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Elucidating The Role of MifS-MifR Two-Component System in Regulating *Pseudomonas aeruginosa* Pathogenicity

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ELUCIDATING THE ROLE OF MIFS-MIFR TWO-COMPONENT SYSTEM IN
REGULATING *PSEUDOMONAS AERUGINOSA* PATHOGENICITY

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Gorakh Digambar Tatke

2016

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Gorakh Digambar Tatke, and entitled Elucidating the Role of MifS-MifR Two-Component System in Regulating *Pseudomonas aeruginosa* Pathogenicity, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2016

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DEDICATION

This work is dedicated to my Family, Teachers and Friends for their constant support, guidance and love.

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ABSTRACT OF THE DISSERTATION

ELUCIDATING THE ROLE OF MIFS-MIFR TWO-COMPONENT SYSTEM IN
REGULATING *PSEUDOMONAS AERUGINOSA* PATHOGENICITY

by

Gorakh Digambar Tatke

Florida International University, 2016

Miami, Florida

Professor Kalai Mathee, Major Professor

Pseudomonas aeruginosa is a Gram-negative, metabolically versatile, opportunistic pathogen that exhibits a multitude of virulence factors, and is extraordinarily resistant to a gamut of clinically significant antibiotics. This ability is in part mediated by two-component systems (TCS) that play a crucial role in regulating virulence mechanisms, metabolism and antibiotic resistance. Our sequence analysis of the *P. aeruginosa* PAO1 genome revealed the presence of two open reading frames, *mifS* and *mifR*, which encodes putative TCS proteins, a histidine sensor kinase MifS and a response regulator MifR, respectively. This two-gene operon was found immediately upstream of the *poxAB* operon, where *poxB* encodes a chromosomal β -lactamase, hinting at the role of MifSR TCS in regulating antibiotic resistance. However, loss of *mifSR* had no effect on the antibiotic resistance profile when compared to *P. aeruginosa* parent PAO1 strain. Subsequently, our phenotypic microarray data (BioLOG) and growth profile studies indicated the inability of *mifSR* mutants to grow in α -ketoglutarate (α -KG), a key

tricarboxylic acid (TCA) cycle intermediate, as a sole carbon source. To date, very little is known about the physiology of *P. aeruginosa* when provided with α -KG as its sole carbon source and the role of MifS and MifR TCS in virulence. Importantly, in the recent years, α -KG has gained notoriety for its newly identified role as a signaling molecule in addition to its conventional role in metabolism. This led us to hypothesize that MifSR TCS is involved in α -KG utilization and virulence in *P. aeruginosa*. Using *mifS*, *mifR* and *mifSR* clean in-frame deletion strains, our study demonstrates that the MifSR TCS modulates the expression *P. aeruginosa* *kgtP* (PA5530) and *pcaT* (PA0229) genes encoding putative α -KG permeases. In addition, our study shows that the MifSR-regulation of these transporters requires functional sigma factor RpoN (σ^{54}). Loss of *mifSR* in the presence of α -KG, resulted in differential regulation of *P. aeruginosa* key virulence determinants including biofilm formation, motility, cell cytotoxicity and the production of pyocyanin and pyoverdine. Involvement of multiple regulators and transporters suggests the presence of an intricate circuitry in the transport of α -KG and its importance in *P. aeruginosa* survival. This is further supported by the α -KG-dependent MifSR regulation of multiple virulence mechanisms. Simultaneous regulation of multiple mechanisms involved in *P. aeruginosa* pathogenesis suggests a complex mechanism of MifSR action. Understanding the physiological cues and regulation would provide a better stratagem to fight often indomitable *P. aeruginosa* infections.

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LIST OF SYMBOLS

SYMBOL	TERM / UNIT
α	Alpha
β	Beta
$^{\circ}\text{C}$	Degrees Celsius
Δ	Delta (deletion)
%	Percent
<i>et al.</i>	et alia
μg	Microgram
μl	Microliter
μm	Micromolar

LIST OF ACRONYMS AND ABBREVIATIONS

ACRONYM/ ABBREVIATION	TERM
A549	Human lung adenocarcinoma epithelial cell line
α -KG	alpha-ketoglutarate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Asp	Aspartate
bp	Base pair
CaCl ₂	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CF	Cystic Fibrosis
CR	Congo red
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
E-test	Epsilometer test
GC	Guanine-cytosine

Glu	Glutamate
Gln	Glutamine
His	Histidine
HK	Histidine kinase
HTH	Helix-turn-helix
J774 E-clone	Murine macrophage cell line
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertini
MFS	Major facilitator superfamily
MgSO ₄	Magnesium sulfate
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
MIC	Minimum inhibitory concentration
Min	Minutes
NaCl	Sodium chloride

Na ₂ HPO ₄	Sodium phosphate dibasic
NH ₄ Cl	Ammonium chloride
NADH	Nicotiamide adenine dinucleotide
OD	Optical Density
OM	Outer membrane
ORF	Open reading frame
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PCR	Polymerase chain reaction
QS	Quorum Sensing
RT-qPCR	Real time quantitative polymerase chain reaction
RR	Response regulator
RNA	Ribonucleic acid
T3SS	Type III secretion system
TCS	Two-component System
μg	Microgram
μl	Microliter
μm	Micromolar

CHAPTER 1

Introduction

Parts of this chapter have been published:

Balasubramanian D., S.K. Murugapiran, E. Silva-Herzog, L. Schneper, X. Yang,

G. Tatke, G. Narasimhan, and K. Mathee. 2013.

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Introduction

“Adaptation is the key to survival”, this is particularly true with bacterial cells as they are often exposed to an array of physiological and environmental changes during their lifecycle. The evolutionary success of bacteria is a testament to its intrinsic capability that helps them to keep pace with the frequently changing environmental conditions, by means of genetic and phenotypic changes. Although they are considered to be the simplest form of life, bacteria have evolved sophisticated molecular machinery that orchestrates multiple functions efficiently. Most bacteria, if not all, live in an extreme and/or dynamic environment, where an adaptive behavior mediated by quick response to ecological variations is a key to existence and proliferation. Bacterial cells achieved this by utilizing numerous signal transduction, information processing systems that incessantly monitor their surroundings for important changes in order to elicit an adaptive response.

Adaptation to environmental stimuli in bacteria is arbitrated predominantly through the expression of transcriptional regulators that include serine-threonine protein kinases (Kalantari *et al.*, 2015), extra-cytoplasmic function (ECF) sigma factors (Mascher, 2013), and two-component signal transduction systems (TCSs) (Stock *et al.*, 2000). These regulators function as cellular interpreters where they directly or with the help of other sensory units, first recognize a specific environmental stimuli at the cell surface and then transmit that extracellular information to the cytoplasm with the help of efficient signaling cascade (mostly phosphorylation and de-phosphorylation reactions) in order to stimulate an adaptive response, mainly by regulating expression of the target genes (Parkinson,

1993, Goudreau & Stock, 1998, Cashin *et al.*, 2006). Amongst these signaling devices, TCS is the predominant signaling mechanism that empowers bacteria with an ability to efficiently sense, respond, and adapt to a wide variety of environments, stressors, and growth conditions (Stock *et al.*, 2000).

Two Component Regulatory Systems

Two-component systems (TCS) are sophisticated signaling mechanisms marked by a highly modular design that have been adapted and integrated into a wide variety of cellular signaling circuits. They are one of the primary means by which bacteria sense and respond to variations in their environments, both intra- and extracellular. The archetypical TCS is composed of a membrane integrated sensory histidine kinase (HK) and a cytoplasmic response regulator (RR) (Figure 1.1). The HK contains a periplasmic N-terminal domain that detects specific stimuli (sensing domain) and a C-terminal cytoplasmic transmitter domain that is comprised of a dimerization domain, a conserved histidine, and an ATP catalytic domain (Raghavan & Groisman, 2010). HKs can have two or more transmembrane domains with little or no periplasmic domain while others are completely cytoplasmic. The cognate response regulator (RR) contains a conserved receiver domain and a variable output domain (Stock *et al.*, 2000). Upon receiving a signal, two HK monomers dimerize and cross-phosphorylate at the conserved histidine residue, the phosphate subsequently being transferred to an aspartate residue in the receiver domain of the cognate RR. The phosphotransfer is catalyzed by the receiver domain and results in a conformational change that activates the output domain, which often binds DNA and modulates gene expression or enzymatic

activity (Stock *et al.*, 2000, Mascher *et al.*, 2006, Gooderham & Hancock, 2009). Variations to this model occur in phosphorelays where a sensor kinase first transfers the phosphoryl group to a RR that has no output domain. The P~RR then transfers the phosphoryl group to a histidine containing phosphotransfer protein (HPT), and this in turn serves as a phosphate donor to a terminal RR which has an output domain that mediates a cellular response. In other cases the sensor kinase and the RR lacking an output domain are fused into one protein (hybrid sensor kinase) (Gao & Stock, 2010). Other variations include the TCS connectors, a group of proteins that modulate the phosphorylation state and activity of sensor HK and RR and establish regulatory links between otherwise independent signal transduction pathways (Mitrophanov & Groisman, 2008).

In general, the mechanism of signal perception mediated by the archetypic bacterial two-component system in order to elicit an adaptive response can be summarized in four steps:

- 1) Signal detection by HK
- 2) ATP-dependent auto phosphorylation of the HK sensor at its C-terminal histidine residue (HK activation)
- 3) Transfer of phosphoryl group to the aspartate residue of the response regulator in the receiver domain (phosphotransfer and RR activation)
- 4) Regulation of target genes by the activated RR (response generation)

TCSs enable bacterial to effectively control their physiological and cellular functions by responding appropriately to a wide range of stimuli such as osmolarity, nutrient availability, temperature, pH and oxidative stress (Albright *et al.*, 1989).

Figure 1.1: Schematic representation of a prototypic two-component system mediated signal transduction. TCS activation is arbitrated through a series of phosphotransfer reactions which typically involves phosphorylation and de-phosphorylation of the sensor histidine kinase (HK) and response regulator (RR). A specific environmental stimulus is detected by the HK through its sensor domain which brings about a conformational change resulting in two HK monomers to dimerize and auto cross-phosphorylate (ATP-dependent) at the conserved histidine residue located at its C-terminal domain. The phosphorylated HK then serves as a phosphate donor for the RR receiver domain, resulting in phosphorylation of a conserved Aspartate residue. The phosphorylation of the aspartate residue subsequently results in the activation of the RR effector domain that mediates the cellular response, usually by differential expression of specific target genes.

***P. aeruginosa* and two-component systems**

Pseudomonas aeruginosa is a ubiquitous, Gram negative bacteria that exhibits extraordinary ability to colonize diverse ecological niches. Its metabolic versatility and adaptability has significantly contributed to its ubiquity (Ramos, 2004). Furthermore, *P. aeruginosa* causes opportunistic infection in humans, and is the primary pathogen responsible for pulmonary exacerbations and mortality in patients with cystic fibrosis (Lyczak *et al.*, 2002). Importantly, it is one of the most recalcitrant nosocomial pathogen, responsible for 10% of all the hospital acquired infections (Sievert *et al.*, 2013). Beyond high infection rate, *P. aeruginosa* has one of the highest case fatality rate amongst all Gram-negative infections (Aliaga *et al.*, 2002). *P. aeruginosa* expresses an arsenal of multi-determinant virulence factors, both cell surface associated (flagella, pili, LPS) and secretory (proteases, exotoxins, phenazines, haemolysins), that play a crucial role in regulating its pathogenicity (Balasubramanian *et al.*, 2013). In addition, a major impediment in treating *P. aeruginosa* infections is its extraordinary intrinsic and acquired resistance to several clinically critical antibiotics (Fraimow & Nahra, 2013). These characteristics together ensures the success of *P. aeruginosa* as an intractable pathogen.

Furthermore, successful infection and disease progression depends significantly on the ability of bacterial pathogens, including *P. aeruginosa*, to adapt efficiently to the host environment. *P. aeruginosa* has evolved complex signal transduction mechanisms that aid in its effective adaptation by regulating and coordinating the temporal expression of various genes, in response to both inter-

and intracellular signaling molecules and environmental cues (Hancock & Speert, 2000, Rodrigue *et al.*, 2000, Ventre *et al.*, 2006, Gooderham & Hancock, 2009, Williams & Camara, 2009, Haussler, 2010, Jimenez *et al.*, 2012). Amongst the gamut of signaling mechanisms identified in *P. aeruginosa*, TCS mediated signal perception play a predominant role in sensing diverse environmental stimuli and eliciting a pertinent and rapid adaptive response (Rodrigue *et al.*, 2000, Stock *et al.*, 2000).

P. aeruginosa, genome encodes one of the largest group of TCS proteins identified in any in any microorganism thus far analyzed (Barakat *et al.*, 2009, Winsor *et al.*, 2011). To date, 62 HK and 73 RR TCS proteins have been identified in *P. aeruginosa* prototypic strain PAO1 (Winsor *et al.*, 2011). This provides the bacterium with a sophisticated capability to regulate diverse metabolic adaptations, virulence and antibiotic resistance processes that are hallmark of *P. aeruginosa* infections. What follows is a brief description of some prominent ones.

PhoQ-PhoP

PhoQ-PhoP is one of the most extensively studied TCS in the γ -proteobacterial species (Miller *et al.*, 1989, Gooderham & Hancock, 2009).). It is composed of the inner membrane histidine sensor kinase (HK) PhoQ and the cytoplasmic response regulator (RR) PhoP (McPhee *et al.*, 2006). This TCS was first identified in *Salmonella enterica* where it was reported to be involved in controlling the expression of non-specific acid phosphatase (Kier *et al.*, 1979). Subsequently, a more profound role of the PhoPQ TCS was identified where it was

involve in regulating several cellular activities including virulence mechanisms, cell cytotoxicity, motility, transport of small molecules, acid tolerance, antibacterial peptide resistance and bacterial surface remodeling (Groisman, 2001, Prost *et al.*, 2008). The PhoQ-PhoP system has been identified as a major component of virulence in other Gram-negative bacteria including *P. aeruginosa*, *Escherichia coli* and *S. enterica* (Gooderham & Hancock, 2009). The PhoPQ TCS is one of the first reported sensory-response mechanism to use extracellular cations as a primary signal (Gooderham & Hancock, 2009).

In *P. aeruginosa*, PhoQ-PhoP TCS has been reported to modulate cellular adaptations to Mg^{2+} -limiting environments (McPhee *et al.*, 2006). It is now well established that sub-micromolar concentrations of Mg^{2+} induce *P. aeruginosa* PhoP-mediated transcription, and millimolar concentrations of Mg^{2+} are known to repress their expression (Gooderham *et al.*, 2009). Furthermore, it is well established that the PhoPQ TCS respond to limiting concentrations of cations and regulate resistance to polymyxin B and cationic antimicrobial peptides through the regulation of the *arnBCDTEF-pmrE* LPS modification operon (Macfarlane *et al.*, 2000, MCPhee *et al.*, 2006) Activation of the genes in the *arnBCADTEF* operon results in the addition of 4-aminoarabinose to the Lipid A moiety of the lipopolysaccharide (LPS). The amalgamation of 4-aminoarabinose and Lipid A is responsible for inducing cytotoxicity in Gram-negative bacteria, because of a reduction of the net negative charge of the LPS. This limits LPS interaction with cationic peptides and cationic antibacterial agents such as polymyxin B (McPhee *et al.*, 2003, Moskowitz *et al.*, 2004). PhoQ is also involved in swarming and

twitching motility as well as in biofilm formation and it is required for virulence without affecting the T3SS or QS systems (Gooderham *et al.*, 2009). It differs from the *Salmonella* PhoQ in that it is also involved in the regulation of transcription of genes outside the PhoP regulon, including *pmrA*, *algU-mucABCD* alginate production operon, Type II secretion proteins HxcQ and XqhA, the quinolone signal biosynthetic genes (*pqsBCD*), iron-scavenging-related genes and siderophore genes as well as energy-metabolism-related genes including *nirO*, *nosR* and cytochrome *o* ubiquinol oxidase operon (Gooderham *et al.*, 2009). Interestingly, *P. aeruginosa* isolates from cystic fibrosis patients have been shown to contain a lipid A structure equivalent to that of those formed by the activation of PhoPQ, and to generate an inflammatory response, suggesting a role of this TCS in eliciting an immune response. Furthermore, HK-PhoQ has been reported to crosstalk with other TCS regulatory proteins (PmrA/PmrB) to modulate several cellular responses (McPhee *et al.*, 2003). MCPhee and coworkers have shown that HK-PhoQ is able to activate the RR-PmrA independently of HK-PmrB suggesting an interaction between these TCSs (McPhee *et al.*, 2003). In addition, increased resistance to antibiotics including polymyxin B, aminoglycosides and quinolones in *phoQ* mutants suggest crosstalk between PhoPQ and other TCS (Kwon & Lu, 2006)

GacS-GacA

The sensor HK-GacS (PA0928) and the RR-GacA (PA2586) are members of a TCS that is present in a wide variety of Gram-negative bacteria (Heeb & Haas, 2001). In *P. aeruginosa*, the GacSA TCS is central to the regulation of the

expression of virulence factors, secondary metabolites, biofilm formation and QS (Kitten *et al.*, 1998, Pessi *et al.*, 2001) and to the switch between acute and chronic infections, as seen in mouse models of cystic fibrosis infection (Rahme *et al.*, 2000, Coleman *et al.*, 2003). GacS is a hybrid sensor HK that contains a HK domain, a RR domain and a histidine phosphotransfer (Hpt) domain (Goodman *et al.*, 2004, Gooderham & Hancock, 2009). The signal transduction pathway from GacA to individual virulence genes is complex. Phosphorylated GacA positively regulates the transcription of two small RNAs, *rsmZ* and *rsmY*, which block the negative regulator RNA-binding protein RsmA, allowing the expression of QS signals, swarming motility, rhamnolipid and lipid biosynthesis (Heeb & Haas, 2001, Wolfgang *et al.*, 2003, Brencic & Lory, 2009). The GacSA TCS is also involved in antibiotic resistance to three different antibiotics: tobramycin, ciprofloxacin and tetracycline (Linares *et al.*, 2006) apparently mediated also by RsmA/RsmZ. The mechanisms of resistance are poorly understood, and susceptibility to other antibiotics or antimicrobial peptides remains unaffected.

