hypothetical, unclassified, unknown
PA2471		5.4	0.002	Hypothetical, unclassified, unknown
PA3773		5.3	0.004	Membrane proteins
PA4710	phuR	5.2	0.008	Transport of small molecules
PA4896		5.2	0.010	Transcriptional regulators
PA0349		5.1	0.008	Membrane proteins
PA1917		5.0	0.009	Hypothetical, unclassified, unknown

PA3286		5.0	0.006	Hypothetical, unclassified, unknown
PA1925		5.0	0.008	Hypothetical, unclassified, unknown
PA4037		4.9	0.010	Transport of small molecules
PA4138		4.9	0.003	Amino acid biosynthesis and metabolism, Translation, post-translational modification, degradation
PA4984		4.9	0.003	Transcriptional regulators
PA4960		4.9	0.002	Amino acid biosynthesis and metabolism
PA5295		4.9	0.010	Hypothetical, unclassified, unknown
PA2257	pvcD	4.8	0.003	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism
PA2128	cupA1	4.8	0.001	Motility & Attachment
PA1923		4.7	0.005	Biosynthesis of cofactors, prosthetic groups and carriers
PA5536	dksA2	4.7	0.005	Transcriptional regulators; Adaptation, Protection
PA4907		4.7	0.004	Putative enzymes
PA0151		4.7	0.000	Transport of small molecules
PA1229		4.7	0.004	Transcriptional regulators
PA5540		4.6	0.002	Hypothetical, unclassified, unknown
PA2885	atuR	4.6	0.000	Transcriptional regulators
PA3597		4.6	0.004	Membrane proteins; Transport of small molecules
PA3952		4.6	0.005	Hypothetical, unclassified, unknown
PA4114		4.6	0.009	Antibiotic resistance and susceptibility, Amino acid biosynthesis and metabolism, putative enzyme
PA4880		4.6	0.010	Central intermediary metabolism
PA4709		4.5	0.004	Putative enzymes
PA1568		4.5	0.001	Hypothetical, unclassified, unknown
PA2336		4.4	0.003	Hypothetical, unclassified, unknown
PA3450		4.4	0.008	Adaptation, Protection
PA4903		4.4	0.004	Membrane proteins; Transport of small molecules
PA1186		4.4	0.010	Hypothetical, unclassified, unknown
PA4167		4.4	0.008	Putative enzymes
PA1257		4.3	0.006	Membrane proteins; Transport of small molecules
PA0135		4.3	0.003	Hypothetical, unclassified, unknown
PA3447		4.3	0.001	Transport of small molecules
PA3219		4.3	0.008	Hypothetical, unclassified, unknown
PA0205		4.2	0.002	Membrane proteins; Transport of small molecules
PA4858		4.2	0.001	Hypothetical, unclassified, unknown
PA1393	cycC	4.1	0.004	Amino acid biosynthesis and metabolism, Central intermediary metabolism, Nucleotide biosynthesis and metabolism
PA4083	cupB4	4.1	0.000	Motility & Attachment; Chaperones & heat shock proteins
PA2058		4.1	0.004	Transport of small molecules
PA0993	cupC2	4.1	0.004	Motility & Attachment; Chaperones & heat shock proteins

PA4883		4.0	0.003	Hypothetical, unclassified, unknown
PA4081	cupB6	4.0	0.007	Motility & Attachment
PA2296		4.0	0.003	Hypothetical, unclassified, unknown
PA2522	czcC	4.0	0.006	Membrane proteins; Transport of small molecules
PA1873		3.9	0.008	Membrane proteins
PA3897		3.9	0.001	Membrane proteins
PA0840		3.9	0.009	Putative enzymes
PA5404		3.8	0.001	Hypothetical, unclassified, unknown
PA3497		3.8	0.003	Hypothetical, unclassified, unknown
PA0287	gpuP	3.8	0.006	Transport of small molecules
PA3006	psrA	3.7	0.007	Transcriptional regulators
PA3909	eddB	3.7	0.001	Hypothetical, unclassified, unknown
PA1258		3.7	0.006	Membrane proteins; Transport of small molecules
PA5324		3.7	0.006	Transcriptional regulators
PA3902		3.7	0.008	Hypothetical, unclassified, unknown
PA1144		3.7	0.003	Membrane proteins; Transport of small molecules
PA1884		3.7	0.001	Transcriptional regulators
PA5293		3.7	0.001	Transcriptional regulators
PA3677		3.6	0.008	Transport of small molecules
PA1909		3.6	0.006	Hypothetical, unclassified, unknown
PA2521	czcB	3.6	0.003	Membrane proteins; Transport of small molecules
PA5467		3.6	0.002	Hypothetical, unclassified, unknown
PA5394	cls	3.5	0.001	Fatty acid and phospholipid metabolism
PA3521		3.5	0.001	Membrane proteins; Transport of small molecules
PA0104		3.4	0.000	Hypothetical, unclassified, unknown
PA5468		3.4	0.003	Transport of small molecules
PA0476		3.4	0.002	Transport of small molecules
PA4514		3.4	0.005	Transport of small molecules
PA3135		3.4	0.002	Transcriptional regulators
PA0680		3.4	0.001	Protein secretion/export apparatus
PA3543	algK	3.4	0.006	Cell wall / LPS / capsule; Adaptation, Protection; Secreted Factors (toxins, enzymes, alginate)
PA3058	pelG	3.4	0.005	Cell wall / LPS / capsule
PA4679		3.4	0.002	Hypothetical, unclassified, unknown
PA0252		3.4	0.010	Hypothetical, unclassified, unknown
PA4176	ppiC2	3.3	0.005	Translation, post-translational modification, degradation, Chaperones & heat shock proteins
PA0689		3.3	0.004	Transport of small molecules
PA4226	pchE	3.3	0.004	Secreted Factors (toxins, enzymes, alginate); Transport of small molecules
PA0707	toxR	3.3	0.005	Transcriptional regulators
PA4028		3.3	0.003	Hypothetical, unclassified, unknown

PA0931	pirA	3.3	0.001	Transport of small molecules
PA0153	pvaH	3.3	0.008	Carbon compound catabolism
PA3319		3.3	0.005	Secreted Factors (toxins, enzymes, alginate)
PA2838		3.3	0.003	Transcriptional regulators
PA2469		3.3	0.005	Transcriptional regulators
PA0234		3.3	0.004	Hypothetical, unclassified, unknown
PA0688		3.3	0.010	Transport of small molecules
PA2121		3.3	0.003	Transcriptional regulators
PA4082	cupB5	3.3	0.008	Motility & Attachment
				Membrane proteins; Antibiotic resistance and susceptibility; Transport of small
PA2055		3.3	0.003	molecules
PA0150		3.2	0.009	Membrane proteins, Transcriptional regulators
PA2064	pcoB	3.2	0.003	Adaptation, Protection
PA3463		3.2	0.001	Hypothetical, unclassified, unknown
				Amino acid biosynthesis and metabolism, Biosynthesis of cofactors, prosthetic
PA4007	proA	3.2	0.008	groups and carriers
PA4989		3.2	0.006	Transcriptional regulators
PA4927		3.2	0.005	Hypothetical, unclassified, unknown
PA5286		3.2	0.000	Hypothetical, unclassified, unknown
PA3591		3.2	0.002	Carbon compound catabolism
PA4156		3.2	0.009	Transport of small molecules
PA0166		3.2	0.000	Membrane proteins; Transport of small molecules
PA2253	ansA	3.2	0.000	Amino acid biosynthesis and metabolism
PA2783		3.2	0.004	Hypothetical, unclassified, unknown
PA5414		3.1	0.005	Hypothetical, unclassified, unknown
PA2583		3.1	0.010	Transcriptional regulators, Two-component regulatory systems
PA3410		3.1	0.001	Transcriptional regulators
PA3449		3.1	0.004	Hypothetical, unclassified, unknown
PA2802		3.1	0.004	Transcriptional regulators
PA2559		3.1	0.003	Hypothetical, unclassified, unknown
PA2310		3.1	0.009	Putative enzymes
PA0557		3.1	0.007	Hypothetical, unclassified, unknown
PA1620		3.1	0.002	Membrane proteins
PA2219	opdE	3.1	0.005	Membrane proteins; Transport of small molecules
PA1264		3.0	0.007	Transcriptional regulators
PA3990		3.0	0.006	Hypothetical, unclassified, unknown
PA2634	aceA	3.0	0.006	Putative enzymes
PA2348		3.0	0.000	Putative enzymes
PA2022		3.0	0.005	Putative enzymes
PA4801		3.0	0.000	Hypothetical, unclassified, unknown
PA0268		3.0	0.009	Transcriptional regulators

PA0206		2.9	0.005	Transport of small molecules
PA5218		2.9	0.009	Transcriptional regulators
PA5477		2.9	0.007	Membrane proteins
PA1956		2.9	0.007	Hypothetical, unclassified, unknown
PA0864		2.9	0.005	Transcriptional regulators
PA0671		2.9	0.007	Hypothetical, unclassified, unknown
PA2384		2.9	0.003	Hypothetical, unclassified, unknown
PA1867	xphA	2.9	0.004	Protein secretion/export apparatus
PA4823		2.8	0.001	Hypothetical, unclassified, unknown
PA5476	citA	2.8	0.003	Membrane proteins; Transport of small molecules
PA2745		2.8	0.002	Putative enzymes
PA4204	ppgL	2.8	0.009	Hypothetical, unclassified, unknown
PA1910	femA	2.8	0.004	Transport of small molecules; Transcriptional regulators
PA3938		2.8	0.002	Transport of small molecules
PA0842		2.8	0.002	Putative enzymes
	gcvH2			
PA2446		2.8	0.002	Amino acid biosynthesis and metabolism
PA2492	mexT	2.8	0.001	Transcriptional regulators
PA0273		2.8	0.006	Membrane proteins; Transport of small molecules
PA3258		2.8	0.009	Hypothetical, unclassified, unknown
PA0824		2.7	0.010	Hypothetical, unclassified, unknown
PA0202		2.7	0.003	Putative enzymes
PA0132	bauA	2.7	0.000	Amino acid biosynthesis and metabolism, Carbon compound catabolism
PA3037		2.7	0.006	Hypothetical, unclassified, unknown
PA5021		2.7	0.003	Membrane proteins, Transport of small molecules
PA0874		2.7	0.006	Hypothetical, unclassified, unknown
PA1412		2.7	0.008	Hypothetical, unclassified, unknown
PA4381		2.7	0.001	Transcriptional regulators, Two-component regulatory systems
PA2766		2.6	0.009	Transcriptional regulators
PA0311		2.6	0.001	Hypothetical, unclassified, unknown
PA0039		2.6	0.006	Hypothetical, unclassified, unknown
PA4822		2.6	0.004	Membrane proteins
PA2511		2.6	0.000	Transcriptional regulators
PA0990		2.6	0.002	Hypothetical, unclassified, unknown
PA5465		2.6	0.002	Hypothetical, unclassified, unknown
PA1868	xqhA	2.6	0.009	Protein secretion/export apparatus
PA1746		2.5	0.008	Hypothetical, unclassified, unknown
PA2487		2.5	0.004	Hypothetical, unclassified, unknown
PA3360		2.5	0.000	Secreted Factors (toxins, enzymes, alginate)
PA3265		2.5	0.003	Membrane proteins, Transport of small molecules
PA1980	eraR	2.5	0.010	Transcriptional regulators; Two-component regulatory systems

PA2929		2.5	0.003	Membrane proteins
PA0682		2.5	0.002	Protein secretion/export apparatus
PA2926				Amino acid biosynthesis and metabolism
,	hisP	2.5	0.003	Transport of small molecules
PA2311		2.5	0.006	Hypothetical, unclassified, unknown
PA0283	sbp	2.5	0.001	Transport of small molecules
PA4031	ppa	2.5	0.000	Central intermediary metabolism
PA4590	pra	2.5	0.004	Transport of small molecules, Carbon compound catabolism
PA0564		2.5	0.001	Transcriptional regulators
PA1364		2.5	0.007	Membrane proteins, transcriptional regulator
PA0790		2.5	0.006	Membrane proteins
PA4355		2.4	0.007	Membrane proteins; Transport of small molecules
PA2356	msuD	2.4	0.004	Carbon compound catabolism; Central intermediary metabolism
PA3926		2.4	0.009	Membrane proteins; Transport of small molecules
PA4072		2.4	0.010	Transport of small molecules
PA2347		2.4	0.010	Putative enzymes
PA5156		2.4	0.003	Hypothetical, unclassified, unknown
PA4144		2.4	0.003	Protein secretion/export apparatus
PA1137		2.4	0.008	Putative enzymes
PA5024		2.4	0.006	Hypothetical, unclassified, unknown
PA4044		2.4	0.001	Biosynthesis of cofactors, prosthetic groups and carriers
PA2094		2.4	0.004	Membrane proteins; Transcriptional regulators
PA4830		2.4	0.003	Hypothetical, unclassified, unknown
PA0288	gpaA	2.4	0.000	Amino acid biosynthesis and metabolism
PA1435		2.4	0.002	Transport of small molecules
PA5309		2.4	0.004	Putative enzymes, Carbon compound catabolism
PA0687		2.4	0.007	Protein secretion/export apparatus
PA0186		2.3	0.006	Transport of small molecules
PA1826		2.3	0.009	Transcriptional regulators
PA3685		2.3	0.009	Hypothetical, unclassified, unknown
PA0044	exoT	2.3	0.006	Secreted Factors (toxins, enzymes, alginate)
PA4682		2.3	0.009	Hypothetical, unclassified, unknown
PA1824		2.3	0.007	Membrane proteins
PA0031	betC	2.3	0.008	Adaptation, Protection
PA0599		2.3	0.007	Hypothetical, unclassified, unknown
PA5505		2.3	0.003	Membrane proteins, Transport of small molecules
PA4160	fepD	2.3	0.003	Membrane proteins; Transport of small molecules
PA3578		2.2	0.009	Hypothetical, unclassified, unknown
PA4390		2.2	0.009	Hypothetical, unclassified, unknown
PA2757		2.2	0.003	Membrane proteins
PA2006		2.2	0.009	Membrane proteins; Transport of small molecules