RetS and LadS

P. aeruginosa utilizes two orphan sensor kinases, RetS (regulator of exopolysaccharide and T3SS; PA4856) and LadS (lost adherence sensor, PA3974), to switch between acute and chronic infections and to regulate virulence mainly through the interaction with GacA-GacS (Goodman *et al.*, 2004, Yahr & Greenberg, 2004). RetS contains one HK domain and two tandem RR receiver domain (Goodman *et al.*, 2004). LadS domain organization is similar to that of RetS, but it does not contain the second C-terminal RR domain (Ventre *et al.*,

2006). LadS regulates both early and late maturation stages of biofilm formation. LadS and RetS are hybrid sensor kinases with a signaling domain consisting of a 7-transmembrane region and a periplasmic sensor domain that mediates homo- and hetero-dimer formation (Goodman *et al.*, 2004). RetS is required for T3SS activation, repression of biofilm formation, and colonization/dissemination in murine acute infection models (Goodman *et al.*, 2004, Laskowski *et al.*, 2004, Zolfaghar *et al.*, 2005). Genes under RetS control are inversely regulated by two other sensor kinases: GacS and LadS, probably through the regulation of the small RNAs RsmZ and RsmA, as suggested by the demonstration that RetS can directly interact with GacS modulating the levels of RsmZ and RsmA (Ventre *et al.*, 2006, Gooderham & Hancock, 2009). LadS regulates biofilm formation probably through the regulation of the *pel* exopolysaccharide operon (Ventre *et al.*, 2006). In a rat model with chronic pulmonary infection, LadS and RetS have been reported to regulate the expression of HcpI secretion island I (required for chronic infection), thus are also thought to have an important role in chronic infection in CF by actively regulating HcpI secretion island I (Potvin *et al.*, 2003). LadS together with RetS and GacA/GacS/RsmZ forms a intricate and dynamic network that directs the expression of *P. aeruginosa* key virulence mechanisms resulting in acute infection (expression of Type III secretion) and chronic infections (biofilm formation) (Goodman *et al.*, 2004, Yahr & Wolfgang, 2006, Gooderham & Hancock, 2009). However, the exact regulatory mechanism as to how LadS and RetS sensors bring about the cascade of signal transduction

by modulating the expression of *rsmZ* and *rsmY* transcription is not known, but it is possible that other response regulators may be involved (Laskowski & Kazmierczak, 2006).

NarL-NarX

P. aeruginosa is often known to assume a biofilm mode of growth that is marked by a substantial metabolic alterations as a consequence of its genetic and phenotypic variations (Waite *et al.*, 2006, Schreiber *et al.*, 2007). The ability of *P. aeruginosa* to grow as biofilms can be largely attributed to its metabolic flexibility and adaptability that enables it to grow in a low nutrient and oxygen-limited environment, a characteristic of biofilms. In particular, anaerobic respiration by *P. aeruginosa* is considered to be a vital metabolic process during its growth as a biofilm in chronic pulmonary infections (Barraud *et al.*, 2006, Palmer *et al.*, 2007). Under anaerobic conditions, *P. aeruginosa* survives and supports its growth either by arginine fermentation or by denitrification (Palmer *et al.*, 2005, Palmer *et al.*, 2007). NarX and NarL TCS pair in *P. aeruginosa* is known to be involved in regulating *P. aeruginosa* denitrification pathways (Schreiber *et al.*, 2007). Denitrification pathways play an important role in anaerobic respiration, where, in the absence of oxygen organism respire nitrate (NO_3^-) or nitrite (NO_2^-). In *P. aeruginosa*, the Anr (PA1544), Dnr (PA0527) regulatory proteins in concert with NarL-NarX TCS control the expression of genes encoding proteins required for denitrification (Benkert *et al.*, 2008). The NarX is a HK which contains a highly conserved nitrate recognition region. This region is termed as a P-box which recognizes environmental presence of nitrate to which NarX responds by

autophosphorylating a conserved histidine residue (Stewart & Bledsoe, 2008). Further, the transfer of high energy phosphate to the conserved aspartate residue of the response regulator NarL results in activation or repression of target operon (Stewart & Bledsoe, 2008). Nitrate metabolism has been linked to motility, biofilm formation and virulence. NarX-HK is reported to be required for swarming motility, although the swarming phenotype has been displayed in a *narL* mutant as a result of overproduction of rhamnolipids (Van Alst *et al.*, 2007). Also, *narLX* double mutants displayed reduced swimming motility in comparison to individual *narL* and *narX* mutants which exhibited normal motility, suggesting that the flagella dependent movement requires both the sensor kinase and the response regulator (Van Alst *et al.*, 2007). Thus, the NarX–NarL TCS is involved in regulation several *P. aeruginosa* virulence related phenotypes including motility and biofilm formation.

Conclusion:

The versatility of two-component regulatory systems in sensing different signals, both extracellular and intracellular, make them an appropriate system for regulating expression of virulence and metabolism. *P. aeruginosa* genome encodes one of the largest groups of TCSs proteins (135), in sequenced bacteria (Winsor *et al.*, 2011). Amongst these only few have been extensively studied and characterized in detail, including PhoP-PhoQ, GacA-GacS, RetS, LadS, and NarL-NarX, that are reported to be responsible for regulating *P. aeruginosa* key virulence determinants. Furthermore, in most of the cases, the environmental signals that influence TCS pathways remains obscure. However, there are a

plethora of uncharacterized putative TCSs in *P.aeruginosa*. Taking into consideration the metabolic adaptability and the plethora of uncharacterized putative TCSs in *P.aeruginosa*, it is most likely that some of these putative systems may have important roles in virulence, antibiotic resistance and metabolism. Further research is required to characterize these role of these putative TCS by elucidate there activating signals, identifying their targets and also various regulatory networks into which TCS are integrated, since these systems can be appropriate drug targets for effective antimicrobial therapeutics.

CHAPTER 2

***Pseudomonas aeruginosa* MifS-MifR two-component system is specific for
 α -ketoglutarate utilization**

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Abstract

Pseudomonas aeruginosa is a Gram-negative, metabolically versatile opportunistic pathogen that elaborates multitude of virulence factors, and is extraordinarily resistance to a gamut of clinically significant antibiotics. This ability, in part, is mediated by two-component regulatory systems (TCS) that play a crucial role in modulating virulence mechanisms and metabolism. MifS (PA5512) and MifR (PA5511) form one such TCS implicated in biofilm formation. MifS is a sensor kinase whereas MifR belongs to the NtrC superfamily of transcriptional regulators that interact with RpoN (σ^{54}). In this study we demonstrate that the *mifS* and *mifR* genes form a two-gene operon. The close proximity of *mifSR* operon to *poxB* (PA5514) encoding a β -lactamase hinted at the role of MifSR TCS in regulating antibiotic resistance. To better understand this TCS, clean in-frame deletions were made in *P. aeruginosa* PAO1 creating $\text{PAO}\Delta\text{mifS}$, $\text{PAO}\Delta\text{mifR}$ and $\text{PAO}\Delta\text{mifSR}$. The loss of *mifSR* had no effect on the antibiotic resistance profile. Phenotypic microarray (BioLOG) analyses of $\text{PAO}\Delta\text{mifS}$ and $\text{PAO}\Delta\text{mifR}$ revealed that these mutants were unable to utilize C₅-dicarboxylate α -ketoglutarate (α -KG), a key tricarboxylic acid cycle intermediate. This finding was confirmed using growth analyses, and the defect can be rescued by *mifR* or *mifSR* expressed in *trans*. These *mifSR* mutants were able to utilize all the other TCA cycle intermediates (citrate, succinate, fumarate, oxaloacetate or malate) and sugars (glucose or sucrose) except α -KG as the sole carbon source. We confirmed that the *mifSR* mutants have functional dehydrogenase complex suggesting a possible defect in α -KG transport. The inability of the mutants to utilize α -KG was rescued by

expressing *PA5530*, encoding C₅-dicarboxylate transporter, under a regulatable promoter. In addition, we demonstrate that besides MifSR and *PA5530*, α -KG utilization requires functional RpoN. These data clearly suggests that *P. aeruginosa* MifSR TCS is involved in sensing α -KG and regulating its transport and subsequent metabolism.

Introduction

Pseudomonas aeruginosa is a metabolically versatile, Gram-negative opportunistic pathogen that is well known for its extensive spatio-temporal distribution (Ramos, 2004). It is a dominant nosocomial pathogen capable of causing acute and chronic infections in immunocompromised and immunosuppressed patients (Ledizet *et al.*, 2012, Turner *et al.*, 2014). In particular, patients with AIDS, severe burn wounds, cystic fibrosis (Brencic *et al.*), chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis and neutropenia are predisposed to *P. aeruginosa* infections (Lyczak *et al.*, 2000, Manfredi *et al.*, 2000, Ramos, 2004, Furukawa *et al.*, 2006, Valderrey *et al.*, 2010). *P. aeruginosa* chronic pulmonary infections are characterized by intensive bronchial neutrophilic inflammation resulting in respiratory failure (Emerson *et al.*, 2002, Cohen-Cymberknoh *et al.*, 2013), a major cause of fatality in CF patients (Lyczak *et al.*, 2002). Moreover, *P. aeruginosa* is associated with keratitis (Dart & Seal, 1988) and chronic suppurative otitis media (Yeo *et al.*, 2007) leading to visual impairment and deafness (Prevatt *et al.*, 2004, Sun *et al.*, 2010). *P. aeruginosa* possess numerous virulence factors, both cell-surface associated and secretory, which significantly contribute to its pathogenesis (Balasubramanian *et al.*, 2013). Effective treatment of *P. aeruginosa* infections is impeded by its extraordinary intrinsic and acquired resistance to numerous clinically important antibiotics (Fraimow & Nahra, 2013). Thus, antibiotic resistance and expression of multi-determinant virulence factors are two critical hallmarks in *P. aeruginosa* infections that make it an intimidating pathogen.

Successful infection and disease progression depends significantly on the ability of any pathogen to effectively utilize available nutrients that are essential for its growth and survival. *P. aeruginosa* is renowned for its extraordinary ability to utilize wide range of organic compounds such as carbohydrates, amino acids, fatty acids, mono- and polyalcohols, di- and tri-carboxylic acids as sources of carbon, nitrogen and energy (Ramos, 2004). However, unlike other bacteria where glucose is the preferred carbon source (Loomis & Magasanik, 1967, Stulke & Hillen, 2000), *P. aeruginosa* preferentially utilizes tricarboxylic acid (TCA) cycle intermediates (Wolff *et al.*, 1991, Collier *et al.*, 1996), specifically, C₄-dicarboxylates of the TCA cycle such as malate, fumarate and succinate (Liu, 1952, Wolff *et al.*, 1991, Collier *et al.*, 1996).

The TCA cycle is an amphibolic pathway that serves two main purposes: energy-generation in aerobic organisms (catabolism), and the generation of intermediates to serve as biosynthetic precursors for fatty acid, amino acid and carbohydrate synthesis (anabolism) (Owen *et al.*, 2002). The metabolic intermediates of the TCA cycle consist of a group of organic anions that include C₄-dicarboxylates (succinate, fumarate, malate and oxaloacetate), C₅-dicarboxylates (alpha-ketoglutarate (α -KG)) and C₆-tricarboxylates (citrate, isocitrate) (Krebs, 1940, Frohman *et al.*, 1951). However, the role of TCA cycle intermediates is not restricted to energy metabolism or to serve as biosynthetic precursors. In the recent years, TCA cycle intermediates, in-particular, succinate and/or α -KG have gained significant importance as biological signaling molecules

in variety of organisms including, bacteria (Ninfa & Jiang, 2005), animals (He *et al.*, 2004) and plants (Feria Bourrellier *et al.*, 2010).

Sensing the available nutrients is a prerequisite for mobilizing the uptake systems. Bacterial two-component systems (TCSs), involving a membrane-bound histidine sensor kinase (HK) and a cytoplasmic response regulator (RR) play an integral part in bacteria's ability to sense physiological cues. In response to stimuli, the sensor autophosphorylates at a conserved histidine residue at the C-terminus, and subsequently the phosphate is transferred to an aspartate residue at the N-terminus of the RR (Stock *et al.*, 1989, Hoch & Silhavy, 1995, Asai *et al.*, 2000). TCSs in *Bacillus subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Klebsiella pneumoniae*, *Rhizobium meliloti* and *Rhizobium leguminosarum* have been shown to regulate extracellular C₄-dicarboxylates and tricarboxylates transport (Jiang *et al.*, 1989, Bott *et al.*, 1995, Reid & Poole, 1998, Golby *et al.*, 1999, Asai *et al.*, 2000, Yamamoto *et al.*, 2000, Brocker *et al.*, 2009). Of these, DctB-DctD in *R. meliloti* is an extensively studied TCS, which in coordination with sigma factor RpoN(σ^{54}) regulates the extracellular transport of C₄-dicarboxylates succinate, fumarate and malate (Ronson *et al.*, 1987, Watson, 1990).

Three TCS protein pairs in *P. aeruginosa* namely, PA5165/PA5166 (DctB/DctD), PA5512/PA5511 (MifS/MifR) and PA1336/PA1335 have been identified to be homologous to the *Rhizobium* C₄-dicarboxylate transport regulatory DctB/DctD (Valentini *et al.*, 2011). Amongst the three, very little is known of PA1336/PA1335. The PA5165/PA5166 (DctB/DctD) TCS has been demonstrated

to regulate the transport of C₄-dicarboxylates, succinate, fumarate and malate in coordination with the sigma factor RpoN (σ^{54}) (Valentini *et al.*, 2011). The SK MifS (65.3 kDa) and RR MifR (49.6 kDa) share 51% and 69% sequence identity to the *R. meliloti* DctB and DctD, respectively (Stover *et al.*, 2000). The RR MifR is involved in regulating the maturation stage of *P. aeruginosa* biofilm formation as *mifR* deficient mutants fail to form microcolonies (Petrova & Sauer, 2009). Later studies reported the interdependence of pyruvate fermentation and functional MifR in supporting microcolony formation (Petrova *et al.*, 2012). However, the mechanism by which MifR is activated in this process remains obscure and no relation with HK MifS has been established. Using clean in-frame deletion mutants of the *mifS*, *mifR* and *mifSR* genes we show that MifSR TCS regulates *P. aeruginosa* α -KG transport and requires functional RpoN.

Materials & Methods

Strains, media and growth conditions

P. aeruginosa wild-type PAO1 (Stover *et al.*, 2000) and its derivatives PAO Δ *mifS*, PAO Δ *mifR*, PAO Δ *mifSR* and PAO Δ *rpoN* or *Escherichia coli* strain DH5 α were used in this study (Table 2.1). *Saccharomyces cerevisiae* strain InvSC1 (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used for *in vivo* homologous recombination (Bascom-Slack & Dawson, 1998). Briefly, all bacterial cultures were grown in Luria Bertani (LB) broth (5 g tryptone, 10 g sodium chloride, and 5 g yeast extract per liter) or agar (LB broth with 1.5% agar) (Difco, NJ, USA) or M9 minimal Media (64 g Na₂HPO₄-7H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5.0 g NH₄Cl, 20 mM MgSO₄, 1 mM CaCl₂ per liter) (Sambrook & Russell, 2001) at 37°C, unless

specified otherwise. Yeast extract-peptone-dextrose media (YEPD: 20 g Bacto Peptone, 10 g yeast extract, 20 g dextrose per liter) was routinely used to culture *S. cerevisiae* and synthetic define agar-uracil media was used as selection media for pMQ30 yeast transformants (Shanks *et al.*, 2006). *P. aeruginosa* competent cells were prepared as previously described (Choi *et al.*, 2006). For growth curve and complementation studies M9 minimal media supplemented with glucose, sucrose or TCA cycle intermediates including citrate, α -KG, succinate, fumarate, malate or oxaloacetate were used as a sole carbon source at 30 mM each unless specified otherwise. Motility assays were performed in LB media (Difco, NJ, USA). For pyocyanin and proverdine production strains were cultivated in King's A medium (Difco, NJ, USA) and King's B medium (King *et al.*, 1954). Cation-adjusted Mueller Hinton broth and agar (Difco, NJ, USA) was used in MIC assays. For plasmid maintenance, antibiotics were added to growth media when appropriate, at the specified concentrations: *E. coli*: ampicillin (Ap) 100 μ g/ml, gentamycin (Siegmond & Wagner) 15 μ g/ml, kanamycin (Km) 20 μ g/ml, *P. aeruginosa*: Gm 75 μ g/ml.

Genetic manipulations

Genetic manipulations were carried out using standard techniques (Sambrook & Russell, 2001). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) and are listed in Table 2.2. Plasmid DNA isolation was carried out using PureLink Hipure Plasmid Miniprep Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and agarose gel fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

RNA and cDNA was made using RNeasy Mini Kit (Qiagen Inc. Venio, Limburg, Netherlands) and SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA). Restriction endonucleases were from New England Biolabs (Ipswich, MA, USA) and DNA sequencing was carried out at Florida International University (FIU) DNA core and at GENEWIZ Inc (South Plainfield, NJ, USA). All other chemicals were purchased from SIGMA-ALDRICH (St. Louis, MO, USA), AMRESCO (Solon, OH, USA) and Fisher Scientific (Waltham, MA, USA).

Table 2.1: Strains and plasmids used in this study.