PA0444		2.2	0.005	Nucleotide biosynthesis and metabolism
PA3979		2.2	0.006	Hypothetical, unclassified, unknown
PA3359		2.2	0.002	Membrane proteins
PA4373		2.2	0.005	Hypothetical, unclassified, unknown
PA0489		2.1	0.004	Putative enzymes
PA4436		2.1	0.002	Transcriptional regulators
PA1993		2.1	0.002	Membrane proteins, Transport of small molecules
PA0348		2.1	0.001	Hypothetical, unclassified, unknown
PA4677		2.1	0.007	Hypothetical, unclassified, unknown
PA5523		2.1	0.002	Putative enzymes
				Membrane proteins; Transport of small molecules; Antibiotic resistance and
PA4990		2.1	0.004	susceptibility
PA5444		2.1	0.001	Membrane proteins
PA3778		2.1	0.004	Transcriptional regulators
PA4478		2.1	0.007	Hypothetical, unclassified, unknown
PA3564		2.1	0.007	Hypothetical, unclassified, unknown
PA1286		2.1	0.002	Membrane proteins; Transport of small molecules
PA0034		2.1	0.007	Transcriptional regulators; Two-component regulatory systems
PA5208		2.1	0.003	Hypothetical, unclassified, unknown
				Membrane proteins; Antibiotic resistance and susceptibility; Transport of small
PA4597	oprJ	2.0	0.002	molecules
PA4622		2.0	0.006	Membrane proteins, Transport of small molecules
PA1845		2.0	0.010	Hypothetical, unclassified, unknown
PA2267		2.0	0.007	Transcriptional regulators
PA2442	gcvT2	2.0	0.001	Central intermediary metabolism, Amino acid biosynthesis and metabolism

Table 5.4 List of statistically significant down-regulated genes of PAO1 $\Delta mvaU$ mutants compared to PAO1 of *P. aeruginosa* (fold change was calculated based on mRNA level of each gene in PAO1 and $\Delta mvaU$ mutants). p -value ≤ 0.01 and fold change ≥ 2.0

Gene ID	Name	Fold Change	<i>P</i> value	PseudoCAP Function Class
PA2667	<i>mvaU</i>	460.7	0.001	Transcriptional regulators
PA4931	<i>dnaB</i>	17.5	0.005	DNA replication, recombination, modification and repair
PA3567		8.5	0.001	Putative enzymes
PA0397		4.9	0.008	Transport of small molecules
PA3734		3.3	0.002	Hypothetical, unclassified, unknown
PA3478	<i>rhlB</i>	2.9	0.009	Secreted Factors (toxins, enzymes, alginate)
PA2901		2.3	0.002	Hypothetical, unclassified, unknown

The distribution of genes according to their functions was examined using *P. aeruginosa* genome data base (<http://www.pseudomonas.com/>) and GO analysis. Subsequently, 15 clusters were determined using functional classification (shown in Figure 5.9B). The genes with fold change (≥ 5.0) were filtered out to exclude the differentially expressed genes with less ratio of mRNA transcript (see table 5-5). Among the highest fold change in up-regulated genes, those involved in PVD biosynthesis including *pvdJ*, *pvdF*, and *pvdG* (in adaptation, protection cluster) were remarkably increased in *mvaU* mutants with the fold changes of 6.3, 6.7, and 7.2, respectively. Accordingly, I hypothesized that *mvaU* might be a negative regulator for the mentioned genes because in the absence of *mvaU*, these genes were remarkably up-regulated.

Figure 5.9 Differentially expressed genes and functional clustering in PAO1 Δ *mvaU* mutants compared to the wild-type *P. aeruginosa*. (A) Total number of statistically differentially expressed genes (p value ≤ 0.01 and fold change ≥ 2.0). Among 247 differentially significant expressed genes, 240 were up-regulated, while seven genes were down-regulated. The function of 77 (up regulated) and two (down-regulated) genes were not identified via *P. aeruginosa* genome data base and GO analysis and were considered unknown. (B) Functional clustering of significantly up-regulated genes in *mvaU* mutants compared to PAO1. The numbers in front of the bars represent the total number of up regulated genes in each cluster.

Table 5.5 List of statistically significant up-regulated and down-regulated genes in PAO1Δ*mvaU* mutants compared with PAO1. *p*-value ≤ 0.01 and fold change ≥ 5.0

ID	Name	Fold change	P value	PseudoCAP Function Class
Up-regulated				
PA5262	fimS	8.7	0.000	Secreted Factors (toxins, enzymes, alginate), Two-component regulatory systems
PA4814	fadH2	7.4	0.003	Fatty acid and phospholipid metabolism
PA2425	pvdG	7.2	0.008	Adaptation, Protection
PA2842		6.8	0.001	Hypothetical, unclassified, unknown
PA2396	pvdF	6.7	0.006	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)
PA3520		6.6	0.004	Hypothetical, unclassified, unknown
PA2400	pvdJ	6.3	0.006	Adaptation, Protection
PA4189		6.1	0.004	Putative enzymes
PA1921		6.1	0.001	Hypothetical, unclassified, unknown
PA0185		6.1	0.007	Membrane proteins, Transport of small molecules
PA0094		6.0	0.001	Hypothetical, unclassified, unknown
PA1907		5.7	0.000	Hypothetical, unclassified, unknown
PA3034		5.7	0.008	Transcriptional regulators
PA0883		5.7	0.001	Putative enzymes
PA4230	pchB	5.6	0.000	Secreted Factors (toxins, enzymes, alginate); Transport of small molecules
PA3580		5.5	0.009	Hypothetical, unclassified, unknown
PA2471		5.4	0.002	Hypothetical, unclassified, unknown
PA3773		5.3	0.004	Membrane proteins
PA4710	phuR	5.2	0.008	Transport of small molecules
PA4896		5.2	0.010	Transcriptional regulators
PA0349		5.1	0.008	Membrane proteins
PA1917		5.0	0.009	Hypothetical, unclassified, unknown
PA3286		5.0	0.006	Hypothetical, unclassified, unknown
PA1925		5.0	0.008	Hypothetical, unclassified, unknown
Down-regulated				
PA2667	mvaU	460.7	0.001	Transcriptional regulators
PA4931	dnaB	17.5	0.005	DNA replication, recombination, modification and repair
PA3567		8.5	0.001	Putative enzymes

Furthermore, based on the analyzed microarray data (Table 5.3) and functional clustering (Figure 5.9), it was aimed to elucidate antibiotic resistance and susceptibility

in *P. aeruginosa*. It was revealed that compared to PAO1, in PAO1 Δ *mvaU* mutants, genes with genome IDs of PA2055, PA4114, PA4597 (*oprJ*), and PA4990 showed significant increase in mRNA transcript levels. These genes are categorized as antibiotic resistance and susceptibility genes using GO analysis. The two genes PA4597 and PA4990 are involved in the production of multidrug efflux outer membrane and multidrug-resistant efflux transporter proteins, respectively (Fraud, Campigotto et al. 2008).