Strain ID	Strain/Plasmid Background	Relevant characteristics	Source
<i>Escherichia coli</i>			
DH5α	<i>E. coli</i>	F ⁻ Φ80/ <i>lacZ</i> ΔM15Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ mk ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	New England Biolabs
<i>Saccharomyces cerevisiae</i>			
INVSc1	<i>S. cerevisiae</i>	MATa <i>his3D1 leu2 trp1-289 ura3-52</i>	Invitrogen
<i>Pseudomonas aeruginosa</i>			
PAO1		Prototypic wild type	(Stover <i>et al.</i> , 2000)
PKM900	PAO1	Δ <i>mifS</i> (PA5512)	PAOΔ <i>mifS</i> ; This study
PKM901	PAO1	Δ <i>mifR</i> (PA5511)	PAOΔ <i>mifR</i> ; This study
PKM902	PAO1	Δ <i>mifSR</i> (PA5511-PA5512)	PAOΔ <i>mifSR</i> ; This study
PAOΔ <i>rpoN</i>	PAO1	Δ <i>rpoN</i> (PA4462)	(Heurlier <i>et al.</i> , 2003)
PAO1Δ <i>rpoN</i> :: <i>rpoN</i>	PAO1	Δ <i>rpoN att</i> Tn7:: <i>rpoN</i> _ <i>aacC1</i>	(Heurlier <i>et al.</i> , 2003)

Plasmids			
pCR2.1 TOPO		Ap ^R , Km ^R ; <i>colE1</i> f1 <i>ori lacZα</i>	Invitrogen
pRK600		Cm ^R ; <i>colE1 tra</i> ⁺ RK2 <i>mob</i> ⁺	(Kessler <i>et al.</i> , 1992)
pRK2013		Km ^R ; <i>colE1 tra</i> ⁺ RK2 <i>mob</i> ⁺	(Figurski & Helinski, 1979)
pEXG2		Gm ^R ; <i>colE1, oriT mob</i> ⁺ <i>sacB</i> ⁺	(Rietsch <i>et al.</i> , 2005)
pMQ30		Gm ^R ; <i>colE1, oriT</i>	(Shanks <i>et al.</i> , 2006)
pPSV37		Gm ^R ; <i>colE1 oriT lacI^q</i> P _{<i>lacUV5</i>}	(Lee <i>et al.</i> , 2010)
pGDT001	pCR2.1 TOPO	Ap ^R ; A ~1.7-kb <i>NheI-XbaI</i> fragment containing <i>mifS</i> ORF (<i>PA5512</i>) amplified from PAO1 genome using HK_ <i>mifSF</i> 1 and HK_ <i>mifSR</i> 1 primers and cloned into pCR 2.1 TOPO	This study
pGDT002	pCR2.1 TOPO	Ap ^R ; A ~1.3-kb <i>NheI-SacI</i> fragment containing <i>mifR</i> (<i>PA5511</i>) ORF amplified from PAO1 genome using GDT_ <i>mifRF</i> 1 and GDT_ <i>mifRR</i> 1 primers and cloned into pCR 2.1 TOPO	This study
pGDT003	pPSV37	Gm ^R ; The <i>mifS</i> ORF subcloned from pGDT001 as an <i>NheI-XbaI</i> fragment into pPSV37	pMifS This study
pGDT004	pPSV37	Gm ^R ; The <i>mifR</i> ORF subcloned from pGDT002 as an <i>NheI-SacI</i> fragment into pPSV37	pMifR This study
pGDT005	pPSV37	Gm ^R ; A ~3.0-kb <i>NheI-SacI</i> fragment containing <i>mifSR</i> (<i>PA5511-PA5512</i>) ORFs amplified from PAO1 genome using HK_ <i>mifSF</i> 1 and GDT_ <i>mifRR</i> 1 primers and cloned directly into <i>NheI-SacI</i> -cut in pPSV37	pMifSR This study
pGDT006	pPSV37	Gm ^R ; A ~1.3-kb <i>NheI-SacI</i> fragment containing	pPA5530 This study

PA5530 ORF amplified
from PAO1 genome using
GDT_*PA5530F1* and
GDT_*PA5530R1* primers
and cloned directly into
NheI-*SacI*-cut in pPSV37

Table 2.2. Primers used in this study.

Primer Name	Sequence
HK <i>mif</i> SUF	5'- GGAATTGTGAGCGGATAACAATTTACACAGGAA ACAGCTTCAGCTCGACTCCGCCGTCG-3'
HK <i>mif</i> SUR	5'- GACGAAGATCACCTGGTCGCCTAGTTAGCTAGCA TCGGCGGATCGAAACGGC-3'
HK <i>mif</i> SDF	5'- GCCGTTTCGATCCGCCGATGCTAGCTAACTAGGC GACCAGGTGATCTTCGTC-3'
HK <i>mif</i> SDR	5'- CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCG TTCTGATACCGCTCTCATGACCGAA-3'
<i>mif</i> RUF1	5'-TTTGAATTTCGCCTGGTCGAGCAGCGCA-3'
<i>mif</i> RUR1	5'-TTTGCTAGCTCGCTCATGTCTG-3'
<i>mif</i> RDF1	5'-TTTAAGCTTCTCGGCTTCGACGCCCAT
<i>mif</i> RDR1	5'-TTTGCTAGCTCGCGAGGCGTC-3'
<i>mif</i> SRUF1	5'- GGAATTGTGAGCGGATAACAATTTACACAGGAA ACAGCTGCGAGCACCAGCGCGCCACT-3'
<i>mif</i> SRUR1	5'- TCTCTGACGCCTCGCGAGGGCTGCTCTAGTTAGC TAGCATCGGCGGATCGAAACGGCGGC-3'
<i>mif</i> SRDF1	5'- GCCGTTTCGATCCGCCGATGCTAGCTAACTAGAG CAGCCCTCGCGAGGCGTCAGAGA-3'
<i>mif</i> SRDR1	5'- CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCG TTCTGATTACGTGTTTCAGCGCGCTG-3'
HK_ <i>mif</i> SF1	5'- <u>GCTAGCAGAAAGGAGA</u> TATACCATGTCCTTGTCCC GTCCGCTG-3'
HK_ <i>mif</i> SR1	5'-TTT <u>TCTAGA</u> TTCATGTCGTTACGCTCGTGTC-3'
GDT_ <i>mif</i> RF1	5'- <u>GCTAGCAGAAAGGAGA</u> TATACCATGAGCGACCAG GTGATCTTCGTCGAC-3'
GDT_ <i>mif</i> RR1	5'-TTT <u>GAGCTC</u> TGCTTCAGGCCGGCTCTTCGC-3'
GDT_PA5530F1	5'- <u>GCTAGCAGAAAGGAGA</u> TATACCATGGAAAGCGCCA AC-3'

GDT_PA5530R1	5'- TTT <u>GAGCTC</u> TCAATCGGTCGTGATCTTCGAGTGC- 3'
GDT_cotransF1	5'-GGTGTTTCAGCCTGATCCTGCCGG-3'
GDT_cotransR1	5'-CCGCTTCGCGGATCGTCGCTTC-3'
GDT_cotransF2	5'-GGATCGTCCACGAACTCGGCGGC-3'
GDT_cotransR2	5'-CAGGCGCACCTCGAAGCCGGAC-3'
GDT_p37_SeqF	5'-GACCCGTTTAGAGGCCCAA-3'
GDT_p37_SeqR	5'-CGTGCTTTACACTTTATGCTTCCGG-3'
<i>mifR</i> _seqF	5'-TGGTGCTGGAGAACCGGC-3'
<i>mifR</i> _seqR	5'-GCAGTTCAGCGCCACGAAC-3'
<i>mifS</i> _SeqF	5'-ATCTGGAACGGCAGTGGAACC-3'
<i>mifS</i> _SeqF2	5'-ATCGACGGCGAGTTGCAGCA-3'
PA5530_seqF	5'-TCGCGGCATGGAAGAGAC-3'
PA5530_seqR	5'-CATGCCGCGACGCAG -3'
DBS_qRT_clpXF	5'- TGCGATTACGATGTGGAGA -3'
DBS_qRT_clpXR	5'- CCCTCGATGAGCTTCAGCA -3'
GDT_qRT_PA5530F	5'-CGCAACGCATCAAGTCGAT-3'
GDT_qRT_PA5530R	5'-AGTCGTACCACTCGACCAGGTT-3'
qRT_ <i>rpoNF</i>	5'-AAATGCGAAAAAGCCATTGAG-3'
qRT_ <i>rpoNR</i>	5'-CCCTGTGCCTCCAGTAAACC-3'
qRT_ <i>icdF</i>	5'-GCGACCGGTGACAAAATCAC-3'
qRT_ <i>icdR</i>	5'-GGGTTCTTCGGTACGCTCAA-3'
qRT_ <i>idhF</i>	5'-GGCGATGATCCGCAACTC-3'
qRT_ <i>idhR</i>	5'-GCATTACCGCCTTGGTGTCT-3'
qRT_ <i>sucAF</i>	5'-CTGCAGCCAGCATCACATG-3'
qRT_ <i>sucAR</i>	5'-CGAGATTGAGGCCCTTCTTG-3'
qRT_ <i>lpd3F</i>	5'-CATGCGGCGGAGATGAAC-3'
qRT_ <i>lpd3R</i>	5'-ACTTCCGGCTGGGTGTAGATG-3'

qRT in the primer name indicates that the primer was designed for qPCR. Broken and continuous lines below the primer sequence indicate ribosome binding and restriction sites respectively.

Construction of *P. aeruginosa* $\Delta mifR$ mutant

An unmarked *mifR* clean in-frame deletion mutant of *P. aeruginosa* was generated by gene splicing (Horton *et al.*, 1990). Upstream and downstream flanking regions of *mifR* were amplified by PCR (GC Rich PCR System, Roche, Indianapolis, IN, USA), using primers listed in Table 2.2. A 754-bp P1 and a 720-bp P2 were amplified using upstream primers *mifRUF1-EcoRI* and *mifRUR1-NheI* and the downstream primers *mifRDF1-NheI* and *mifRDR1-HindIII* (Table 2.2), respectively from PAO1 genomic DNA. After sequencing to ensure fidelity, P1 and P2 were spliced together to obtain a 1474-bp deletion fragment with a deletion of *mifR* containing stop codons at its junction (inserted as part of *NheI* site in the primer). This was then sequenced and subcloned into a *P. aeruginosa* non-replicative plasmid pEXG2 (Rietsch *et al.*, 2005) as a *EcoRI-HindIII* fragment and moved into the wild-type PAO1 strain by allelic replacement (Schweizer & Hoang, 1995) using pRK600 and pRK2013 as the helper plasmids (Figurski & Helinski, 1979, Kessler *et al.*, 1992). Clones were screened for Gm sensitivity ($75 \mu\text{g ml}^{-1}$) and sucrose resistance (8% sucrose) corresponding to a double cross-over recombination event and replacement of the target gene with the deletion product. The presence of the deletion in PAO $\Delta mifR$ (PKM901) was confirmed by PCR amplification and sequencing of the deletion product (data not shown).

Construction of *P. aeruginosa* $\Delta mifS$ and $\Delta mifSR$ mutants

The unmarked *mifS* and *mifSR* deletion in PAO1 was generated by using the yeast system of double-stranded gap repair and homologous recombination (Shanks *et al.*, 2009). Briefly, the *mifS* and *mifSR* upstream and downstream

flanking regions were amplified by PCR using primers listed in Table 2.2. To create a *mifSR* deletion, an upstream 933-bp P1 and a downstream 1115-bp P2 were amplified using primer pairs *mifSRUF1-mifSRDF1* and *mifSRUR1-mifSRDR1*, respectively. Similarly, to create *mifS* deletion, an upstream 703-bp P1 and a downstream 653-bp were amplified using primer pairs HK*mifSUF*-HK*mifSDF* and HK*mifSUR*-HK*mifSDR*, respectively. HK*mifSUF* and *mifSRUF1* primers had stretches of homologous DNA, 5'-GGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCT-3' and 5'-CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT-3', respectively, to target recombination of the amplicons with pMQ30 vector. These primer pairs also had complementing sequences at the 3' end to facilitate joining to create the P3 fragment, as well as stop codons (CTAGTTAGCTAG) to prevent any run off translation. The pMQ30 vector has double selection markers *URA3* for yeast and gentamycin for *E. coli* (Shanks *et al.*, 2006). Yeast cells were transformed with the P1, P2 and linearized pMQ30 (*Bam*HI digested) using standard protocols and (Shanks *et al.*, 2009) colonies were selected on sucrose-uracil plates.

The yeast colonies were checked for the presence of P3 constructs for *mifS* and *mifSR* deletions by amplification using upstream forward (*mifSRUF1* and HK*mifSUF*, respectively) and downstream reverse (*mifSRDR1* and HK*mifSDR*, respectively) primers. Yeast DNA was isolated from the positive colonies as described earlier (Shanks *et al.*, 2009). *E. coli* was transformed with the recombinant pMQ30 plasmids containing P3s and screened for gentamycin resistance. The amplified P3s from the recombinant plasmids were sequenced to

ensure fidelity. The constructs were then moved into PAO1 strain using tri-parental mating and screened for single and double crossovers using counter selection with sucrose and gentamycin as described earlier (Balasubramanian *et al.*, 2012, Kumari *et al.*, 2014). The presence of the gene deletions in all the mutants were confirmed using standard molecular methods (PCR and DNA sequencing of the locus). These strains are henceforth referred to as PAO Δ *mifS* (PKM900) and PAO Δ *mifSR* (PKM902).

Construction of complementing plasmids

DNA fragments from *P. aeruginosa* PAO1 with *mifS* (~1.77 kb) and *mifR* (~1.35 kb) were PCR amplified using primer pairs HK_*mifSF*1-HK_*mifSF*1R1, GDT_*mifRF*1-GDT_*mifRR*1, respectively. In order to ensure expression of the genes, the primers are designed such that the ORF will juxtapose against a strong ribosome binding site (Lehninger *et al.*, 2013). The PCR amplified products were cloned into pCR2.1 TOPO (Invitrogen, Life Technologies, Carlsbad, CA, USA) using manufacturers protocol to generate plasmids pGDT001 and pGDT002, respectively. The fidelity of the PCR amplified product was confirmed by sequencing. The fragments carrying *mifS* and *mifR* were moved into a broad host range pPSV37-Gm plasmid (Lee *et al.*, 2010) as a *Nhe*I-*Sac*I fragments, downstream of an inducible P_{*lacUV5*} promoter to generate plasmids pGDT003 and pGDT004, respectively. Henceforth, these plasmids are referred to as pMifS and pMifR.

DNA fragments from PAO1 with *mifSR* (~3.12 kb) and *PA5530* (~1.3 kb) were PCR amplified using primer pairs HK_*mifSF*1-GDT_*mifRR*1 and

GDT_PA5530F1-GDT_PA5530R1 (Table 2.2), respectively. The PCR amplified products were cloned directly into pPSV37-Gm plasmid as *NheI*-*SacI* fragments, downstream of an inducible P_{lacUV5} promoter to generate plasmids pGDT005 and pGDT006, respectively. Sequence fidelity was confirmed by sequencing using the primers GDT_p37_SeqF-R, *mifR*_seqF-R, *mifS*_seqF-F2 and *PA5530*_seqF-R (Table 2.2). Henceforth, these plasmids are referred to as pMifSR and pPA5530.

These expression plasmids were then introduced into wild-type PAO1, PAO Δ *mifS*, PAO Δ *mifR*, PAO Δ *mifSR* and PAO Δ *rpoN* deletion mutants by electroporation (Choi *et al.*, 2006) and gentamycin resistant colonies were selected.

Phenotypic microarray

Comparative phenotypic microarray profiles of wild-type PAO1 with PAO Δ *mifR* and PAO Δ *mifS* mutant were performed at BioLOG Inc. (Hayward, CA, USA). Phenotypic profiling was carried out in triplicate and data analyses was done using OmniLog PM Software.

Growth curves

P. aeruginosa PAO1 and its derivatives were grown overnight at 37°C in LB broth with or without antibiotics. Overnight cultures were washed with sterile 0.85% NaCl (wt/vol) solution to remove spent and residual media. Cultures were diluted in fresh M9 minimal media to obtain equal optical densities (OD₆₀₀) of 0.025. Growth of the cultures was assessed in LB broth and in M9 minimal media supplemented with glucose (30 mM), sucrose (30 mM) or TCA cycle intermediates including citrate, α -KG, succinate, fumarate, malate or oxaloacetate (at 30 mM,

unless specified otherwise) as a sole carbon source in 48 and 96 well plates (*Falcon*). Growth was monitored by determining absorbance at 600 nm using BioTek Synergy HT (Winooski, VT, USA) plate reader for 18-24 h at 37°C. All experiments were performed multiple times in triplicate.

Pyocyanin and pyoverdine production

Extracellular pyocyanin was quantified by extracting the pigment from culture supernatants using the chloroform-HCL method as described previously (Essar *et al.*, 1990). Briefly, 5 ml culture supernatants from stationary-phase cultures (~18 h) grown in King's A medium was extracted with 3 ml chloroform. Pyocyanin was then re-extracted into 1 ml of 0.2 N HCl, resulting in a pink color, indicating the presence of pyocyanin that was read at 520 nm. The concentration is expressed as µg of pyocyanin produced per ml of culture (µg/ml), by multiplying the optical density OD₅₂₀ by 17.072 (Essar *et al.*, 1990).

To measure pyoverdine production, cells were grown overnight at 37°C in King's B medium (King *et al.*, 1954). Pyoverdine in the supernatant was read at 405 nm and normalized to the initial cell density (OD₆₀₀). Pyoverdine levels were expressed as a ratio of OD₄₀₅/OD₆₀₀ (Shen *et al.*, 2002).

Minimum Inhibitory Concentration

MICs were determined using the E-test as per the manufacturers protocol (BioMerieux, USA) and/or by standard broth microdilution method . The assays were performed in triplicate, each with technical triplicate, for each antibiotic in cation-adjusted Mueller Hinton broth.

RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from *P. aeruginosa* wild-type PAO1, PAO Δ *mifR*, PAO Δ *mifS* and PAO Δ *mifSR* strains grown in LB broth followed by 1 h treatment with 30 mM α -KG. Briefly, overnight cultures grown in LB broth at 37°C were washed with sterile 0.85% saline solution to remove spent media and were subcultured at 37°C, 200 rpm in LB media. LB broth was used as a carbon source for initial growth of cultures since PAO Δ *mifR*, PAO Δ *mifS*, PAO Δ *mifSR* and PAO Δ *rpoN* strains exhibit growth defects in the presence of α -KG alone. When the cells reached an optical density at 600 nm (OD₆₀₀) of 0.6-0.7 all the cultures were treated with 30 mM α -KG for 1 h. Post treatment, RNA was stabilized by addition of phenol-ethanol mixture (Brencic *et al.*, 2009). Stabilized RNA was then isolated using RNeasy Mini Kit (Qiagen, Inc Venio, Limburg, Netherlands) as per manufacturer's protocol. Residual genomic DNA contamination was removed using RQ1 Rnase-free DNase (Promega, Madison, WI, USA) and RNA was repurified using Rneasy Mini Kit (Qiagen, Inc Venio, Limburg, Netherlands). Quality of purified RNA was assessed on a denaturing agarose gel (NorthernMax Gly, Ambion, Life Technologies, Carlsbad, CA, USA) and quantified at 260 nm (BioTEK, Synergy HT, Winooski, VT, USA). cDNA was then synthesized by annealing NS5 random primers to total purified RNA and subsequent extension was carried out using SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA).

qRT-PCR to study expression levels of *PA5530* under α -KG induction was performed using Applied Biosystems Step One cycler and detection system with

PowerSYBR Green PCR MasterMix with ROX (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). In addition RNA was isolated from PAO1, PAO Δ *mifR*, PAO Δ *mifS* and PAO Δ *mifSR* strains grown in M9 Minimal media supplemented with citrate (30 mM) without α -KG treatment, as described previously. qRT-PCR to study expression levels of genes encoding sigma-54 *rpoN* (PA4462), iso-citrate dehydrogenase (*idh* (PA2623) and *icd* (PA2624)), α -KG dehydrogenase complex (*sucA* (PA1585) and *lpd3* (PA4829)) were done essentially as described above. The cycling conditions used were 95°C/2 minutes (holding); 40 cycles of 95°C/15 sec, 60°C/1 min (cycling); 95°C/15 sec, 60°C/1 min, 95°C/15 sec (0.6°C ramp) (melt curve). Expression was normalized to *cpx* (PA1802), whose expression was determined to remain constant between the samples and conditions tested (Balasubramanian *et al.*, 2012).

Bioinformatic Analyses

Sequence analyses and domain organization studies were performed using the Simple Modular Architecture Research Tool (SMART) (Schultz *et al.*, 1998) and InterPro domain prediction database (Hunter *et al.*, 2012). *mifS* (P_{*mifS*}) and PA5530 (P_{PA5530}) promoter analyses and motif search was done using the ensemble learning method SCOPE and GLAM2 (Gapped Local Alignment of Motifs) (Chakravarty *et al.*, 2007, Frith *et al.*, 2008). Multiple sequence alignment was generated using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and www.pseudomonas.com (Winsor *et al.*, 2011).

Statistical Analyses

All data were analyzed for statistical significance using the Student's *t*-test on GraphPad or Analysis of Variance (ANOVA) with post-hoc testing when appropriate, on IBM SPSS Statistics 22.0 statistical analysis software. Differences were considered to be significant at *p*- values < 0.05.

Results

***mifS* and *mifR* are a part of a two-gene operon**

In eubacteria, the genes that encode a HK and its cognate RR are often linked and are co-transcribed (Stock *et al.*, 1989). Our sequence analysis of *P. aeruginosa* PAO1 genome revealed that *mifS* (PA5512) and *mifR* (PA5511) are adjacent to each other, in the same orientation. The predicted translation start site of *mifR* ORF overlaps with *mifS* translation termination codon indicating that they are cotranscribed (Figure 2.1A and 2.1B). To determine if these two genes form an operon, cDNA across the intergenic regions spanning *mifS* and *mifR* was amplified using GDT_cotransF1-R1 and GDT_cotransF2-R2 primers (see Materials and Methods)x. As expected, 200 bp and 100 bp products were detected when using primers that span the overlapping region (Figure 2.1C, Lane 3 and Lane 4). These results confirm that *mifS* and *mifR* are a part of a two-gene operon. As controls, the *mifSR* genes were also amplified (Figure 2.1C, Lane 2).

Loss of *mifS* and *mifR* did not affect antibiotic resistance

To identify the role of MifSR TCS, clean in-frame deletion mutants of *mifS*, *mifR* and *mifSR* were constructed in the prototypic *P. aeruginosa* PAO1. Henceforth they will be referred to as PAO Δ *mifS*, PAO Δ *mifR* and PAO Δ *mifSR*,

respectively. For complementation studies, recombinant plasmids containing the entire *mifR*, *mifS* and *mifSR* genes were constructed. The complementing plasmids with the genes are called pMifS, pMifR and pMifSR. These plasmids were introduced into the respective mutant strains.

Figure 2.1: Genome organization of the *mifSR* gene locus. In *P. aeruginosa* PAO1 the *mifR* (PA5511) ORF has a translation start codon (Briard *et al.*) overlapping the *mifS* (PA5512) termination codon (TGA), denoted in red (B), suggesting that the *mifS* and *mifR* genes are physically linked. The cDNA amplification of the intergenic region spanning the *mifS* and *mifR* genes using GDT_cotrans F1-R1 and GDT_cotrans F2-R2 primers (Table 1) confirm that the two genes *mifS* and *mifR* are co-transcribed and form an operon (C).

Previous studies in our lab postulated that the MifSR TCS system, found 81-bp upstream of the *pox* operon, may contribute to *P. aeruginosa* β -lactam resistance (Kong *et al.*, 2005) as the genes regulated by TCS tend to be co-located on the chromosome (Stock *et al.*, 1989). However, MIC analyses using E-test and micro-dilution methods showed that the loss of these genes did not affect the antibiotic resistance profile when compared to the parent strain, *P. aeruginosa* PAO1 (Data not shown). Further, qRT-PCR studies showed that deletion of *mifS*, *mifR* and *mifSR* had no effect on the expression of *poxB* compared to the parent PAO1 (Figure 2.2).

Figure 2:2 Expression of *poxB* (PA5514) in *mifSR* mutants. Expression of *poxB* (PA5514) was tested in *mifSR* mutants relative to PAO1. Data was normalized to expression in PAO1. Bars above or below the line represents up- and down-regulation, respectively and the bars indicate standard errors. The *clpX* gene (PA1802) was used as the housekeeping control. There was no statistically significant difference (p -value > 0.05) between the wild type PAO1 and *mifSR* mutant strains as determined by one-way ANOVA and student's unpaired *t* test.

***mifS*, *mifR* and *mifSR* mutants failed to grow in the presence of α -KG**

The $\text{PAO}\Delta\textit{mifS}$, $\text{PAO}\Delta\textit{mifR}$ and $\text{PAO}\Delta\textit{mifSR}$ mutants exhibited no discernible phenotype compared to the parent PAO1 when tested for growth, swimming, swarming, twitching motility (LB media), pyocyanin production (LB & King's A media), pyoverdine production (LB & King's B Media), congo red binding assay (CR media) and antibiotic resistance (MH media) (Data not shown). Hence, a comparative phenotypic microarray analysis was performed with the wild-type PAO1, $\text{PAO}\Delta\textit{mifR}$ and $\text{PAO}\Delta\textit{mifS}$ mutants (BioLOG Inc.). Out of approximately 2000 metabolic and chemical sensitivity assays tested, $\text{PAO}\Delta\textit{mifR}$ exhibited four gain-of-function and 29 loss-of-function phenotypes whereas $\text{PAO}\Delta\textit{mifS}$ exhibited two gain-of-function and 23 loss-of-function phenotypes (Figure 2.3A). A single gain of function phenotype shared between $\text{PAO}\Delta\textit{mifS}$ and $\text{PAO}\Delta\textit{mifR}$, was the ability to utilize L-methionine. When metabolism and chemical sensitivity were compared, the mutants appear more sensitive to various antibiotics (Figure 2.3B). However, none of these were reproducible in the lab in the MH media. The loss of *mifS* and *mifR* resulted in differential phenotype in the presence of six metabolites, amongst which, two were common to both *mifS* and *mifR* mutants (Figure 2.3B). The shared metabolic phenotypes involved the utilization of L-methionine and α -KG (Figure 2.3C). Compared to the parent PAO1, the mutants did not exhibit any growth increase when provided with L-methionine (Figure 2.4). This could be simply due to the difference in culture conditions and BioLOG proprietary media.

The inability to utilize α -KG by $\text{PAO}\Delta\textit{mifS}$ (Figure 2.5A) and $\text{PAO}\Delta\textit{mifR}$ (Figure 2.5B) in the BioLOG assay was reproduced in M9 minimal media

supplemented with 30 mM α -KG (Figure 2.5C). In fact, all three mutant strains, *PAO Δ mifR*, *PAO Δ mifS* and *PAO Δ mifSR* failed to grow in the presence of α -KG (Figure 2.5C). To rule out potential toxicity, the wild-type *P. aeruginosa* PAO1 and the mutants were cultured in M9 minimal media with varying concentrations of α -KG, ranging from 1 to 80 mM (Figure 2.6). The mutants exhibited no growth in the presence α -KG after 24 h at 37°C, whereas the wild-type PAO1 exhibited an increase in growth that was proportional to α -KG concentration (Figure 2.6B). All subsequent experiments were done with 30 mM α -KG. The growth defect exhibited by *PAO Δ mifS*, *PAO Δ mifR* and *PAO Δ mifSR* could be restored to the wild-type levels by introducing *mifR* and *mifSR* genes into the respective mutants (Figure 2.5D and Figure 2.7A).

Figure 2.3: *mifS* and *mifR* dependent phenotypes. To identify the role of *P. aeruginosa mifSR* TCS, comparative phenotypic microarray of PAOΔ*mifS*, PAOΔ*mifR* mutants and wild-type PAO1 strain was performed at BioLOG Inc. (Hayward, CA, USA). Venn diagram of differentially regulated phenotypes of the mutants compared to their isogenic parent PAO1, showing gain of function or loss of function phenotypes (A). Phenotypic differences were further classified based on metabolic and chemical sensitivity properties (B). The phenotypes common to both *mifS* and *mifR* mutants are listed (C).

Figure 2.4: Growth curve analysis in the presence of methionine. Growth curves of *P. aeruginosa* wild-type PAO1 and *mifSR* mutants in M9 minimal media supplemented with glucose (30 mM) and methionine (5 mM) as carbon and nitrogen source.

Figure 2.5: Phenotypic microarrays of *PAOΔmifS* and *PAOΔmifR* mutants.

The loss of *mifS* and *mifR* results in a growth deficient phenotype in the presence of α-KG as a sole carbon source, as depicted by BioLOG plate PM1, well D6 (A and B). Loss of growth phenotype was confirmed by growing PAO1, *PAOΔmifS*, *PAOΔmifR* and *PAOΔmifSR* mutants in M9 minimal media with α-KG (30 mM) for 18 to 24 h at 37°C (C). The growth defect was rescued by expressing *mifR* and *mifSR* genes (D) and the gene encoding the α-KG specific transporter PA5530 (E) in *trans*.

Figure 2.6: Growth profile in the presence of varying concentrations of α-KG. PAO1 and its isogenic *mifSR* mutants, PAOΔ*mifS*, PAOΔ*mifR* and PAOΔ*mifSR* were grown in M9 minimal media with varying concentrations of α-KG (1 to 80 mM) as the sole carbon source. Growth was monitored by measuring absorbance at 600 nm (OD₆₀₀) over a period of 24 h at 37°C. OD₆₀₀ at 0 h (A) and 24 h (B) is plotted against α-KG concentration. Results shown are mean with standard deviation of three biological replicates. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** *p*-value < 0.001. in *trans*.

Figure 2.7: Rescue of α -KG-dependent growth phenotype of *mifSR* mutants. Growth curves of *P. aeruginosa* wild-type PAO1, *mifSR* single and double deletion mutants and its complementing clones (A) and in the presence of pPA5530 (B) in M9 minimal media with α -KG (30 mM).

***mifSR* mutants exhibit α -KG dependent growth defect**

α -KG is a key TCA cycle intermediate (Figure 2.8) and plays an important role in regulating carbon and nitrogen metabolism (Doucette *et al.*, 2011). It has been previously shown that *P. aeruginosa* preferentially utilizes TCA cycle intermediates as a carbon source over other compounds (Liu, 1952, Wolff *et al.*, 1991, Suh *et al.*, 2002). To test if the growth defect exhibited by the loss of *mifS* and *mifR* is restricted to α -KG utilization, the mutants and the complementing strains were grown in the presence of TCA cycle intermediates citrate, succinate, fumarate, malate and oxaloacetate at 30 mM each. No difference in growth was observed between wild type PAO1 and its isogenic mutants in the presence of other TCA cycle intermediates except for α -KG (Table 2.3). This is not surprising as *P. aeruginosa* can use the glyoxylate shunt pathway to bypass the need for α -KG (Figure 2.8) (Campbell *et al.*, 1953). Furthermore, no difference in the growth profile of the wild type PAO1 and *mifSR* mutants was observed when grown in the presence of sugars, glucose and sucrose (30 mM each) (Data not shown). To reconfirm that the presence of α -KG is not toxic, the cells were grown in the presence of citrate and succinate combined in equal concentration with α -KG. The mutants and the wild type shared similar early exponential growth (Figure 2.9). However, the mutants reached stationary phase earlier as compared to the parent strain PAO1. This suggests that the presence of excess carbon source in the form of α -KG further contributes to the growth of PAO1. These analyses indicate that *mifSR* mutants are only defective in α -KG utilization.

Figure 2.8: Tricarboxylic acid (TCA) cycle and its related reactions. Enzymes converting iso-citrate to α -KG (iso-citrate dehydrogenase: Icd, Idh), α -KG to succinyl-coA (α -KG dehydrogenase complex: SucA, SucB, Lpd3) and those involved in the glyoxylate shunt (isocitrate lyase (AceA) and malate synthase G (GlnB)) are shown in bold. Green boxes indicate the amino acid biosynthetic precursors of α -KG involved in the anaplerotic reaction.

Figure 2.9: Growth curves in presence of α -KG in combination with succinate and citrate. To determine if α -KG is toxic to the cells, wild-type PAO1 and *mifSR* mutants were grown in the presence of α -KG in combination with succinate (A) and citrate (B) at 30 mM each. In comparison to the wild-type PAO1, *mifSR* mutants shared a similar exponential phase but reached stationary phase earlier, suggesting that it has depleted usable C-source. This suggests that PAO1 can efficiently utilize excess carbon source in the form of α -KG contributing to its increased growth.

Table 2.3. Growth properties of *mifSR* mutants in presence of TCA cycle intermediates.

Carbon Source	PAO1	$\Delta mifS$	$\Delta mifR$	$\Delta mifSR$
Pyruvate	+++	+++	+++	+++
Oxaloacetate	+++	+++	+++	+++
Citrate	+++	+++	+++	+++
Succinate	+++	+++	+++	+++
Fumarate	+++	+++	+++	+++
Malate	+++	+++	+++	+++
α-Ketoglutarate	+++	---	---	---

+++ , growth; ---, no growth

Growth of the wild type PAO1 and *mifSR* mutants was tested in M9 minimal media supplemented with different TCA cycle intermediates at 30 mM each, as the sole carbon source. Cells were cultured for 18 to 24 h at 37 °C and their growth was monitored by measuring the absorbance at 600 nm. No difference was observed in the growth rate of *mifSR* mutants compared to the parent PAO1 strain. Data is represented in terms of growth and no growth phenotype.

***mifSR* mutants are defective in α -KG transport**

The absence of growth in the presence of exogenous α -KG could be due to either failure to enter the cells or loss of the mutants' ability to convert α -KG to succinate. The latter is likely if the mutants failed to express a functional α -KG dehydrogenase complex. The ability of *mifSR* mutants to grow effectively in the presence of citrate and succinate suggests that these mutants are likely to harbor a functional α -KG dehydrogenase complex, unless the mutants bypass it using the glyoxylate shunt (Figure 2.8). The former is likely as qPCR analysis of genes encoding isocitrate dehydrogenase (*idh*, *icd*) and α -KG dehydrogenase complex (*sucA*, *sucB*, *lpd3*) revealed no difference in the expression levels in the wild-type PAO1 and *mifSR* mutants (Figure 2.10).

α -KG is a hub for anaplerotic reactions, a process for replenishing TCA cycle intermediates. In this process glutamate, glutamine, proline and arginine act as precursor molecules for α -KG synthesis (Kornberg, 1966). Growth studies in the presence of these amino acids would serve as another indirect measure to test the functionality of α -KG dehydrogenase complex in *mifSR* mutants. To test this hypothesis, PAO1, PAO Δ *mifR*, PAO Δ *mifS* and PAO Δ *mifSR* mutants were cultured in the presence of glutamate, glutamine, proline and arginine (Table 2.4). The parent PAO1 and the isogenic mutants exhibited similar growth phenotype. From the expression studies and growth analyses we deduce that the *mifSR* mutants are impaired in α -KG transport.

Figure 2.10: Quantification of *rpoN*, *acnA*, *idh*, *icd*, *sucA*, and *lpd3* mRNA by qRT-PCR. RNA was isolated from cells grown in M9 minimal media supplemented with citrate (30 mM), reverse transcribed to cDNA and the presence of specific transcripts was analyzed by qPCR using gene-specific primers (Table 5). The expression of genes encoding aconitate hydratase 1 (*acnA* (PA1562)) isocitrate dehydrogenase (*idh* (PA2623)) isocitrate dehydrogenase, α -KG dehydrogenase complex (*icd* (PA2623)), *sucA* (PA1585) and *lpd3* (PA4829), and σ^{54} (*rpoN* (PA4462)) were analyzed in *mifSR* mutants relative to PAO1 ($\log_{10}$ RQ = 1). Bars above or below the line represents up- and down-regulation, respectively and the bars are standard errors. The *clpX* (PA1802) gene was used as the housekeeping control. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test. Difference in the expression levels of genes is not statistically significant at p -value < 0.05.

Table 2.4. Growth profile analysis of the *mifSR* mutants in presence of amino acids.

Carbon Source	PAO1	$\Delta mifR$	$\Delta mifS$	$\Delta mifSR$
Glutamate	+++	+++	+++	+++
Glutamine	+++	+++	+++	+++
Proline	+++	+++	+++	+++
Arginine	+++	+++	+++	+++

+++ , growth; ---, no growth

Cells were grown in the M9 minimal media with the indicated amino acids (30 mM each). Data is represented in terms of growth and no growth phenotype.

***mifSR* TCS genes regulate extracellular α -KG transport**

In a recent study using transposon mutagenesis; PA5530 was identified as the functional α -KG transporter (Lundgren *et al.*, 2014). To confirm the role of *P. aeruginosa* PA5530 in α -KG uptake and identify the role of *mifSR* genes, the gene was amplified and subcloned downstream of the inducible P_{lacUV5} promoter. The plasmid pPA5530 was introduced into PAO1 and the *mifSR* mutants. Expression of PA5530 in *trans* in PAO $\Delta mifS$, PAO $\Delta mifR$, PAO $\Delta mifSR$ mutants restored their growth to a level similar to the wild-type PAO1 in M9 minimal media with α -KG (30 mM) as the sole carbon source (Figure 2.7B). Expression of an extra copy of PA5530 gene in the wild-type PAO1 did not affect its growth (Figure 2.5E). This finding suggests that expression of PA5530 is likely regulated by MifSR and/or α -KG. In fact, expression of PA5530 is regulated by α -KG, as seen in qRT-PCR analysis when PAO1 was grown in M9 media with varying amounts α -KG (Figure 2.11A). The loss of *mifS*, *mifR* and *mifSR* results in a significant decrease in PA5530 expression as compared to the wild type PAO1 in the presence of α -KG (Figure 2.11B). Thus, α -KG-dependent PA5530 expression requires MifSR.