Moreover, PA4114 and PA2025 were previously predicted to be involved in antibiotic resistance by mediating in transportation of the major facilitator superfamily (Small, Chang et al. 2007). Accordingly, it was hypothesized that *mvaU* might contribute in the repression of the antibiotic resistance genes in PAO1. To verify our hypothesis, initially, a simple and primary antibiotic sensitivity assay called antibiotic sensitivity disk diffusion was performed to see whether *mvaU* deletion shows any difference in antibiotic susceptibility compared to the wild type. According to the literatures, more common antibiotics were selected (listed in Table 5.6) and the antibiotic sensitivity test was conducted by inoculating single colonies of PAO1 Δ *mvaU* mutants and PAO1 in 5 mL of Mueller-Hinton broth (Thermo Scientific, USA) with Sm (50 μ g mL⁻¹) for *mvaU* mutants, incubated at 37°C and 250 rpm shaking overnight. Cells were then diluted and incubated at the same conditions to get OD₆₀₀ of 1.0 (1-1.5 $\times$ 10⁸ CFU mL⁻¹). Around 200 μ L of each cell culture was spread on Mueller-Hinton agar (Thermo Scientific, USA). The antibiotic disks (Thermo Scientific, USA) were placed on the

plate using sterilized tweezers, and the plates were incubated at 35°C for 16 h prior to the determination of results. After appropriate incubation time, the plates were observed based on the zone of inhibition for each antibiotic disk. The inhibition zone was measured to the nearest millimeter. The diameter of zones was then compared with the standard tables provided by the company, and the susceptibility of cells to each antibiotic was determined qualitatively as susceptible, intermediate, or resistant. The disk diffusion comparison of both mutant and wild-type strains showed no differences in antibiotic susceptibility between these two groups of cells (see Figure 5.10).

Table 5.6 Name of antibiotics used in this study for antibiotic susceptibility disk diffusion

Name of Antibiotic				
Gentamicin	Amoxicillin	Erythromycin	Vancomycin	Ampicillin
CN	AML	E	VA	AMP
Meropenem	Aztreonam	Piperacillin	Levofloxacin	Cefotaxime
MEM	ATM	PRL	LEV	CTX
Neomycin				
N

Figure 5.10 Disk diffusion assay with PAO1 Δ *mvaU* and wild-type PAO1 cell cultures.

5.4.3 qRT-PCR

Quantitative real time PCR (qRT-PCR) was carried out to validate the microarray data.

The primers used in this experiment targeting *pvdJ*, *pvdF*, *pvdG*, and *rpoD* (reference gene) are shown in Table 5-2. I measured the expression levels of *pvdJ*, *pvdF*, and *pvdG* genes in wild-type, complementary, and mutant strains. Fold change expression was normalized to the control (wild-type PAO1) and was measured as shown in Table 5.7. The data from qRT-PCR were corroborated with microarray analysis, indicating that the expression levels of *pvdJ*, *pvdF*, and *pvdG* significantly increased in *mvaU* mutants in contrast with the wild-type and complementary strains that did not show any expression in mRNA levels of *pvdJ*, *pvdF*, and *pvdG* (Figure 5.11 and Figure 5.12).

In regard with these results, I hypothesized that *mvaU* might be involved in these three

PVD biosynthesis genes. The regulatory role of *mvaU* could be considered as a repressor. For further investigation, I conducted PVD measurements in wild-type, complementary and mutant strains.

Table 5.7 Real-time PCR gene expression. The expressions of three target genes from microarray data were measured using qRT-PCR, and the normalized fold change of each gene in wild-type, deleted mutant and complementary strains was calculated using " $2^{-\Delta\Delta C_T}$ " formula.

Name of sample	Gene ID (gene name)		
	PA2396 (<i>pvdF</i>)	PA2400 (<i>pvdJ</i>)	PA2495 (<i>pvdG</i>)
	$2^{-\Delta\Delta C_T}$		
PAO1 (wild type)	0.14	0.12	0.49
PAO1 Δ <i>mvaU</i> (<i>mvaU</i> mutant)	7.0	8.54	2.02
PAO1 Δ <i>mvaUC</i> (complementary)	0.06	0.07	0.42

The functions of PvdF and PvdJ were known to be mediating in PVD biosynthesis, while the function of PvdG has not been determined yet (Smith, Sims et al. 2005). Table 5.8 indicates the identified genes involved in PVD biosynthesis or transportation. From the literatures, it was revealed that *pvdJ* and *pvdF* are involved in the important PVD synthesis genes through the encoding of the peptide synthetase enzymes (Visca, Serino et al. 1992, Ravel and Cornelis 2003).

Figure 5.11 Quantitative real-time PCR (qRT-PCR) analysis of (A) *pvdF*, (B) *pvdJ*, and (C) *pvdG* genes in PAO1 (wild type), PAO1 Δ *mvaU* (deleted *mvaU* mutant), and PAO1 Δ *mvaUC* (complementary) strains.

Figure 5.12 Summary of quantitative real-time PCR (qRT-PCR) analysis of *pvdF*, *pvdJ*, and *pvdG* genes in PAO1 (wild type), PAO1 Δ *mvaU* (deleted *mvaU* mutant), and PAO1 Δ *mvaUC* (complementary) strains.

Table 5.8 List of genes involved in PVD biosynthesis and transportation

Gene ID	Name	PseudoCAP Function Class	Reference
PA1754	cysB	Transcriptional regulators	Imperiet <i>et al</i> 2010
PA2398	fpvA	Transport of small molecules	Poole <i>et al</i> 1993
PA2387	fpvI	Transcriptional regulators, Transport of small molecules	Beareet <i>et al</i> 2003
PA2388	fpvR	Transcriptional regulators, Transport of small molecules, Membrane proteins	Lamont <i>et al</i> 2002, Beareet <i>et al</i> 2003
PA2254	pvcA	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintziet <i>et al</i> 1996,
PA2255	pvcB	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintziet <i>et al</i> 1996, 1999
PA2256	pvcC	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintziet <i>et al</i> 1996,
PA2257	pvcD	Secreted Factors (toxins, enzymes, alginate), Amino acid biosynthesis and metabolism	Stintziet <i>et al</i> 1996,
PA2386	pvdA	Adaptation, Protection	Viscaet <i>et al</i> 1994
PA2399	pvdD	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)	Merriman <i>et al</i> 1995
PA2397	pvdE	Membrane proteins, Transport of small molecules, Adaptation, Protection	McMorran <i>et al</i> 1996
PA2396	pvdF	Adaptation, Protection, Secreted Factors (toxins, enzymes, alginate)	McMorranet <i>et al</i> 2001
PA2425	pvdG	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2413	pvdH	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2402	pvdI	Putative enzymes	Lehoux <i>et al</i> 2000
PA2400	pvdJ	Adaptation, Protection	Lehoux <i>et al</i> 2000
PA2424	pvdL	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2393	pvdM	Central intermediary metabolism	Lamont <i>et al</i> 2003
PA2394	pvdN	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2395	pvdO	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2392	pvdP	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2385	pvdQ	Adaptation, Protection	Lamont <i>et al</i> 2003
PA2389	pvdR	Transport of small molecules	Imperiet <i>et al</i> 2009
PA2426	pvdS	Transcriptional regulators	Cunliffe ; Miyazaki <i>et al</i> 1995
PA2390	pvdT	Membrane proteins, Transport of small molecules	Imperi <i>et al</i> 2009
PA5531	tonB1	Transport of small molecules	Shirley <i>et al</i> 2009

5.4.4 Pyoverdine measurements

As a complementary experiment, we measured the PVD concentration in cell lysates of PAO1 (WT), PAO1 $\Delta mvaU$ (mutant), and PAO1 $\Delta mvaUC$ (complementary) strains. Regarding the fluorescent properties of PVD, the intracellular and supernatant concentration of PVD was determined by fluorometric measurements. The results of PVD measurement in cell lysates revealed that the concentration of PVD in *mvaU* mutants was significantly higher. On the other hand, *mvaU* mutants accumulated a higher amount of PVD, about four- and two-fold, more than PAO1 and PAO1 $\Delta mvaUC$ strains, respectively (Figure 4A). Similarly, more PVD was detected in the supernatant of *mvaU* mutants compared to the wild type and PAO1 $\Delta mvaUC$ (Figure 4B), while this difference in supernatant PVD concentration was not as significant as the PVD concentration in lysate. In line with microarray data and qRT-PCR results, PVD measurements in lysate and supernatant samples supported our hypothesis indicating that *mvaU* could be proposed as a potential negative regulator for our three target genes (*pvdJ*, *pvdF* and *pvdG*) mediating in PVD biosynthesis.

Figure 5.13 Intracellular PVD measurement in PAO1 (wild type), PAO1 Δ *mvaU* (deleted *mvaU* mutant), and PAO1 Δ *mvaUC* (complementary) strains.

Figure 5.14 Supernatant PVD measurement in PAO1 (wild type), PAO1 Δ *mvaU* (deleted *mvaU* mutant) and PAO1 Δ *mvaUC* (complementary) strains.

5.5 Conclusion

I carried out a microarray experiment on *mvaU* mutants and wild type of *P. aeruginosa* PAO1. The transcriptome profiling analysis showed that in *mvaU* mutants the mRNA transcript levels of three PVD biosynthesis genes *pvdF*, *pvdG*, and *pvdJ* were significantly increased compared with wild-type PAO1. These validated data by qRT-PCR motivated me to think about regulatory role of *mvaU* in PVD-related genes. I proposed that these PVD related genes can be affected by a global regulator, the *mvaU* gene, in *P. aeruginosa*. The phenotypic change of the *mvaU* mutant cells within incubation at 37°C indicated the production of fluorescent molecules such as PVD or pyocyanin in this mutant strain, whereas wild-type and complementary strains were not found with any color changes in the cultural cell. In line with these experiments, PVD was measured in supernatant and cell lysate of deleted *mvaU* mutants (PAO1- Δ *mvaU*) as well as wild-type and complementary (PAO1- Δ *mvaUC*) strains. The PVD measurement demonstrated that PVD was significantly higher in *mvaU* mutant cells in contrast with the wild type and complementary cells. For further analysis as a future work, I can conduct the visualization of PVD molecule in *mvaU* and wild type of PAO1 using confocal microscopy. In addition, more molecular-basis studies is proposed, including a study on operons to identify genes located on the same operon as my three target genes in PVD pyocin biosynthesis. It has been revealed that genes located in the same operon show a correlation with their neighbor genes.

I also suggest performing a microarray analysis in mutant and wild type strains upon pyocin treatment (shown in the appendix 2) to discover any linkage between the effect of pyocin treatment in transcriptome profiling and gene expression in wild-type PAO1 as well as mutant *mvaU* strain. I conducted the microarray experiment on deleted *mvaU* of PAO1 strain with pyocin treatment and compared these data with untreated *mvaU* mutants. The results were derived after using GeneSpring GX 11 and GO analysis as shown in the appendix 2. The primary analysis showed that *pvdG* was down-regulated in *mvaU* mutants upon exposure to pyocin S5. This finding could be a promising hint to lead us to investigate the mechanism of the regulatory role of *mvaU* in pyocin production. As shown in the appendix 2B, pyocin S5 may affect the probable regulatory role of *mvaU* in PVD biosynthesis.

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

Pseudomonas aeruginosa is a Gram-negative bacterium with the ability of producing virulence factors including proteases, toxins, and siderophores such as pyoverdine (PVD). Because of its resistance towards many antibiotics, in particular in patients with immune deficiency disorders such as cystic fibrosis or cancer, this bacterium is ranked as one of the most causes of death in these patients. The resistance of *P. aeruginosa* to a wide range of synthetic antibiotics forces scientists to think of developing effective strategies to treat its lethal infections. This bacterium is able to produce natural antibacterial peptides called pyocins. Pyocins protect producing strain against other relative competitors. The producing strain is resistant to its own pyocin because of the probable immunity gene.

In this study, chapter 3 and chapter 4 mostly focus on the pyocin-producing properties of *P. aeruginosa*. To summarize, chapter 3 provided the first experimental evidence indicating that the nucleic acid sequences at positions 1067817– 1066321 encode a pyocin S5 (PA0985). Furthermore, the killing activity of pyocin S5 against seven clinical isolates of *P. aeruginosa* strains (from cystic fibrosis patients) was elucidated.

In this chapter, it was further showed that the antimicrobial mechanism of pyocin S5

results in membrane damage to a pyocin S5-sensitive strain (DWW3), evidenced by the leakage of intracellular materials, increase of membrane permeability, and cell surface disruption. With regard to the importance of the discovery of effective antimicrobial molecules against the clinical isolates of *P. aeruginosa*, this study may suggest the potential use of pyocin S5 in *P. aeruginosa* therapeutic purposes. In addition, this research could be expanded to investigate specific receptors for pyocins including pyocin S5. Moreover, the killing mechanism of pyocins can be exploited for developing a novel treatment regimen with a low probability of antibiotic resistance and higher specificity in *P. aeruginosa* targeting. Our group has successfully engineered cells that are able to specifically release pyocin S5 upon sensing the presence of *P.aeruginosa* (Saeidi, Wong et al. 2011). Subsequently, this narrow antimicrobial spectrum can be applied for broader applications by developing chimeric pyocins.