Figure 2.11: Expression of *PA5530* in response to α -KG. *PA5530* gene expression was determined in the wild type PAO1 with varying concentrations of α -KG (1 h) (A). In addition, the expression of *PA5530* was tested in *mifSR* mutants relative to PAO1, with cells exposed to 30 mM α -KG for 1 h (B). Data was normalized to expression in PAO1 under the respective conditions. Bars above or below the line represents up- and down-regulation, respectively and the bars indicate standard errors. The *clpX* gene (*PA1802*) was used as the housekeeping control. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001

RpoN (σ^{54}) is required for α -KG utilization

The closest *P. aeruginosa* MifS and MifR homologs are *R. meliloti* DctB and DctD (Stover *et al.*, 2000). In fact, MifR is 69% similar to *R. meliloti* DctD that belongs to the Sigma 54 (σ^{54}) dependent NtrC family of transcriptional regulators (Stover *et al.*, 2000, Valentini *et al.*, 2011). Thus, it is likely that MifR has the conserved domains found among NtrC family of regulators, an N-terminal regulatory, a central σ^{54} activation and a C-terminal DNA binding domains (Morett & Segovia, 1993, Osuna *et al.*, 1997). MifR analysis using the simple modular architecture research tool (SMART) (Schultz *et al.*, 1998) and InterPro (Hunter *et al.*, 2012) revealed the presence of three domains: CheY-homologous receiver/regulatory, a central AAA⁺ region required for σ^{54} activation, and the DNA binding helix-turn-helix domains (Figure 2.12A). The central AAA⁺ domain contains seven conserved regions designated C1 to C7 (Morett & Segovia, 1993) that are characteristic of σ^{54} -dependent transcriptional regulators. Sequence analysis of MifR revealed the presence of all the seven conserved regions in the AAA⁺ domain between amino acid residues 144 to 373 (Figure 2.12B).

Since MifR exhibits high identity to σ^{54} -dependent transcriptional regulators, we hypothesized that *P. aeruginosa* *rpoN* mutants should exhibit a α -KG-dependent phenotype, similar to the *mifSR* mutants. To verify this hypothesis, we tested the ability of PAO Δ *rpoN* mutant to grow in the presence of α -KG (30 mM) (Table 2.5). As expected, PAO Δ *rpoN* failed to grow in the presence of α -KG (Table 2.5). The growth of the *rpoN* mutant was restored in PAO Δ *rpoN*::*rpoN* complementing strain. Further, in *trans* expression of *mifR* and *mifSR* in

PAO Δ *rpoN* mutant failed to restore their growth in the presence of α -KG (Table 2.5). This data confirms that MifR regulatory function requires functional RpoN (σ^{54}).

The small 81-bp *mifSR* promoter has no obvious RpoN sigma factor -12/-24 consensus sequence: 5'-TGGCACG-N4-TTGCW-3' in which W stands for either A or T (Figure 2.13A) (Barrios *et al.*, 1999). In fact, it appears to have a potential -10 (consensus: TATAAT) but lacked -35 (consensus: TTGACA) for sigma-70 promoter (Figure 2.13A) (Paget & Helmann, 2003). On the other hand, the promoter region of *PA5530* is 315-bp long with strong -12 and -24 boxes upstream of the predicted transcription start site (Figure 2.13B). We hypothesized that the inability of *rpoN* mutant to utilize α -KG can be rescued by expressing *PA5530* under a regulatable promoter P_{lacUV5} . As expected, the growth of the *rpoN* mutant was restored when the plasmid harboring the transporter *PA5530* was expressed in *trans* (Table 2.5). This suggests that expression of *PA5530* requires both MifSR TCS and RpoN.

The presence of a common motif, GATCGGCGGATt/gTCC, in the P_{mifS} and P_{PA5530} (Figure 2.13A and 2.13B) suggest that these two operons share some common regulatory mechanism. In addition, both promoters possess multiple motifs: P_{mifS} has two sets of large overlapping inverted repeats, and P_{PA5530} has three sets of direct repeats (Figure 2.13A and 2.13B). However, the role of these motifs remains to be elucidated.

Table 2.5. Growth properties of PAO1 Δ *rpoN* and its derivatives in the presence of α -KG and LB.

Strain	Plasmid	α -KG	LB
PAOΔ<i>rpoN</i>	-	---	+++
	Vector	---	+++
	pRpoN	+++	+++
	pMifR	---	+++
	pMifSR	---	+++
	pPA5530	+++	+++

+++ , growth; ---, no growth

Growth of PAO1 Δ *rpoN* mutant and its derivatives was tested in the M9 minimal media supplemented with α -KG (30 mM) and in the LB media at 37°C for 24h.

Figure 2.13: *In silico* analysis of *mifS* (P_{mifS}) and *PA5530* (P_{PA5530}) promoter sequences. Motif search was done using the ensemble learning method SCOPE and GLAM2 (Gapped Local Alignment of Motifs) (Chakravarty *et al.*, 2007, Frith *et al.*, 2008). (A) Sequence analysis of the 81-bp (P_{mifS}) (black) indicates a putative σ^{70} -dependent -10 consensus (TATAAT). However, it lacks the -35 consensus (TTGACA) for σ^{70} promoter (Potvin *et al.*, 2008). Arrows represent the long 17-bp direct and inverted repeats in P_{mifS} with a consensus GGAT/cAGCGACATCGGCG. (B) The 315-bp promoter region of *PA5530* showing strong -12 and -24 σ^{54} -dependent promoter like element and the proposed transcription start site (+1). Dashed line (blue) depicts a common motif in P_{mifS} and P_{PA5530} suggesting a common regulatory mechanism (A and B). The three pairs of direct repeats in P_{PA5530} are represented by green, blue and orange arrows. P_{PA5530} possess the signature sequence (AAc/uAAc/uAA) for catabolite repression control (Crc) protein (brown box) (Sonnleitner *et al.*, 2009). The uncharacterized small antisense RNA (asRNA) identified in the P_{PA5530} region (Wurtzel *et al.*, 2012) is indicated by marked line.

Figure 2.14: Proposed model for α-KG utilization in *P. aeruginosa*. HK-MifS senses the extracellular α-KG to undergo phosphorylation. The phosphate is transferred to the RR-MifR. The phosphorylated MifR in coordination with RpoN (σ⁵⁴) activates the expression of α-KG specific transporter gene *PA5530*. *PA5530* thus enables the influx of α-KG to meet the metabolic and energy demands of the cells. *PA5530* promoter (P_{PA5530}) region has a Crc binding site (Figure 2.13), suggesting that it is under the catabolite repression control by Crc/CrcZ. The P_{PA5530} also shows the presence of another uncharacterized small non-coding asRNA indicating a multilayered and complex regulation of the α-KG transport system.

Discussion

P. aeruginosa pathogenicity relies significantly on its metabolic flexibility. However, establishment of successful infection and its progression requires more than just meeting nutritional demands. Precision in sensing environmental signals concomitant with a quick and appropriate response is the key to efficient bacterial adaptation and survival. An arsenal of TCSs encoded in its genome has furnished *P. aeruginosa* with a sophisticated capability to regulate diverse metabolic and virulence processes, ensuring its success as a pathogen (Nishijyo *et al.*, 2001, Gooderham & Hancock, 2009, Winsor *et al.*, 2011). *P. aeruginosa* genome encodes one of the largest groups of TCS proteins identified in any sequenced bacterial species (Barakat *et al.*, 2009, Winsor *et al.*, 2011). Bacterial TCS's sense and respond to a variety of external cues such as nutrient availability, osmolarity, redox state, temperature, and concentrations of other extracellular molecules (Stock *et al.*, 2000). However, very few TCS signaling molecules have been identified to date. In this study we suggest that the *P. aeruginosa* MifSR TCS exclusively senses α -KG, a C₅ dicarboxylate and a key component of TCA cycle.

***P. aeruginosa* antibiotic resistance is independent of MifSR TCS**

A common feature of bacterial genomes is a close association between the functionally related genes and their location on the chromosome (Dandekar *et al.*, 1998, Overbeek *et al.*, 1999). Typically, genes encoding functionally related HKs and RRs are often physically linked and are co-transcribed as an operon (Stock *et al.*, 1989, Chen *et al.*, 2004). Indeed, our *in silico* analysis (Figure 2.1A and 2.1B) and cDNA amplification (Figure 2.1C) revealed that *mifS-mifR* genes are co-

transcribed and form an operon. This also suggests that HK-MifS and RR-MifR are functionally related and work as a TCS pair. In addition, TCS proteins are known to regulate expression of genes in their immediate vicinity (Stock *et al.*, 1989). The *mifSR* genes are 81 bp upstream of the two-gene *poxAB* (PA5513-5514) operon. Due to the proximity of *mifSR* to *poxB* which encodes for a β -lactamase, we postulated that *mifSR* TCS regulates antibiotic resistance. However, our initial results nullified this hypothesis in which comparative MIC's (Data not shown) and qRT-PCR data (Figure 2.2) showed no difference in antibiotic resistance profiles or *poxB* expression between the wild-type PAO1 and *mifSR* single and double deletion mutants.

MifSR TCS regulates *P. aeruginosa* α -KG utilization

A previous transcriptome study of the wild-type PAO1 and a *mifR* deletion mutant cultivated under biofilm-specific conditions showed significant alteration in the expression of genes involved in regulating *P. aeruginosa* metabolism, small molecule transport and amino acid biosynthesis (Petrova *et al.*, 2012). The majority of the changes observed in phenotypic microarrays of the *mifS* and *mifR* mutant strains cultivated under planktonic conditions were associated with chemical sensitivity and not with metabolism (Figure 2.3B). Only 12-16% of phenotypic changes were associated with metabolism. This confirms the significant metabolic differences in the rich planktonic versus anaerobic mode of biofilm growth in *P. aeruginosa* (Waite *et al.*, 2006).

Petrova *et al.* (2012) have also demonstrated that genes involved in energy metabolism, including anaerobic metabolism and fermentative pathways using

arginine (*arcDABC*) and pyruvate, were expressed significantly less in $\Delta mifR$ mutant biofilms as compared to its parent PAO1 (Petrova *et al.*, 2012). Though pyruvate is needed for biofilm formation, it cannot compensate for the loss of *mifR* (Petrova *et al.*, 2012). Interestingly, the biofilm phenotype associated with the loss of *mifR* can be complemented by *ldhA* encoding D-lactate dehydrogenase to wild type levels of biomass accumulation and microcolony formation (Petrova *et al.*, 2012). These findings suggest that MifR somehow regulates expression of *ldhA*, a second gene in a three-gene operon *gacS-ldhA-PA0926* (Winsor *et al.*, 2011). Importantly, analyses of the promoters reveal the presence of a shared motif in P_{mifS} (GATCCGCCGATGTCC) and P_{PA5530} (GATCGGCGGATTTCC) (Figure 2.13) and P_{gacS} (AATCCGCCGGGCTGC) suggesting a possible coordinate regulation, and that need to be verified.

Our phenotypic microarray analyses and growth experiments suggested that *P. aeruginosa* α -KG utilization requires MifS and MifR (Figure 2.5 and 2.7A). The ability of $PAO\Delta mifR$, $PAO\Delta mifS$ and $PAO\Delta mifSR$ to grow in the presence of α -KG was restored by in *trans* expression of *mifR* and *mifSR* (Figure 2.7A). Interestingly, the $PAO\Delta mifS$ was complemented by pMifR and pMifSR (Figure 2.5D) but not by pMifS alone. To rule out that gene expression may have been compromised, the *mifS* gene was cloned downstream of the inducible P_{lacUV5} promoter. Though the expression of stable protein was visible in a protein gel, it failed to complement $PAO\Delta mifS$ mutant (data not shown). This suggests that cis-expression of *mifS* and *mifR* is critical for MifS-function. Other researchers have encountered similar problems involving histidine kinases (Yeung *et al.*, 2011).

Moreover, complementation of the PAO Δ *mifS* with pMifR suggests that either phosphorylation is not required or there is a potential crosstalk between MifR and other non-cognate HKs. Alternatively, phosphorylation of MifR can occur through small molecule phosphor-donors, like acetyl phosphate, carbamoyl phosphate and phosphoramidate (Deretic *et al.*, 1992). Such phenomenon is observed with other TCS RRs (Lukat *et al.*, 1992, Wanner & Wilmes-Riesenberg, 1992, Varughese, 2002). However, this has to be verified.

The C₅-dicarboxylate α -KG is an important intermediate in the energy-generating TCA cycle (Figure 2.8) and plays a key role in regulating carbon and nitrogen metabolism (Doucette *et al.*, 2011). Similar to other bacteria (Janausch *et al.*, 2002), TCS's in *P. aeruginosa* have been reported to regulate transport and utilization of TCA cycle intermediates such as succinate, fumarate, malate and citrate (Nishijyo *et al.*, 2001, Valentini *et al.*, 2011). The *R. meliloti* DctB/DctD system is a well-characterized TCS that controls the transport of TCA cycle C₄-dicarboxylates succinate, fumarate and malate (Janausch *et al.*, 2002). Though *P. aeruginosa* MifS/MifR proteins are homologous to *R. meliloti* DctB/DctD TCS proteins, the *mifSR* mutants efficiently utilized citrate, succinate, fumarate, malate, oxaloacetate, sucrose and glucose but exclusively failed to grow in the presence of α -KG (Table 2.3). This was further supported by another parallel study that shows that α -KG utilization requires MifR (Lundgren *et al.*, 2014). Thus, the *P. aeruginosa* MifSR TCS is specifically and uniquely involved in C₅-dicarboxylate α -KG utilization.

MifSR TCS modulates *P. aeruginosa* α -KG transport

The inability to utilize α -KG suggested that the *mifSR* mutants either have a defective α -KG dehydrogenase complex (inability to convert α -KG to succinyl-coA, Figure 2.8), or they are deficient in the transport of α -KG into the cell. The former was ruled based upon multiple findings: unchanged expression levels of genes encoding α -KG dehydrogenase, *lpd3* (PA4829) and *sucA* (PA1585) (Figure 2.10); the ability to use C₄ and C₆ dicarboxylates (Table 2.3) and C5 family of amino acids such as arginine, proline, glutamine, and histidine (Table 2.4). The C5 family of amino acids act as biosynthetic precursors of glutamate that ultimately are converted to α -KG by a transamination reaction or through the action of glutamate dehydrogenase (Lehninger *et al.*, 2013). These findings strongly argued that the *mifSR* mutants were defective in their ability to transport α -KG into the cell.

To date, among the identified carboxylate transporters, the C₄-dicarboxylate transporters have been reasonably well characterized. Based on protein sequence similarity analysis, bacterial C₄-dicarboxylate transporters are classified into five families, namely, dicarboxylate transport (DctA); dicarboxylate uptake (DcuAB), (DcuC) and (CitT) and the tripartite ATP-independent periplasmic (TRAP) families (Janausch *et al.*, 2002). Amongst these, DctA transporters, a subgroup of the dicarboxylate/amino acid:cation symporter (DAACS) family (Busch & Saier, 2002, Busch & Saier, 2004, Saier *et al.*, 2006), are extensively studied and are implicated in the transport of C₄-dicarboxylates in *Escherichia coli* (Davies *et al.*, 1999), *Bacillus subtilis* (Asai *et al.*, 2000), *Rhizobium meliloti* (Yarosh *et al.*, 1989, Watson, 1990), *Rhizobium leguminosarum* (Finan *et al.*, 1981, Ronson *et al.*, 1987) and

Corynebacterium glutamicum (Youn *et al.*, 2009). As we were trying to identify the MifSR-dependent transporter Lundgren *et al.*, reported that PA5530 is involved in α -KG transport (Lundgren *et al.*, 2014). As predicted, in *trans* expression of PA5530 was able to restore the ability of *mifR*, *mifS* and *mifSR* mutants to grow in α -KG (Figure 2.5E). This is further confirmed by the increase in PA5530 expression in PAO1 in the presence of α -KG (Figure 2.11A). PA5530 shares no homology with the *P. aeruginosa* C₄-dicarboxylate transporter PA1183 (DctA). However, it does have conserved protein domain family PRK10406 implicated in α -KG transport and shares ~70% homology to *E. coli* and *Erwinia* spp. α -KG permease KgtP (Seol & Shatkin, 1991, Marchler-Bauer *et al.*, 2015). A common feature in the transport of C₄-dicarboxylates and other carbon sources in different bacteria is the involvement of TCS mediated regulatory mechanism. Involvement of TCSs, a stimulus-response coupled mechanism, in the transport of C₅-dicarboxylates suggests a more profound role of α -KG as a signaling molecule.

***P. aeruginosa* α -KG transport requires functional RpoN (σ^{54})**

P. aeruginosa RpoN (σ^{54}) is involved in a myriad of functions including expression of virulence factors and nutrient uptake (Potvin *et al.*, 2008). Functional RpoN is reported to be critical for maintaining a carbon-nitrogen balance in *Pseudomonads* (Kohler *et al.*, 1989, Nishijyo *et al.*, 2001, Cases *et al.*, 2003, Li & Lu, 2007, Zhang & Rainey, 2008). Sequence analysis of MifR indicated a requirement of functional RpoN in modulating *P. aeruginosa* α -KG utilization. Our study confirms that α -KG utilization in *P. aeruginosa* PAO1 requires functional RpoN (Table 2.5). This phenotype is not strain-specific as phenotypic microarray

profiling (BioLOG) of *P. aeruginosa* PA14 *rpoN* mutant exhibited a similar phenotype, a significant difference in the ability to utilize α -KG as a carbon source as compared to the wild-type PA14 (Behrends *et al.*, 2013). An RpoN-dependent phenotype was also observed with citrate and 4-hydroxyphenylacetate utilization (Behrends *et al.*, 2013). Similarly, utilization of C₄-dicarboxylates succinate, fumarate and malate in *R. meliloti* and *P. aeruginosa* also requires the sigma factor RpoN (σ^{54}) (Ronson *et al.*, 1987, Ronson *et al.*, 1987, Valentini *et al.*, 2011).

The need for RpoN (σ^{54}) to utilize α -KG in *P. aeruginosa* can be bypassed by expressing *PA5530* encoding for the transporter under a regulatable promoter but not MifS and MifR. Consistent with the need for RpoN (σ^{54}), the promoter for *PA5530* has the requisite signature sequences (Figure 2.13). Like most complex RpoN-dependent promoters (Merrick, 1993), the region is long with multiple motifs that include a signature sequence (AAc/uAAc/uAA) for catabolite repression control (Crc) protein, a post-transcriptional inhibitor that binds the mRNA preventing translation (Moreno *et al.*, 2007, Sonnleitner *et al.*, 2009, Browne *et al.*, 2010). Expression of *crc* is in-turn regulated by RpoN-dependent non-coding RNA CrcZ (Sonnleitner *et al.*, 2009) whose absence in *rpoN* mutant can also lead to reduced expression of *PA5530*. Also, analysis of *P. aeruginosa* PA14 transcripts indicates that the *PA5530* promoter is under a small non-coding antisense RNA (asRNA) regulation (Wurtzel *et al.*, 2012). Though the role of Crc, CrcZ and the asRNA in α -KG transport has to be verified experimentally, it suggests an additional layer of regulation superimposed on the need for MifS and MifR on the expression of the C₅-dicarboxylate transporter *PA5530*.