In chapter 4, I strived to demonstrate the role of PA0984, a colicin immunity protein of *P. aeruginosa* named S5I. Towards this aim, pyocin S5 immunity protein in *P. aeruginosa* was compared with immunity proteins of *E. coli* using protein sequence, secondary structure, and transmembrane domains analysis. Based on the detected similarities of this protein and *E. coli* immunity proteins, S5I protein was predicted as a trans-membrane protein probably with an immunizing role against pyocin S5. Accordingly, DWW3 (that is devoid of *S5I* gene) was selected as a pyocin-sensitive strain of *P. aeruginosa*, to examine the extended immunity introducing by an

exogenous *S5I* expression. Coherently, the immunity role of the *S5I* gene in immunizing pyocin S5-producing strains was determined. Therefore, this study successfully confirmed the protective role of the *S5I* gene in *P. aeruginosa*, where the sensitivity of the cells to pyocin S5 is determined by the presence or absence of this gene. Furthermore, here, I implied that when a pyocin S5-sensitive strain such as DWW3 was engineered to express the *S5I* gene, it could become resistant to pyocin S5. This work can be further extended in the mechanism of *S5I* and investigate how *S5I* can protect pyocin S5-producing strains against their own pyocins. According to colicin immunity studies, the *E. coli* immunity proteins tightly bind to their colicin receptors and protect cells from the lethal activity of colicins (Cascales, Buchanan et al. 2007, Jakes and Cramer 2012). Similarly, with regard to the homology in *S5I* and colicin immunity protein structure (determined in this work), *S5I* may interrupt the pore-forming activity of pyocin S5 by preventing the translocation and insertion of the pore-forming domain of pyocin S5 to the outer membrane surface. Although elucidating the mechanism of *S5I* gene assuredly requires further investigation, the findings presented in this thesis accredit the potential role of *S5I* as a therapeutic target that can sensitize *P. aeruginosa* to pyocin S5 treatment. Future efforts might include screening for *S5I* inhibitors, for an S5 combinatorial therapeutic strategy to target S5-resistant strains.

Thus, by combining a strategy to use pyocin S5 as an antibiotic agent and simultaneously recruit an approach to reverse or silence the immunity of *S5I* in target

cells (*P. aeruginosa*), these bacterial cells will be destroyed by their own pyocins, and a more refined and effective treatment against *P. aeruginosa* can be accomplished.

Chapter 5 aimed to investigate the regulatory role of the *mvaU* gene as a global regulator in *P. aeruginosa*. Towards this aim, the *mvaU* gene was knocked out in *P. aeruginosa* PAO1 strain, and a microarray experiment was conducted to study the transcriptome profiling in mutant and wild type strains. Following this experiment, the differentially expressed genes were further analyzed using bioinformatics algorithms, such as GO analysis. This study revealed that the deletion of *mvaU* from *P. aeruginosa* PAO1 results in the up regulation of three PVD-related genes such as *pvdF*, *pvdJ*, and *pvdG*. In addition, the phenotypic change of *mvaU* mutants within incubation at 37°C indicates the production of fluorescent molecules such as PVD in this mutant strain, whereas the wild type and complementary strains did not show a significant phenotypic change in their cultured cells. In line with microarray data, PVD measurements demonstrated the production of PVD in the *mvaU* mutant strain in contrast with wild type. This study led me to focus on PVD-related genes as candidates that can be regulated by the *mvaU* gene in PAO1. PVD is known as a virulence factor in *P. aeruginosa*. This molecule is required for the pathogenicity of *P. aeruginosa* because of its role as an iron source provider. Moreover, PVD is an inducer for other virulence factors in *P. aeruginosa*. These properties make PVD as an important factor in biofilm formation and hence an interesting and promising subject to be expanded in the *Pseudomonas* research fields. As discussed earlier, previous studies showed that PVD is involved in biofilm

formation and pathogenicity in *P. aeruginosa*. In this study, it was shown that the production of PVD has been remarkably decreased in *mvaU* defected-PAO1 strain compared with that of the wild typ. Therefore, impeded biofilm formation is expected in *mvaU* mutant cells, and the mutants will most likely show the lack of biofilm formation. Additional application of confocal microscopy will be helpful to illustrate reduction of biofilm development, and to visualize PVD accumulation to demonstrate difference in PVD amount in both wild type and *mvaU* mutant strains.

Besides, more molecular-basis experiments including a study on operons was proposed to achieve further investigations in PVD regulation via the *mvaU* gene and identification of the genes that are located in the same operon as *pvdF*, *pvdJ*, or *pvdG* genes. It has been shown that genes located on the same operon are expected to be in correlation with their neighbors. Overall, I suggest performing a microarray analysis in the *mvaU* mutant and the wild type of *P. aeruginosa* PAO1 strain upon pyocin treatment to discover probable linkage between the effects of pyocin treatment in transcriptome profiling and gene expression in wild-type PAO1 as well as the mutant *mvaU* strain. I carried out the microarray experiment on deleted *mvaU* strain with pyocin treatment and the data was compared with untreated *mvaU* mutants. The results were derived after using GeneSpring GX11 and GO analysis as shown in the appendix 2. This analysis will be continued for further investigation.

In summary, this thesis verified the antimicrobial mechanism of pyocin S5 against clinical isolates of *P. aeruginosa* in cystic fibrosis patients. Moreover, the efficacy and the mode of action of *S5 immunity* gene were elucidated, which renders the host resistance to pyocin S5. In addition, transcriptome profiling revealed that *mvaU*, a global regulatory gene, is involved in pyoverdine regulation. Therefore, the achievement of this project is an effort that investigates virulence genes in *P. aeruginosa* PAO1 and aids in screening of *S5I* inhibitors, for an S5 combinatorial therapeutic strategy to target S5-resistant strains of *P. aeruginosa*. The findings in this study, including the relationship between PVD-biosynthesis genes and *mvaU* regulatory gene and the affirmation of S5 and S5I role, provide valuable insights in understanding virulence of *P. aeruginosa* and expanding therapeutic approaches beyond antibiotic treatment.

APPENDIX

Appendix 1

Appendix 1. The vector maps of (A) pyocin S5 protein expression (B) *S5I* (immunity) gene transformation to DWW3 (C) *mvaU* gene deletion in *P. aeruginosa* PAO1.

Appendix 2

Table A. List of significantly up-regulated genes of PAO1 Δ mvaU (*P. aeruginosa*) in response to 30 min pyocin S5 treatment, compared with untreated samples. p -value ≤ 0.05 and fold change ≥ 2.0

Gene ID	Name	fold change	p -value	PseudoCAP Function Class
PA3582	glpK	4.8	0.04	Glycerolipid metabolism
PA3584	glpD	2.9	0.01	Energy metabolism
PA3194	edd	2.8	0.02	Pentose phosphate cycle
PA3193	glk	2.7	0.03	Energy metabolism
PA3183	zwf	2.5	0.02	Pentose phosphate cycle
PA5435		2.1	0.02	Pyruvate metabolism
PA3580		2.0	0.03	Hypothetical, unclassified, unknown

Table B. List of significantly down-regulated genes of PAO1 Δ mvaU (*P. aeruginosa*) in response to 30 min pyocin S5 treatment, compared with untreated samples. p -value ≤ 0.05 and fold change ≥ 2.0

GeneName	Name	fold change	p -value	PseudoCAP Function Class
PA4880		2.2	0.01	Central intermediary metabolism
PA5324		2.2	0.01	Transcriptional regulators
PA2425	pvdG	2.1	0.04	Adaptation, Protection
PA3296	phoA	2.1	0.04	Central intermediary metabolism
PA5123		2.1	0.04	Hypothetical, unclassified, unknown
PA2398	fpvA	2.0	0.03	Transport of small molecules
PA2209		2.0	0.04	Hypothetical, unclassified, unknown
PA0154	pcaG	2.0	0.01	Carbon compound catabolism