Conclusion

In eukaryotic cells, the mitochondria serve as a hub and reservoir of the TCA cycle and its intermediates, respectively. Bacterial pathogens can be highly virulent intruders of the host tissue, causing significant damage leading to cellular aberrations and injury. Mitochondrial dysfunction, a consequence of cell injury, results in efflux of TCA cycle intermediates leading to an increase in their extracellular concentrations (Hebert, 2004). It is known that TCA cycle intermediates (C_4 , C_5 , and C_6 dicarboxylates) are present at micromolar (μM) concentrations in blood that increase with tissues damaged (He *et al.*, 2004, Hebert, 2004). α -KG can also act as a reactive oxygen species scavenger, especially for hydrogen peroxide, protecting both host and pathogen (Long & Halliwell, 2011). For pathogenic bacteria such as *P. aeruginosa*, efficient uptake of TCA intermediates from the host is crucial for its survival, especially when it is bombarded with host reactive oxygen species, and requires the activity of bacterial carboxylate transport proteins. The transport proteins could be specific for C_4 , C_5 , and C_6 intermediates and may use a cognate TCS. This study suggests a complex regulatory cascade in modulating *P. aeruginosa* C_5 -dicarboxylate, α -KG uptake involving the PA5530 transporter, the MifS/MifR TCS and the sigma factor RpoN (Figure 2.14). It appears that MifS senses the presence of α -KG and signals MifR. The activated MifR in concert with RpoN initiates the transcription of α -KG-specific transporter gene PA5530. Analyses of the published data suggests that the PA5530 promoter is under several layers of regulation including catabolite repression mediated by Crc/CrcZ (Sonnleitner *et al.*, 2009) and the small non-

coding asRNA (Wurtzel *et al.*, 2012). Though the asRNA has been identified (Wurtzel *et al.*, 2012), it has not been characterized. It is not surprising that the PA5530 expression is potentially regulated by Crc, as it would allow control of transporter(s) in response to the presence of carbon sources in the environment. In addition to MifSR (PA5512/PA5511), PA1336/PA1335 have been identified to be homologous to the *Rhizobium* C₄-dicarboxylate transport regulatory DctB/DctD TCS (Stover *et al.*, 2000, Valentini *et al.*, 2011). However, the role of PA1336/PA1335 remains to be elucidated. The *P. aeruginosa* genome also encodes 19 other paralogs of PA5530 dicarboxylate transporters, most of which have share less than 50% similarity except for PA0229 (PcaT). PA0229 and PA5530 have 73% similarity. Future studies will determine if the transporters are preferentially or hierarchically upregulated depending on the carbon source. It is also important to note that much of bacterial physiology, particularly of pathogens such as *P. aeruginosa* remains a mystery. Metabolic versatility, expression of virulence factors and antibiotic resistance together makes *P. aeruginosa* an portentous pathogen. Thus, understanding the physiological cues and regulation would provide a better stratagem to fight the often indomitable infections.

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CHAPTER 3

Contemporary role for the ancient metabolite α -ketoglutarate in regulating *Pseudomonas aeruginosa* pathogenicity

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Abstract

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen responsible for causing incapacitating infections in individuals with impaired immunity. Successful infection and disease progression relies significantly on the ability of any pathogen to effectively utilize available nutrients that are essential for its growth and survival. Unlike other pathogenic bacteria where glucose is the preferred carbon source, *P. aeruginosa* preferentially utilizes tricarboxylic acid (TCA) cycle intermediates as carbon, nitrogen and energy sources. In *P. aeruginosa*, two-component system (TCS) signaling proteins have played an integral part in regulating the uptake of TCA cycle intermediates, specifically, C₄-dicarboxylates such as succinate, fumarate and malate. We recently identified a TCS protein pair PA5512/PA5511 (MifS/MifR) that in- tandem with the sigma factor RpoN (σ^{54}) facilitate the uptake of extracellular C₅-dicarboxylate α -ketoglutarate (α -KG) by activating the expression of *P. aeruginosa* α -KG-specific permease PA5530 (KgtP). In the present study, we demonstrate that besides KgtP, *P. aeruginosa* genome encodes a second α -KG transporter PA0229 (PcaT) whose expression is independent of the MifSR TCS, suggesting an additional layer of regulation involved in *P. aeruginosa* α -KG uptake. Importantly, in the recent years, α -KG has become the focal point of research because of its newly identified role as a signaling molecule in-addition to its conventional role in metabolism. To date, very little is known of the physiology of *P. aeruginosa* when provided with α -KG as the sole carbon source and their role in pathogenesis. Using PAO Δ mifS, PAO Δ mifR and PAO Δ mifSR mutants expressing the α -KG transport protein PcaT

in trans, we additionally investigated the role of MifSR TCS and α -KG in regulating *P. aeruginosa* virulence. Multiple virulence phenotypes were tested in the presence of α -KG, citrate and/or succinate, as a sole carbon source. In comparison to the wild-type PAO1 strain, *mifSR* deletion strains exhibited differential regulation of biofilm formation, pyocyanin and pyoverdine production, motility, and cell cytotoxicity in the presence of α -KG. Subsequently, *mifSR* mutant derivatives exhibited no discernible phenotypes compared to the parent PAO1 when α -KG was replaced with citrate and/or succinate, as the sole carbon source. Thus, MifSR TCS regulates the expression of *P. aeruginosa*'s key virulence determinants in α -KG-dependent manner. Simultaneous regulation of multiple mechanisms involved in *P. aeruginosa* pathogenesis suggests a complex mechanism of MifSR action. Understanding the physiological cues and regulation would provide a better stratagem to fight the often indomitable *P. aeruginosa* infections.

Introduction

Despite continuous efforts in the development of new antimicrobial drugs, bacterial infections are frequently associated with high morbidity and mortality rates. In particular, infections caused by the multidrug resistant bacteria, including, but not restricted to the so called "ESKAPE" pathogens (E*nterococcus faecium*, S*taphylococcus aureus*, K*lebsiella* species, A*cinetobacter baumannii*, P*seudomonas aeruginosa*, and E*nterobacter* species) (Rice, 2008), represent a major public health concern (Tommasi *et al.*, 2015) and economic burden worldwide (Boucher *et al.*, 2009). Amongst these organisms, *P. aeruginosa* is

frequently implicated in causing injurious infections (Rice, 2008), and has gained significant attention over the years as one of the most portentous pathogen.

P. aeruginosa is a Gram-negative, γ -proteobacteria that is widely distributed in nature (Green *et al.*, 1974). It is well-known for its nutritional and ecological resilience, which enables it to endure and thrive in diverse environmental settings, both natural and artificial (Ramos, 2004). Although, *P. aeruginosa* is a multi-host pathogen infecting insects (Bulla, 1975), nematodes (Mahajan-Miklos *et al.*, 1999), plants (Sing & Schroth, 1977, Rahme *et al.*, 1995) and mammals (Stevens *et al.*, 1994), it is the epitome of opportunistic human infections (Ramos, 2004). It is one of the most feared nosocomial pathogen, accounting for 10% of all the hospital acquired infections (Hancock & Speert, 2000, Sievert *et al.*, 2013). *P. aeruginosa* can colonize different tissue types leading to multifactorial clinical and pathological manifestations including pneumonia (Driscoll *et al.*, 2007), urinary tract infection (Driscoll *et al.*, 2007), gastrointestinal infection (Markou & Apidianakis, 2014), chronic suppurative otitis media (Yeo *et al.*, 2007, Mittal *et al.*, 2015), endocarditis (Fish *et al.*, 1937, Sandre & Shafran, 1996), osteomyelitis (Carek *et al.*, 2001), keratitis (Dart & Seal, 1988), bacteremia (Kerby, 1947) and soft-tissue infection (Moet *et al.*, 2007). The largest cohorts of *P. aeruginosa* infected patients are the ones with AIDS, severe burn wounds, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), diabetes, cardiovascular disease, cancer and those with acquired or primitive immunological abnormalities (Manfredi *et al.*, 2000, Ramos, 2004, Driscoll *et al.*, 2007, Vento *et al.*, 2008, Valderrey *et al.*, 2010). Also, beyond high infection rate, *P. aeruginosa*

has the highest case fatality rate of all Gram-negative infections (Aliaga *et al.*, 2002). *P. aeruginosa* is extensively armed with a set of multi-determinant virulence factors, both cell surface associated (flagella, pili, LPS) and secretory (proteases, exotoxins, phenazines, haemolysins), which modulates its pathogenic potential. In addition to its ability to dodge host immunity, a major setback in effectively treating *P. aeruginosa* infections is its extraordinary intrinsic and acquired resistance to a gamut of clinically critical antibiotics (Breidenstein *et al.*, 2011). Expression of diverse virulence factors and antibiotic resistance together, makes *P. aeruginosa* an indomitable pathogen.

Metabolic versatility is the key to bacterial pathogenesis and disease progression. In order to survive and multiply in the host systems, bacterial pathogens have developed mechanisms to regulate their metabolism according to the nutrient composition and availability (Silby *et al.*, 2011). Carbon and nitrogen are the two major nutrients required by all living organisms to perform fundamental cellular activities. *P. aeruginosa* uses an eclectic range of organic compounds including carbohydrates, amino acids, fatty acids, mono- and polyalcohols, di- and tri-carboxylic acids as carbon, nitrogen and energy source (Ramos, 2004). Although efficiently metabolized via the Entner-Doudoroff pathway, sugars are less preferred carbon source (Entner & Doudoroff, 1952, Lessie & Phibbs, 1984). Instead, *P. aeruginosa* has a predilection for tricarboxylic acid (TCA) cycle intermediates, specifically, C₄-dicarboxylates of the TCA cycle such as malate, fumarate and succinate (Liu, 1952, Collier *et al.*, 1996).

The TCA cycle, classical (Krebs, 1940) or the modified form (Romano & Conway, 1996, Hugler *et al.*, 2005, Zhang & Bryant, 2011), is one of the most important central metabolic pathway used by all organisms to generate energy. Essentially, the TCA cycle plays a dual role in cell metabolism: 1) it produces carbon dioxide and reduced electron carriers (NADH and FADH₂) by oxidation of acetyl-CoA to generate energy (catabolism), and 2) provides cells with precursor metabolites as building blocks for the synthesis of carbohydrates, fatty acids, nucleic acids and amino acids (anabolism) (Owen *et al.*, 2002). Because of its amphibolic nature and ubiquity, it is not surprising that different organisms use and modulate TCA cycle according to their metabolic requirements, thus giving rise to a considerable diversity in its way of operating. Moreover, it is becoming evident that the TCA cycle is embedded in a more complex network of metabolic pathways, and its intermediates, beyond their canonical function play a profound role as intra- and extracellular signals (Benit *et al.*, 2014, Huergo & Dixon, 2015, Haas *et al.*, 2016).

TCA cycle intermediates are organic acids, that based on the number of carbon atoms are classified into three groups C₄-dicarboxylates (succinate, fumarate, malate and oxaloacetate), C₅-dicarboxylate (α -Ketoglutarate (α -KG)) and C₆-tricarboxylates (citrate and iso-citrate) (Krebs, 1940). Amongst these, the C₅-dicarboxylate α -KG has gained significant attention because of its newly established role as a signaling molecule in-addition to its conventional role in metabolism. There is mounting evidence that corroborates the role of α -KG as a signaling molecule in all three domains of life, archaea (Dodsworth *et al.*, 2005),

bacteria (Ninfa & Jiang, 2005) and eukaryota (Lancien *et al.*, 2000, He *et al.*, 2004). Indeed, it is now known that α -KG plays a key role in many signaling pathways including, but not limited to, regulation of carbon/nitrogen metabolism (Doucette *et al.*, 2011), cyclic AMP (cAMP) synthesis (Rabinowitz & Silhavy, 2013), paracrine mediator in regulating renal acid-base balance (Tokonami *et al.*, 2013) and in epigenetic regulation mediated through histone modification (Letouze *et al.*, 2013, Carey *et al.*, 2015) and α -KG-dependent oxygenases (Loenarz & Schofield, 2008).

In bacteria, intracellular levels of α -KG acts as a key indicator of the cellular C/N status (Doucette *et al.*, 2011). α -KG provides the major carbon skeleton for nitrogen assimilation pathways (Commichau *et al.*, 2006). High levels of intracellular α -KG signals nitrogen starvation, thereby activating the transcription of nitrogen assimilation genes (Leigh & Dodsworth, 2007). It is now well established that α -KG directly regulates nitrogen metabolism by binding and modulating the activity of either the nitrogen regulatory PII protein GlnB in *E. coli* (Ninfa & Jiang, 2005) and/or the transcription factor NtcA in cyanobacteria (Tanigawa *et al.*, 2002, Vazquez-Bermudez *et al.*, 2002). Signal perception mechanisms mediated by α -KG, nitrogen regulatory PII and NtcA proteins has been studied and reviewed extensively (Forchhammer, 2008, Forchhammer, 2010, Huergo *et al.*, 2013, Huergo & Dixon, 2015). Furthermore, endogenous α -KG acts as a reactive oxygen species scavenger, particularly of hydrogen peroxide thus acting as a protectant against oxidative damage (Mailloux *et al.*, 2009, Lemire *et al.*, 2010) and cyanide poisoning (Kunz *et al.*, 1998). Recent studies have reported the use of exogenous α -KG as a preferred carbon source during the

course of infection by pathogenic bacteria (Guo *et al.*, 2012, Cai *et al.*, 2013). This together suggests a more intricate and intense role of α -KG in regulating bacterial physiology and metabolism.

Sensing the available nutrient is a prerequisite for bacterial growth and virulence. Bacteria have evolved various host-adapted nutrient acquisition strategies to modulate the uptake systems. One canonical mechanism by which most bacteria sense and respond to the available nutrient is the two-component systems (TCSs). TCSs are made of two signal transduction proteins, a membrane-bound histidine sensor kinase (HK) and a cytoplasmic response regulator (RR), usually a DNA binding protein (Gross *et al.*, 1989). In response to stimuli, the sensor autophosphorylates at a conserved histidine residue at the C-terminus, and subsequently the phosphate is transferred to an aspartate residue at the N-terminus of the RR (Stock *et al.*, 1989, Stock *et al.*, 2000). The phosphorylated RR then modulates the expression of various genes required to bring about the appropriate response (Bourret *et al.*, 1989). Given the importance of TCA cycle intermediates as a preferred carbon source in numerous bacteria (Finan *et al.*, 1983, Collier *et al.*, 1996, Tang *et al.*, 2005), it is not surprising that the TCSs are involved in regulating their uptake. In both, Gram-negative and Gram-positive bacteria TCS proteins have been implicated in regulating the transport and utilization of TCA cycle intermediates, particularly the C₄-dicarboxylates (succinate, fumarate and malate) and the C₆-tricarboxylate (citrate) (Asai *et al.*, 2000, Janausch *et al.*, 2002). However, not much is known about the assimilation of the C₅-dicarboxylate α -KG.

The TCS proteins responsive to extracellular α -KG has been identified in limited number of bacterial species, including *Escherichia coli* (Cai *et al.*, 2013), *Rhizobium tropici* (Batista *et al.*, 2009) and most recently in *P. aeruginosa* (Tatke *et al.*, 2015). In *P. aeruginosa*, a TCS protein pair PA5512/PA5511 (MifS/MifR) together with the sigma factor RpoN (σ^{54}) regulates the transport of extracellular α -KG by activating the expression of the α -KG-specific permease PA5530, a Major Facilitator Superfamily (MFS) of secondary active transporter protein (Lundgren *et al.*, 2014, Tatke *et al.*, 2015). *P. aeruginosa* MifS/MifR pair is homologous to the *Rhizobium meliloti* C₄-dicarboxylate transport regulatory DctB/DctD TCS (Winsor *et al.*, 2011). No role of *P. aeruginosa* MifS and MifR in the utilization of TCA cycle C₄-C₆ di and tricarboxylates (succinate, fumarate, malate, oxaloacetate and citrate) has been established (Tatke *et al.*, 2015). Thus, *P. aeruginosa* MifSR TCS is specifically and uniquely involved in α -KG utilization.

Previously, the MifR protein has been identified to be essential for regulating *P. aeruginosa* biofilm formation (Petrova & Sauer, 2009) and pyruvate fermentation (Petrova *et al.*, 2012). Additionally, in *P. aeruginosa*, nutritional cues such as individual carbon and nitrogen sources have been known to influence several clinically relevant virulence phenotypes such as motility, biofilm development, quorum sensing and pigment production (Kohler *et al.*, 2000, Klausen *et al.*, 2003(a), Palmer *et al.*, 2005, Shrout *et al.*, 2006, Palmer *et al.*, 2007). To date, very little is known about the physiology of *P. aeruginosa* when provided with α -KG as the sole carbon source. Here we show that, MifS/MifR TCS and the C₅-dicarboxylate α -KG plays a perspicacious role in regulating *P.*

aeruginosa pathogenicity by modulating its key virulence traits, including, motility, pigment production, biofilm formation and cell cytotoxicity. This study is one of the first to report a novel role of the regulatory mechanism exerted by MifSR TCS and significantly highlights the potential role of α -KG as a signaling molecule in *P. aeruginosa*.

Materials & Methods

Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. Briefly, strains of *Pseudomonas aeruginosa* and *Escherichia coli* were routinely cultured in Luria Bertani (LB) broth (5 g tryptone, 10 g sodium chloride, and 5 g yeast extract per liter) or agar (LB broth with 1.5% agar) (Difco, NJ, USA) or in M9 minimal Media (64 g Na₂HPO₄·7H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5.0 g NH₄Cl, 20 mM MgSO₄, 1 mM CaCl₂ per liter) (Sambrook & Russell, 2001) at 37°C, unless specified otherwise. *P. aeruginosa* competent cells were prepared using the sucrose (300 mM) method as previously described (Choi *et al.*, 2006). Growth curves, complementation studies and phenotypic assays were performed in M9 minimal media supplemented with TCA cycle intermediates, α -KG, succinate and/or citrate (at 30 mM each), as described previously (Tatke *et al.*, 2015). For selection and maintenance of plasmids, antibiotics when used, were at the following specified concentrations, unless specified otherwise: *E. coli*: ampicillin (Ap) 100 µg/ml, gentamycin 15 µg/ml, kanamycin (Km) 20 µg/ml, *P. aeruginosa*: Gm 75 µg/ml.

Cell culture and media

A549, a human lung adenocarcinoma epithelial cell line was provided by Dr. Rahul Mittal (University of Miami, Miller School of medicine, Miami, FL). The J774 E-clone, a murine macrophage cell line, also a generous gift from Dr. Philip Stahl (Washington University Medical school, St Louis, MO) and the human epithelial adenocarcinoma cell line was obtained from ATCC (ATCC[®]CCL-2[™]). J774 E-clone cell lines were maintained under a 5% CO₂ atmosphere in Dubelco's Minimum Essential Medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 Units/mL penicillin and 100 µg/mL of streptomycin. A549 cells were grown in Roswell Park Memorial Institute Medium (RPMI) 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 24 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 Units/mL penicillin, 100 µg/mL of streptomycin and incubated at 37°C in 5% CO₂. HeLa cells were cultured in DMEM supplemented with 10% FCS and 2 mM L-glutamine and 1% non-essential amino acids in presence of 5% CO₂ at 37°C. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Genetic manipulations

Genetic manipulations were carried out as previously described using standard techniques (Green *et al.*, 2012). Polymerase Chain Reaction (PCR) amplification was performed using the AccuPrime high-fidelity Taq DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA). Primers for PCR and sequencing were synthesized by Integrated DNA Technologies, Inc.

(Coralville, IA, USA) and are listed in Table 3.2. DNA purification and plasmid DNA isolation was carried out using nucleic acid purification kits (Promega, Madison, WI, USA) and PureLink Hipure Plasmid Miniprep Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Wizard® SV Gel and PCR Clean-Up System kits (Promega, Madison, WI, USA) were used for PCR product and agarose gel fragment purification. RNA extraction and cDNA synthesis was performed using RNeasy Mini Kit (Qiagen Inc. Venio, Limburg, Netherlands) and SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA). Restriction enzymes were from New England Biolabs (Ipswich, MA, USA), and DNA sequencing was carried out at Florida International University (FIU) DNA core and at GENEWIZ Inc. (South Plainfield, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Amresco (Solon, OH, USA) and ThermoFisher Scientific (Waltham, MA, USA), unless otherwise specified.

PCR amplification and cloning of *pcaT*

DNA fragments from PAO1 with *pcaT* (~1.3 kb) and *PA0703* (~1.3 kb) were PCR amplified using primer pairs GDT_*pcaTF1*-GDT_*pcaTR1* (Table 3.2), respectively. The primers are designed such that the ORF is contiguous to a strong ribosome binding site (AGGAGA), to ensure expression of the genes (Lehninger *et al.*, 2013). The PCR amplified *pcaT* was cloned directly into a broad host range pPSV37-Gm (Lee *et al.*, 2010) plasmid as a *NheI*-*SacI* fragments, downstream of an inducible P_{lacUV5} promoter to generate plasmids pGDT007, respectively. The fidelity of the PCR product was confirmed by sequencing using the primers

GDT_p37_SeqF-R, *pcaT*_seqF-R and *PA0703*_seqF-R (Table 3.2). Henceforth, this plasmid is referred to as pPcaT, respectively.

The expression plasmid pPcaT was subsequently introduced by electroporation (Choi *et al.*, 2006) into PAO1 and its isogenic mutants PAO Δ *mifS*, PAO Δ *mifR*, PAO Δ *mifSR* and PAO Δ *rpoN*.

Growth curves

Growth curves were performed as described previously (Tatke *et al.*, 2015). Briefly, *P. aeruginosa* wild type PAO1 and its isogenic mutant derivatives were grown overnight in LB broth with or without antibiotics at 37°C. After overnight growth, spent and residual media was removed by centrifugation and the cultures were washed with sterile 0.85% NaCl (wt/vol) solution. Cultures were then diluted in fresh M9 minimal media supplemented with TCA cycle intermediates including α -KG, succinate and/or citrate (at 30 mM each) as a sole carbon source to obtain equal optical densities (OD₆₀₀) of 0.025. Growth was monitored in 48-96 well plates (Corning-Falcon, NY, USA) by determining absorbance at 600 nm using BioTek Synergy HT plate reader for 14-18 h at 37°C. The experiments were performed multiple times in triplicates.

Motility assays

Motility assays were examined on 0.3%, 0.6% and 1% (wt/vol) M9 minimal media agar plates as a described previously (Deziel *et al.*, 2001). All cultures were grown overnight in LB media at 37°C. Overnight cultures were washed with sterile 0.85% NaCl (wt/vol) solution and diluted to OD₆₀₀ of 1. Swimming and swarming motilities were evaluated by spotting 1-2 μ l cultures onto 0.3% and 0.6% (wt/vol)

M9 minimal media agar plates with α -KG or citrate (at 30 mM each) as a sole carbon source. Twitching motility was evaluated by stab inoculating OD₆₀₀ normalized cultures onto 1% (wt/vol) M9 minimal media agar plates with α -KG or citrate (at 30 mM each) as a sole carbon source. Plates were incubated at 37°C and motility patterns were assessed 24 to 48 h post-incubation. Diameters of the swim or twitch zones were measured in millimeter (mm) and swarming was assessed by determining the surface coverage on agar plates post incubation. Each form of motility was assessed multiple times with three replicates for each mutant.

Rhamnolipid assay

P. aeruginosa wildtype PAO1, PAO1(pPcaT), PAO Δ *mifS*(pPcaT), PAO Δ *mifR*(pPcaT) and PAO Δ *mifSR*(pPcaT) strains were assayed for rhamnolipid production using the cetyltrimethylammonium bromide (CTAB) methylene blue agar plate assay as described previously (Siegmund & Wagner, 1991). Briefly, all cultures were grown overnight at 37°C in LB broth. Spent and residual media from the overnight culture was removed by centrifugation, and the cultures were washed with sterile 0.85% NaCl (wt/vol) solution. Rhamnolipid production was evaluated by spot inoculating OD₆₀₀ normalized cultures on M9 minimal media supplemented with 30 mM α -KG (unless otherwise specified), 0.0005% (wt/vol) methylene blue and 0.02% (wt/vol) CTAB. Plates were incubated at 37°C for 24 h followed by incubation at room temperature for at least 48 h until a blue halo appeared around the colonies, indicating rhamnolipid production (Siegmund & Wagner, 1991). The

diameter of the blue halo (in millimeter (mm)) around the colonies was measured as a mark of rhamnolipid production.

Pyocyanin production

Pyocyanin produced was measured by extracting the pigment from culture supernatants using the chloroform-hydrochloric acid (HCL) method as described previously (Essar *et al.*, 1990). Briefly, 5-ml culture supernatant from the stationary-phase cultures (~18 h) grown in M9 minimal media supplemented with TCA cycle intermediates including α -KG, citrate and succinate (30 mM each) individually as a sole carbon source was mixed with 3 ml chloroform to extract pyocyanin into the organic phase. Pyocyanin from the organic phase was then re-extracted into 1 ml of 0.2 N HCl. The resulting pink color formation, an indication of the presence of pyocyanin was read at 520 nm. The concentration is expressed as μg of pyocyanin produced per ml of culture ($\mu\text{g}/\text{ml}$), by multiplying the optical density OD_{520} by 17.072 (Essar *et al.*, 1990). Absorption spectra analysis (250 nm to 800 nm) to detect pyocyanin from the cell free culture supernatants, was performed using the Beckman Coulter Inc., DU 800 spectrophotometer.

Pyoverdine production

To measure pyoverdine production, cells were grown overnight at 37°C in M9 minimal media supplemented with α -KG, succinate and/or citrate, individually (at 30 mM each) as a sole carbon source. Extracellular pyoverdine from the culture supernatant was read at 405 nm and normalized to the initial culture density (OD_{600}). Pyoverdine levels were expressed as a ratio of $\text{OD}_{405}/\text{OD}_{600}$ (Shen *et al.*, 2002). Absorption spectra analysis (250 nm to 800 nm) to detect pyoverdine from

the cell free culture supernatants, was performed using the Beckman Coulter Inc., DU 800 spectrophotometer.

Cytotoxicity assay

P. aeruginosa wild-type PAO1 and its isogenic PAO Δ *mifR*, PAO Δ *mifS*, PAO Δ *mifSR*, mutant strains and its derivatives were used for the cytotoxicity assay. Their cytotoxicity was assessed using a human lung adenocarcinoma epithelial cell line (A549), a murine macrophage cell line (J774 E-clone) and HeLa cells. Bacterial cytotoxicity was quantified by measuring the lactate dehydrogenase (LDH) enzyme activity from the culture supernatant post 24 h infection with *P. aeruginosa* strains (Hauser & Engel, 1999). Briefly, monolayers of J774 E-clone, A594 and HeLa cells (10^6 cells/mL) were incubated with bacterial suspension containing 10^8 CFU/mL of bacteria. Overnight bacterial cells were subcultured into M9 minimal media with α -KG (30 mM) to a final OD_{600 nm} of 0.1. The infection was synchronized by centrifugation for 5 minutes at 1000 x *g* and incubated for 24 hours at 37°C in 5% CO₂. Cell-free culture supernatant were then collected and the amount of lactate dehydrogenase (LDH) enzyme activity was assayed in a 96-well plate using the Sigma kit as per manufacturer's instructions (Sigma Aldrich, St. Louis, MO). Triplicate wells were used for each test, and all tests were repeated at least three times.

Biofilm formation

Biofilm formation was assessed on 96-well polystyrene flat-bottomed (Costar, Corning NY) and/or on non-tissue culture-treated 96-well polyvinyl chloride (PVC) U-bottom (Falcon) microtiter plates, as described previously

(Friedman & Kolter, 2004, O'Toole, 2011). Briefly, PAO Δ *mifS*(pPcaT), PAO Δ *mifR*(pPcaT), PAO Δ *mifSR*(pPcaT), PAO1 (pPcaT) as well as the control strains PAO1(wildtype), PAO Δ *retS* (positive control, hyperbiofilm producer) and PAO Δ *pel* Δ *psl* Δ *algD* (negative control, no biofilm producer) were grown in LB broth overnight at 37°C. Cultures were then diluted to an OD₆₀₀ of 0.0025 in M9 minimal media supplemented with α -KG, succinate or citrate individually (at 30 mM each). The plates were incubated under static condition at room temperature for 24, 48 and 72 hours. At each time point, the culture was decanted and the plates were washed by gently submerging them in a small water tub in order to remove the unattached cells, and the plates were dried for 10-15 minutes at room temperature. To stain the biofilm, 0.1% solution of crystal violet (St. Louis, MO, USA) in water was added to each well (200 μ L). After 20-minute incubation at room temperature, crystal violet was decanted, and the plates were washed 7 to 8 times in a small water tub. Plates were then inverted and left to dry overnight. Quantification of the attached and stained cells was done after solubilization of the dye with absolute ethanol (200 μ L). The optical density of the solution reflecting the relative biofilm formation was measured at 590 nm using BioTek Synergy HT (Winooski, VT, USA) and expressed as relative biofilm formation (OD₅₉₀ mutant / OD₅₉₀ wildtype) (Ueda & Wood, 2009). The experiments were performed multiple times in triplicates.

RNA isolation and cDNA synthesis

P. aeruginosa wild-type PAO1 and its isogenic PAO Δ *mifR*, PAO Δ *mifS*, PAO Δ *mifSR*, PAO Δ *rpoN* mutant strains and its derivatives were used for RNA extraction. Total RNA was extracted from cells grown to mid-exponential phase

with or without α -KG (at 30 mM) treatment. Briefly, cultures were grown overnight in LB broth at 37°C. Post incubation overnight cultures were washed with sterile 0.85% saline solution to remove spent media and were subcultured at 37°C, 200 rpm in LB media. LB media was used as a preferred nutrient source for initial growth of cultures since *PAO Δ mifR*, *PAO Δ mifS*, *PAO Δ mifSR* and *PAO Δ rpoN* mutants exhibit growth defects in the presence of α -KG alone. When the cells reached an OD₆₀₀ of 0.6-0.7, all the cultures were treated with 30 mM α -KG for 2 h. Post treatment, the RNA was stabilized by adding phenol-ethanol mixture (Brencic *et al.*, 2009). The stabilized RNA was then isolated using RNeasy Mini Kit (Qiagen Inc. Venio, Limburg, Netherlands) as per manufacturer's protocol. Residual genomic DNA contamination was removed using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and RNA was repurified using RNeasy Mini Kit (Qiagen Inc. Venio, Limburg, Netherlands). RNA quality was preliminarily checked on a denaturing agarose gel (NorthernMax Gly, Ambion) followed by analysis on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA Integrity Numbers of 8.0 or above were subsequently used for cDNA synthesis. cDNA was synthesized by annealing NS5 random primers to total purified RNA and subsequent extension was carried out using SuperScript III reverse transcriptase, as per the manufacturers protocol (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Quantitative real-time PCR

P. aeruginosa genes encoding α -KG transporter proteins (*pcaT* and *PA5530* (*kgtP*)) and pyocyanin synthesis (*phzA1*, *phzB1*, *phzA2*, *phzM* and *phzS*)

were amplified (qRT-PCR) under α -KG induced condition at 30 mM, using the ABI 7500 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) cyclor with Power SYBR Green PCR MasterMix with ROX (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The gene-specific primers used to quantitate the expression levels of the transcript are listed in Table 3.2. Ten nanograms of cDNA was used per reaction well in the qRT-PCR assays. The cycling conditions used were 95°C/2 minutes (holding); 40 cycles of 95°C/15 sec, 60°C/1 min (cycling); 95°C/15 sec, 60°C/1 min, 95°C/15 sec (0.6°C ramp) (melt curve). The reading was normalized to *P. aeruginosa clpX* (PA1802), whose expression was determined to remain constant between the samples and conditions tested. The qRT-PCR assays for each gene was repeated twice in triplicate, and the mean value of the quantification was calculated. Melting curves were determined to ensure primer specificity.

Bioinformatic and statistical analyses

Sequence analyses and multiple sequence alignments were generated using ClustalW2 <http://www.ebi.ac.uk/Tools/msa/clustalw2/> and www.pseudomonas.com (Winsor *et al.*, 2011). Student's *t*-test or Analysis of Variance (ANOVA) with post-hoc testing, when applicable, was used to analyze statistical differences. The *t*-test and ANOVA were performed using GraphPad and IBM SPSS statistics 22.0 statistical analysis software. A *p*-value of < 0.05 was considered statistically significant.

Table 3.1: Strains and plasmids used in this study.

Strain ID	Strain/Plasmid Background	Relevant characteristics	Source
<i>Escherichia coli</i>			
DH5α	<i>E. coli</i>	F ⁻ Φ80/ <i>lacZ</i> ΔM15Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ mk ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	New England Biolabs
<i>Pseudomonas aeruginosa</i>			
PAO1		Prototypic wild type	(Stover <i>et al.</i> , 2000)
PKM900	PAO1	Δ <i>mifS</i> (PA5512)	PAOΔ <i>mifS</i> (Tatke <i>et al.</i> , 2015)
PKM901	PAO1	Δ <i>mifR</i> (PA5511)	PAOΔ <i>mifR</i> (Tatke <i>et al.</i> , 2015)
PKM902	PAO1	Δ <i>mifSR</i> (PA5511-PA5512)	PAOΔ <i>mifSR</i> (Tatke <i>et al.</i> , 2015)
Plasmids			
pPSV37		Gm ^R ; <i>colE1 oriT lacI^q P_{lacUV5}</i>	(Lee <i>et al.</i> , 2010)
pGDT004	pPSV37	Gm ^R ; The <i>mifR</i> ORF subcloned from pGDT002 as an <i>NheI</i> - <i>SacI</i> fragment into pPSV37	(Tatke <i>et al.</i> , 2015)
pGDT005	pPSV37	Gm ^R ; A ~3.0-kb <i>NheI</i> - <i>SacI</i> fragment containing <i>mifSR</i> (PA5511-PA5512) ORFs amplified from PAO1 genome using HK_ <i>mifSF</i> 1 and GDT_ <i>mifRR</i> 1 primers and cloned directly into <i>NheI</i> - <i>SacI</i> -cut in pPSV37	(Tatke <i>et al.</i> , 2015)
pGDT006	pPSV37	Gm ^R ; A ~1.3-kb <i>NheI</i> - <i>SacI</i> fragment containing PA5530 ORF amplified from PAO1 genome using GDT_PA5530F1 and GDT_PA5530R1 primers	(Tatke <i>et al.</i> , 2015)

pGDT007	pPSV37	and cloned directly into <i>NheI</i> - <i>SacI</i> -cut in pPSV37 Gm ^R ; A ~1.3-kb <i>NheI</i> - <i>SacI</i> fragment containing <i>pcaT</i> (PA0229) ORF amplified from PAO1 genome using GDT_ <i>pcaTF</i> 1 and GDT_ <i>pcaTR</i> 1 primers and cloned directly into <i>NheI</i> - <i>SacI</i> -cut in pPSV37	This study
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Table 3.2: Primers used in this study.