Table C. List of significantly up-regulated genes of PAO1 (*P. aeruginosa*) in response to 30 min pyocin S5 treatment. *p*-value ≤ 0.05 and fold change ≥ 2.0

Gene				
ID	Name	Fold change	p value	PseudoCAP Function Class
PA3584	glpD	12.5	0.04	Central intermediary metabolism; Energy metabolism
PA1940		4.5	0.01	Hypothetical, unclassified, unknown
PA5517		4.4	0.00	Hypothetical, unclassified, unknown
PA1243		4.4	0.02	Two-component regulatory systems
	exbD			
PA0694	2	4.2	0.04	Membrane proteins,Transport of small molecules
PA5343		3.7	0.02	Hypothetical, unclassified, unknown
PA2338		3.7	0.03	Transport of small molecules
PA3582	glpK	3.5	0.04	Central intermediary metabolism
PA2358		3.5	0.01	Hypothetical, unclassified, unknown
PA0145		3.3	0.02	Hypothetical, unclassified, unknown
PA1215		3.2	0.02	Hypothetical, unclassified, unknown
	cupB			
PA4081	6	3.1	0.04	Motility & Attachment
PA3444		3.1	0.04	putative enzyme
PA0792	prpD	2.9	0.03	Carbon compound catabolism
PA1286		2.9	0.04	Membrane proteins,Transport of small molecules
PA0103		2.8	0.00	Membrane proteins,Transport of small molecules
PA1380		2.8	0.03	Transcriptional regulators
PA3726		2.7	0.02	Hypothetical, unclassified, unknown
PA2719		2.7	0.05	Hypothetical, unclassified, unknown
PA2334		2.6	0.04	Transcriptional regulators
PA4113		2.6	0.02	Membrane proteins; Transport of small molecules
PA0216		2.5	0.02	Membrane proteins,Transport of small molecules
PA2914		2.4	0.05	Membrane proteins; Transport of small molecules
PA4165		2.4	0.00	Transcriptional regulators
PA1857		2.2	0.02	Membrane proteins
PA1013.1		2.2	0.01	Non-coding RNA gene
PA2871		2.1	0.03	Hypothetical, unclassified, unknown
PA0089	tssG1	2.1	0.01	Protein secretion/export apparatus
				Membrane proteins; Amino acid biosynthesis and metabolism;
PA5170	arcD	2.1	0.03	Transport of small molecules
PA3749		2.1	0.00	Membrane proteins; Transport of small molecules
PA0883		2.0	0.04	Putative enzymes

Table D. List of significantly down-regulated genes of PAO1 (*P. aeruginosa*) in response to 30 min pyocin S5 treatment. p -value ≤ 0.05 and fold change ≥ 2.0

Gene ID	Name	Fold change	p value	PseudoCAP Function Class
PA3426		7.0	0.04	phenylacetate catabolic process
PA0578		6.0	0.04	Hypothetical, unclassified, unknown
				Central intermediary metabolism, translation, post translation modification,
PA4563	rpsT	5.8	0.02	degradation
PA1031		5.2	0.04	Hypothetical, unclassified, unknown
PA1969		5.2	0.01	Hypothetical, unclassified, unknown
PA3821	secD	5.1	0.01	Membrane proteins, protein secretion/export apparatus
PA4268	rpsL	3.5	0.01	Translation, post-translational modification, degradation
PA4690.				
5		3.4	0.03	Non-coding RNA gene
PA3103	xcpR	3.2	0.03	Protein secretion/export apparatus
PA3133.				
2		3.2	0.02	Non-coding RNA gene
PA4661	pagL	3.1	0.00	Hypothetical, unclassified, unknown
PA1942		3.0	0.03	Hypothetical, unclassified, unknown
PA1311	phnX	3.0	0.04	Carbon compound catabolism
PA2735		3.0	0.01	Putative enzymes
PA3117	asd	2.9	0.01	Amino acid biosynthesis and metabolism
PA0981		2.8	0.04	Hypothetical, unclassified, unknown
PA2395	pvdO	2.7	0.04	Adaptation, Protection
PA4439	trpS	2.7	0.03	Amino acid biosynthesis and metabolism
PA3010		2.7	0.05	Hypothetical, unclassified, unknown
	pauD			
PA1742	2	2.6	0.02	Putative enzymes,carbon compound catabolism
PA3406	hasD	2.5	0.01	Transport of small molecules
PA0652	vfr	2.5	0.03	Transcriptional regulators
PA3347		2.4	0.02	Motility & Attachment,Two-component regulatory systems
PA2291		2.4	0.03	Transport of small molecules
PA0758		2.4	0.05	Hypothetical, unclassified, unknown
PA3057		2.3	0.04	Hypothetical, unclassified, unknown
PA1538		2.3	0.04	Putative enzymes
PA2991	sth	2.3	0.01	Nucleotide biosynthesis and metabolism
PA2379		2.3	0.04	Putative enzymes
PA3815	iscR	2.3	0.03	Adaptation, Protection
PA0665		2.2	0.04	Hypothetical, unclassified, unknown,iron-sulfur cluster assembly
PA1158		2.2	0.02	Two-component regulatory systems
PA2081	kynB	2.2	0.01	Amino acid biosynthesis and metabolism,Hypothetical, unclassified

PA5548		2.1	0.02	Membrane proteins,Transport of small molecules
PA5552	glmU	2.1	0.05	Cell wall / LPS / capsule
PA1096		2.1	0.01	Hypothetical, unclassified, unknown
PA0961		2.1	0.03	Transcriptional regulators, response to cold
PA2010		2.0	0.00	Transcriptional regulators
PA1528	zipA	2.0	0.02	Cell division
PA3647		2.0	0.00	Membrane proteins,Transport of small molecules
PA0581		2.0	0.03	Hypothetical, unclassified, unknown, Predicted membrane protein
PA0760		2.0	0.05	Hypothetical, unclassified, unknown

Appendix 3

DNA sequence of pyocin S5 (PA0985)

ATGTCCAATGACAACGAAGTACCTGGTTCCATGGTTATTGTGCGACAAGGTCCAGACGATC
AATACGCATACGAGGTTCCCCCTATCGATAGCGCGGCCGTTGCCGGAATATGTTTGGCGA
CTTAATTCAAAGAGAAATATATCTACAGAAAAACATTTATTATCCAGTCCGATCTATTTTA
ACAAGGAACAAAAGAAAAGAAGGAGATCAACAAGAAAGTATCTGATCAAGTCGATGGCTT
GCTAAAGCAGATCACTCAAGGAAAAAGGGAGGCCACAAGGCAAGAGCGAGTCGATGTCAT
GTCGGCAGTCCTGCACAAGATGGAATCTGATCTTGAAGGATACAAAAAGACCTTTACCAAA
GGCCCATTCATTGACTACGAAAAGCAGTCAAGCCTCTCCATCTATGAGGCCTGGGTCAAGA
TCTGGGAGAAGAAGTCTTGGGAAGAAAGAAAGAAAGTACCCTTTTCAGCAGCTTGTTAGAAT
GAACTGGAGCGGGCGGTTGCCTACTACAAACAAGATTCATCTCTGAAGCGGTAAAAGTGC
TAAGACAGGAGCTCAACAAGCAAAAAGCGCTAAAGGAAAAAGAGGACCTCTCTCAACTGG
AGCGGGACTACAGAACCCGAAAGGCGAATCTCGAGATGAAAGTACAATCCGAGCTTGATC
AAGCGGGAAGTGCTTTGCCTCCATTGGTCAGTCCAACGCCAGAGCAATGGCTTGAACGTGC
CACAAGACTGGTTACGCAAGCAATTGCTGATAAAAAGCAGCTGCAGACCACAAACAATAC
TCTTATCAAGAATTCCCCAACCCCTCTAGAAAAGCAGAAAGCCATCTACAATGGTGAGCTA
CTTGTGGATGAGATAGCCAGTCTACAGGCCCGCTTAGTTAAGCTGAACGCCGAAACGACAC
GACGCAGGACAGAAGCAGAACGCAAGGCGGCCGAGGAACAAGCGTTGCAAGATGCTATTA
AATTTACTGCCGACTTTTATAAGGAAGTAAGTGAAGAAATTTGGCGCACGAACATCGGAGAT
GGCGCGCCAACTGGCCGAAGGCGCCAGGGGGAAAAATATCAGGAGTTCGGCGGAAGCAAT
CAAGTCGTTTGAAAAGCACAAGGATGCGTTAAATAAAAAACTTAGCCTTAAAGATAGGCA
AGCCATTGCCAAAGCCTTTGATTCTCTAGACAAGCAGATGATGGCGAAGAGCCTTGAGAAA
TTTAGCAAAGGCTTTGGAGTTGTAGGCAAAGCTATTGACGCCGCCAGCCTGTACCAAGAGT
TCAAGATATCTACGAAACCGGGGACTGGAAACCATTCCTTTGTAAAAATTGAAACACTAGC
TGCTGGTGCGGCCGCCAGTTGGCTTGTGGGTATTGCATTTGCCACGGCAACAGCCACTCCT
ATAGGCATTCTGGGGTTTCGCACTGGTAATGGCAGTTACCGGGGGCGATGATTGACGAAGACC
TTCTAGAAAAAGCAAACAATCTTGTAAATATCCATTTAA

DNA sequence of pyocin S5 immunity (PA0984)

TTGAGCTTTAAATACTATTGGGCTAAGTTTTTCTGGGGAGCTTTCTTCTTTGTTTTAGTGG
CTTGAAAGGCTCCGTATTTCCCAGCCTGGCATCAGTTAACCCTTGGTAGTGGCTGGGC
TCAGTACTATCCTGTTTCCTTTCTCGGTAAAGCTTGTTGAAGACTTCGCTTTAAATATAC
GGAAAGAGAGTTCTGGGTCACGGGTTTTTTCTCCGAAACCCCTGCCAAAACAGGTTTGT
ATGCAGTCTTTTATTTGCTTGTTATTTGTTTTCAATTCCCTTGGGGATGGTTTTTTTATTC
TATAAGTATGGAAAGGCCTCGTAG

DNA sequence of mvaU (PA2667)

ATGTCCAAACTTGCCGAGTTCCGCGAGGCAGAGCGCAAACTTCAGGAGCAACTGGCCCTGC
TGGAAGAGCTGAAAAGCGACAGCAGCCTGAAGCAGGAACTGGAATTCAAGGACAAGTTGC
AGGCGTTGATGGACAAGTACGGCATGACCCTGCACAACATCATCGCCATCCTCGACCCCAA
GGCTCCGGTCACCGTCAGCGCCGCTCCGAGCGCCGTGCCCGCGCCCTGAAGGTCTACAAG

AACCCGAACAACGGTGAAGTCGTCGAAACCAAGGGCGGCAACCACAAGGTTCTGAAAGCC
TGGAAAGAACAGTACGGTTCGAAACCGTCGAATCCTGGCTGCAACGCTAA

Pyocin S5 amino acid sequence (PA0985)

MSNDNEVPGSMVIVAQGPDDQYAYEVPPIDSAAVAGNMFGDLIQREIYLQKNIYYPVRSIFE
QGTKEKKEINKKVSDQVDGLLKQITQGKREATRQERVDVMSAVLHKMESDLEGYKKTFTK
GPFIDYEKQSSLSIYEAWVKIWEKNSWEERKKYPFQQLVRDELERAVAYYKQDSLSEAVKV
LRQELNKQKALKEKEDLSQLERDYRTRKANLEMKVQSELDQAGSALPPLVSPTPEQWLER
TRLVTQAIADKKQLQTTNNTLIKNSPTPLEKQKAIYNGELLVDEIASLQARLVKLNAETTRRR
TEAERKAAEEQALQDAIKFTADFYKEVTEKFGARTSEMARQLAEGARGKNIRSSAEAIKSFE
KHKDALNKKLSLKDRQAIKAFDSLQMMAKSLEKFSKGFGVVGKAIDAASLYQEFKIST
ETGDWKPFVFIETLAAGAAASWLVGIAFATATATPIGILGFALVMAVTGAMIDEDLLEKAN
NLVISI

Colicin Ia amino acid sequence (ZP_02783400.1)

MSDPVRITNPGAESLGYDSGHEIMAVDIYVNPVRVDVFHGTTPPAWSSFGNKTIWGGNEWV
DDSPTRSDIEKRDKEITAYKNTLSAQKQENENKRTEAAKRLSAAIAAREKDENTLKTLRAGN
ADAADITRQEFRLLQAELEYGFRTEIAGYDALRLHTESRMLFADADSLRISPREARSLIEQA
EKRQKDAQNADKKAADMLAEYERRKGILDTRLSELEKNGGAALAVLDAQQARLLGQQTR
NDRAISEARNKLSSVTESLNTARNALTRAEQQLTQQKNTPDGKTIVSPEKFPGRSSTNHSIVV
SGDPRFAGTIKITSAVIDNRANLNYLLTHSGLDYKRNLNDRNPVVTEDEGDKKIYNAEV
AEWDKLRQRLLDARNKITSAESAVNSARNNLSARTNEQKHANDALNALLKEKENIRNQLA
GINQKIAEEKRKQDELKATKDAINFTEFLKSVSEKYGAKAEQLAREMAGQAKGKKIRNVE
EALKTYEKYRADINKKINAKDRAAIAAALESVKLSDISSNLNRFSGGLGYAGKFTSLADWITE
FGKAVRTENWRPLFVKTEAIIAGNAATALVALVFSILTGSALGIIGYGLLMAVTGALIDESLV
EKANKFWGI

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Ling. H., Saeidi. N, **Rasouliha. B. H.** and Chang. M. W (2010). "A predicted S-type pyocin shows a bactericidal activity against clinical *Pseudomonas aeruginosa* isolates through membrane damage." FEBS letters **584** (15): 3354-3358.

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