Primer Name	Sequence
GDT_ <i>pcaTF</i> 1	5'- <u>GCTAGCAGAAAGGAGA</u> TATACCATGACTGCCCCTG TTCAC-3'
GDT_ <i>pcaTR</i> 1	5'-TTT <u>GAGCTC</u> CCGTCAGGGCTCGTCGTT-3'
GDT_p37_SeqF	5'-GACCCGTTTAGAGGCCCAA-3'
GDT_p37_SeqR	5'-CGTGCTTTACACTTTATGCTTCCGG-3'
<i>pcaT</i> _seqF	5'-TTCGTGATCGGCGCGATC -3'
<i>pcaT</i> _seqR	5'-CAGCCAGAGGGCGATCAC-3'
DBS_qRT_clpXF	5'- TGCGATTACGATGTGGAGA -3'
DBS_qRT_clpXR	5'- CCCTCGATGAGCTTCAGCA -3'
GDT_qRT_ <i>pcaTF</i>	5'- TGCGATTACGATGTGGAGA -3'
GDT_qRT_ <i>pcaTR</i>	5'- CCCTCGATGAGCTTCAGCA -3'
GDT_qRT_PA5530F	5'-CGCAACGCATCAAGTCGAT-3'
GDT_qRT_PA5530R	5'-AGTCGTACCACTCGACCAGGTT-3'
qRT_ <i>phzA1F</i>	5'- GGCACAACGTGCGGATCT -3'
qRT_ <i>phzA2R</i>	5'- CGCACTCGACCCAGAAGTG-3'
qRT_ <i>phzA2F</i>	5'- GCCCTGCGCGAATACG -3'
qRT_ <i>phzA2R</i>	5'- CCATTCCTCTTTTCCCGTTCT -3'
qRT_ <i>phzSF</i>	5'- GCCCTGCGCGAATACG -3'
qRT_ <i>phzSR</i>	5'- CCATTCCTCTTTTCCCGTTCT -3'
qRT_ <i>phzMF</i>	5'- GAGCGCATCCATCGACTGAT -3'
qRT_ <i>phzMR</i>	5'- GGTATCGCCCTGGAAGATCTC -3'

Results and Discussion

Acquisition of energetically favorable carbon and energy sources from the extracellular milieu of nutrients is of paramount importance to bacterial survival and disease progression. The C₅-dicarboxylate α -KG is a key intermediate of the energy-generating TCA cycle and plays an important role in regulating the cellular carbon and nitrogen metabolism (Doucette *et al.*, 2011). An important aspect of the nutrient acquisition process is their transport from the environment into the cells via the transporter proteins. The transport of α -KG has been studied in only a handful of bacteria including *P. aeruginosa* (Lundgren *et al.*, 2014, Tatke *et al.*, 2015), *E. coli* (Seol & Shatkin, 1991, Cai *et al.*, 2013), *R. tropici* (Batista *et al.*, 2009), *X. oryzae* (Guo *et al.*, 2012) and *Staphylococcus aureus* (Tynecka *et al.*, 2001). In *P. aeruginosa*, *E. coli* and *R. tropici* extracellular α -KG transport is arbitrated by TCS proteins MifS/MifR (Tatke *et al.*, 2015), KguS/KguR (Cai *et al.*, 2013) and KgtS/KgtR (Batista *et al.*, 2009), respectively. The present study further explores the role of *P. aeruginosa* *mifSR* encoding the sensor kinase MifS and response regulator MifR in regulating α -KG transport. Furthermore, using the PAO Δ *mifS*, PAO Δ *mifR* and PAO Δ *mifSR* mutants and their derivatives, we additionally investigated the role of MifSR TCS and the C₅-dicarboxylate α -KG in regulating *P. aeruginosa* virulence.

***P. aeruginosa* possess multiple α -KG transport proteins**

The *E. coli* transporter KgtP is the most extensively studied α -KG permease (Seol & Shatkin, 1991, Seol & Shatkin, 1992, Seol & Shatkin, 1993). A homology search in *P. aeruginosa* PAO1 genome revealed the presence of two ORFs

PA0229 (*pcaT*) and PA5530 encoding the putative dicarboxylic acid transport proteins that are homologs of *E. coli* α -KG permease KgtP. After here we name *P. aeruginosa* PA5530 as KgtP. In recent studies, KgtP has been characterized and identified as the functional α -KG transporter, whose expression is regulated by the MifSR TCS in coordination with the alternate sigma factor RpoN (σ^{54}) (Lundgren *et al.*, 2014, Tatke *et al.*, 2015). In *P. putida*, PcaT has been implicated in the transport of β -ketoadipate (Ondrako & Ornston, 1980). However, in *P. aeruginosa* PcaT function was not previously explored.

Our sequence analyses of *P. aeruginosa* PcaT and KgtP with *E. coli* KgtP using BLASTP revealed that *P. aeruginosa* PcaT (PA0229) is the closest *E. coli* KgtP homolog with 88% similarity and 70% identity, suggesting a similar function. *P. aeruginosa* PcaT and KgtP share 72% similarity and 55% identity, and have the conserved protein domain family PRK10406 implicated in α -KG transport (Marchler-Bauer *et al.*, 2015). This data strongly suggests that *P. aeruginosa* PcaT is capable of transporting α -KG into the cells. To confirm the role of *P. aeruginosa* *pcaT* in α -KG uptake, *pcaT* was amplified and subcloned downstream of the inducible P_{lacUV5} promoter to generate the plasmid pPcaT. The plasmid pPcaT was then introduced into the wild-type PAO1 and its isogenic *mifSR* mutant strains. The PAO Δ *mifS*, PAO Δ *mifR* and PAO Δ *mifSR* mutants exhibit an α -KG-dependent growth defect due to the deficiency in the transport of extracellular α -KG (Tatke *et al.*, 2015). Expression of *pcaT in trans* in the *mifSR* mutants restored their growth to a level comparable to the wild-type PAO1 in M9 minimal media with α -KG (30 mM) as the sole carbon source (Figure 3.1).

Figure 3.1: Growth of *mifSR* mutants in the presence of α -KG and pPcaT. Growth curves of *P. aeruginosa* wildtype PAO1 and *mifSR* mutants expressing *pcaT*, encoding the putative carboxylic acid transporter *in trans* in M9 minimal media with α -KG (30 mM). Results shown are mean with standard deviation of three biological replicates.

Table 3.3: Growth Properties of the *mifSR* mutants and its derivatives in the presence of α -KG

	PAO1	$\Delta mifR$	$\Delta mifS$	$\Delta mifSR$
EV	+++	---	---	---
pPcaT	+++	+++	+++	+++
pPA5530	+++	+++	+++	+++

Furthermore, growth of the wild-type PAO1 was not affected by the expression of an extra copy of the *pcaT* gene (Figure 3.1). This finding confirms the role and ability of *P. aeruginosa* PcaT in transporting extracellular α -KG into the cells.

Biochemical studies have hinted to the presence of inducible α -KG transporters in *Pseudomonas* spp (Campbell & Stokes, 1951, Edwards *et al.*, 1979). This was supported by our recent findings where the expression of *P. aeruginosa* *kgtP* gene encoding the α -KG permease was significantly induced by α -KG (Tatke *et al.*, 2015). To test if the expression of *pcaT* gene is induced by the presence of extracellular α -KG, qRT-PCR analysis was performed. Expression of *pcaT* was tested in the wild-type PAO1 grown with varying amounts of α -KG (1 – 80 mM). The presence of α -KG resulted in the activation of *pcaT* expression in a concentration independent manner (Figure 3.2). This data suggests that *pcaT* expression is induced by α -KG.

The *mifSR* mutants failure to grow in the presence of α -KG is the result of a defect in the expression of α -KG transporter proteins (Tatke *et al.*, 2015). This growth deficient phenotype can be rescued by uncoupling the MifSR regulatory control over *pcaT* and *kgtP* by expressing these genes under a regulatable promoter (Table 3.3). This suggests that MifSR TCS, similar to *kgtP*, regulates *pcaT* expression. To determine if this is true, we looked at the *pcaT* and *kgtP* RNA levels in the $\Delta mifS$, $\Delta mifR$ and $\Delta mifSR$ mutant strains. Corroborating our previous findings (Tatke *et al.*, 2015), expression of *kgtP* was downregulated in the *mifSR* deficient strains (Figure 3.3). However, surprisingly, *pcaT* expression was upregulated in the absence of *mifSR* and the presence of α -KG (Figure 3.3). This

suggests that MifSR TCS negatively regulates *pcaT* expression. In the experimental set up, the regulatory control of *pcaT* was uncoupled by expressing it under P_{lacUV5} . In the *mifSR* mutants, there is an intact *pcaT*, however it is unable to rescue the α -KG dependent growth defect (Table 3.3), despite the increased expression (Figure 3.3). This implies that perhaps *pcaT* expression is controlled post-transcriptionally by an un-identified factor which requires intact MifSR. These findings strongly suggests that MifSR TCS inversely regulates the expression of *P. aeruginosa* *pcaT* and *kgtP*.

Our sequence analysis of the *pcaT* upstream promoter (P_{pcaT}) region revealed the presence of a putative weak RpoN (σ^{54}) sigma factor binding -24/-12 sequence: TGGCAC-N7-TCGCC (Barrios *et al.*, 1999) and an A-rich motif adjacent to the ribosome binding site with a signature sequence (AAc/uAAc/A) for the catabolite repression control (Crc) protein (Figure 3.4) (Sonnleitner *et al.*, 2009, Browne *et al.*, 2010). Crc is a post- transcriptional inhibitor that prevents translation initiation by binding to the mRNA (Moreno *et al.*, 2007, Sonnleitner *et al.*, 2009). In addition, we identified four putative GGA trinucleotide motifs in the P_{pcaT} , a characteristic binding site recognized by the post-transcriptional control RsmA family of proteins (Brencic & Lory, 2009). Also, in the predicted secondary structure of the *pcaT* leader sequence using Mfold we found that the GGA motifs are exposed in the steam-loop structures which could acts as a substrate for RsmA binding, as has been observed for several known RsmA targets (Figure 3.4). Though the role of Crc and RsmA in α -KG transport has to be verified

experimentally, it suggests an additional layer of regulation controlling *pcaT* expression.

Figure 3.2: *pcaT* gene expression in response to α -KG. qRT-PCR was used to measure the relative quantities of *pcaT* mRNA after *P. aeruginosa* PAO1 cells were induced for an hour with varying concentration of α -KG (1 to 80 mM). Bars above or below the line represents up- and down-regulation, respectively, and the error bars indicate standard errors. Data were normalized to expression in PAO1 uninduced cells. The *clpX* gene (*PA1802*) was used as the housekeeping control. Statistically significant difference as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001 compared with expression in the uninduced condition.

Figure 3.3: Quantification of *pcaT* mRNA in *mifSR* mutants. The expression of *pcaT* was tested in the *mifSR* single and double deletion mutants relative to the wildtype PAO1, with cells exposed to α -KG (30 mM) for an hour. Data was normalized to expression in PAO1. Bars above or below the line represents up- and down-regulation, respectively, and the error bars indicate standard errors. Furthermore, expression of the recently reported PA5530 gene encoding the α -KG transporter protein [Our Reference] was tested. The *clpX* gene (*PA1802*) was used as the housekeeping control. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001.

Figure 3.4 Promoter analysis of α -KG permease encoding *pcaT* gene. Genome organization of *pcaT* gene locus (A). Sequence analysis of the 148-bp (P_{pcaT}) showing weak -12 and -24 RpoN (σ^{54}) dependent promoter like element (Purple). P_{pcaT} possess the signature sequence (AAC/uAAc/uAA) for catabolite repression control (Crc) protein (brown box). Yellow box depicts the four putative GGA trinucleotide motifs in the P_{pcaT} , a characteristic binding site recognized by the post-transcriptional control RsmA family of proteins.

MifS/MifR TCS regulates *P. aeruginosa* virulence

Success of *P. aeruginosa* as a recalcitrant pathogen relies significantly on its metabolic flexibility, which is exhibited by its ability to utilize a myriad of organic compounds as carbon and energy source for its survival and growth. Furthermore, *P. aeruginosa* is conspicuous for its ability to cause both acute and chronic infections in immunocompromised patients (Bodey *et al.*, 1983). This versatility is manifested in part by expression of a vast array of virulence determinants, both cell-associated and secretory, that are capable of overpowering the host cell defenses and catabolizing macromolecules (Gellatly & Hancock, 2013). It is therefore not surprising that the synthesis and regulation of multiple virulence factors is controlled by nutrient availability. Carbon and nitrogen sources have been shown to influence several virulence related phenotypes in *P. aeruginosa* including biofilm development (Klausen *et al.*, 2003(a), Shrout *et al.*, 2006), motility (Kohler *et al.*, 2000), pigment production (Palmer *et al.*, 2007, Dandekar *et al.*, 2012) and cell cytotoxicity (Bains *et al.*, 2012). To date, very little is known of the physiology of *P. aeruginosa* when provided with α -KG as the sole carbon source and its role in virulence. Importantly, it is now known that extracellular α -KG is a primary carbon source for some pathogenic bacteria during infection (Guo *et al.*, 2012, Cai *et al.*, 2013). Considering these findings, we further explored if α -KG has a more profound physiological role in regulating *P. aeruginosa* virulence and if MifSR TCS play any role. An impediment in performing experiments with *mifSR* mutants in the presence of α -KG was there inability to grow in media with α -KG as the sole carbon source (Table 3.3). To circumvent the problem, all the virulence

related phenotypic assays were performed using $\Delta mifS$, $\Delta mifR$ and $\Delta mifSR$ mutants harboring pPcaT plasmid, unless specified otherwise. Thus, in the *mifSR* mutants, *in trans* expression of the α -KG transporter PcaT ensures the influx of extracellular α -KG into the cells while manifesting the effect of *mifSR* deletions on *P. aeruginosa* virulence phenotypes.

Enhanced biofilm formation in the absence of *mifSR*

Biofilm mode of growth is one of the hallmark of *P. aeruginosa* chronic infections (Tolker-Nielsen, 2014). Subsequently, biofilm infections are difficult to eradicate as a result of biofilm's inherited tolerance to clinically significant antibiotics (Costerton *et al.*, 1999). It has been long known that TCS signaling proteins play a crucial role in modulating *P. aeruginosa* biofilm formation (Mikkelsen *et al.*, 2011). The response regulator MifR, has been implicated in regulating *P. aeruginosa* biofilm formation, as *mifR*-deficient strains failed to form mature biofilms due to a defect in microcolony formation (Petrova & Sauer, 2009). Furthermore, previous studies have reported the interdependence of pyruvate fermentation and requirement of functional MifR in supporting microcolony formation (Petrova *et al.*, 2012). Considering the above results in the light of previous findings (Tatke *et al.*, 2015) we investigated the impact of MifSR TCS on *P. aeruginosa* biofilm formation in the presence of α -KG (30 mM) as the sole carbon source. As mentioned before, because of the inability of the $PAO\Delta mifR$, $PAO\Delta mifS$ and $PAO\Delta mifSR$ mutants to grow in the presence of α -KG (Table 3.3) (Tatke *et al.*, 2015), biofilm formation was assessed using *mifSR* mutant strains harboring the pPcaT plasmid, unless otherwise specified. Also, to rule out the

possible role of pPcaT affecting biofilm formation, the wild-type strain PAO1 expressing PcaT *in trans* was used as a control.

Compared to the wild-type PAO1 strain, PAO Δ *mifR*(pPcaT), PAO Δ *mifS*(pPcaT) and PAO Δ *mifSR*(pPcaT) mutants exhibited an increase in biofilm formation in the presence of α -KG as carbon source (Figure 3.5A). The difference was significant at all time points (P -value > 0.005) tested over a period of 72 hours. Importantly, expression of the wild-type *mifR* and *mifSR* genes *in trans* in the PAO Δ *mifR* and PAO Δ *mifSR* mutants restored the biofilm formation to the wild-type PAO1 levels (Figure 3.5A). Furthermore, the presence of pPcaT itself did not have any adverse effect on biofilm formation, as PAO1(pPcaT) formed comparable level of biofilm as that of the wild-type PAO1 without pPcaT (Figure 3.5A). Additionally, our positive control PAO Δ *retS* a hyper biofilm forming strain and the negative control PAO Δ *pel* Δ *psl* Δ *algD* that forms no biofilms showed the expected results (Figure 3.5).

We have previously shown that MifSR TCS is specific for regulating *P. aeruginosa* α -KG transport, as *mifS*, *mifR* and *mifSR* mutants exhibited no growth difference compared to the wild-type PAO1 in the presence of TCA cycle C₄-dicarboxylate (succinate, fumarate, malate) and/or the C₆-tricarboxylate (citrate) intermediates (Tatke *et al.*, 2015). It was of interest to investigate if the enhanced biofilm formation phenotype of *mifSR*(pPcaT) mutants is α -KG specific. To test this, biofilm formation ability of the *mifSR*(pPcaT) mutants was evaluated in the presence of citrate and/or succinate (30 mM each) as the sole carbon source. No difference in biofilm formation between the *mifSR*(pPcaT) mutant strains and the

wild-type PAO1 was observed over a period of 72 hours in the presence of succinate (Figure 3.5B) or citrate (Figure 3.5C) as a carbon source. These findings suggests that MifSR TCS negatively regulates biofilm formation in an α -KG dependent manner.

It is now well established that the type and availability of nutrients significantly impact biofilm development and composition (Bowden & Li, 1997, Sauer *et al.*, 2004). In *P. aeruginosa*, biofilm formation and development progresses distinctly in the presence of different carbon and nitrogen sources (Klausen *et al.*, 2003(a), Klausen *et al.*, 2003(b), Shrout *et al.*, 2006). Most of the studies to date have looked at the influence of citrate, succinate, pyruvate, glutamate, casaminoacids, and sugars on the formation of *P. aeruginosa* biofilms (De Kievit *et al.*, 2001, Klausen *et al.*, 2003(a), Klausen *et al.*, 2003(b), Shrout *et al.*, 2006, Petrova *et al.*, 2012). The present study is one of the first to report α -KG mediated two-component signaling in regulating *P. aeruginosa* biofilm formation.

Figure 3.5: α -KG dependent regulation of biofilm formation by *mifSR* mutants. Biofilm formation of the wild-type PAO1 and *mifSR* mutants expressing *pcaT* *in trans*, hyperbiofilm producing PAO Δ retS (positive control), and less biofilm producing PAO Δ pel Δ psl Δ algD (negative control) in M9 minimal media with α -KG (A), succinate (B) and citrate (C) at 30 mM each, as a sole carbon source. The data shown is the relative biofilm formation of each mutant and complementing strain as compared to that of the wild type PAO1 as described in material and methods. Each result represents the mean with standard deviation of three biological replicates. The inset, taken at 72 hours, demonstrates the superior biofilm formation capacity of PAO Δ mifS(pPcaT), PAO Δ mifR(pPcaT), PAO Δ mifSR (pPcaT) mutants compared to PAO1 in the presence of α -KG (D). Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001.

***mifSR* deletion mutants are defective in swarming motility**

In pathogenic bacteria including *P. aeruginosa*, motility facilitates successful invasion, survival and colonization within a host, increases nutrient acquisition efficiency and helps challenging or evading the host defenses, thus playing an indispensable role in its virulence (Kazmierczak *et al.*, 2015). Depending on the growth conditions and medium, *P. aeruginosa* exhibits three types of motility, namely swimming, swarming and twitching (Rashid & Kornberg, 2000). The motility is controlled by two cell-surface appendages, a single polar flagellum and type-IV pili (Harshey, 2003). The flagellum enables swimming in liquid environments (Toutain *et al.*, 2005) whereas type-IV pili mediates twitching across solid surfaces (Burrows, 2012). Swarming is a coordinated rapid movement over semi-solid or viscous surfaces requiring both flagella and type-IV pili (Kohler *et al.*, 2000). In *P. aeruginosa*, motility plays a crucial role in facilitating the transition from a free-living planktonic growth to the more sessile biofilm mode of growth (Sauer *et al.*, 2002).

On the basis of our findings that *P. aeruginosa mifSR*-deficient strains form hyper-biofilms in the presence of α -KG, we hypothesized that MifSR TCS is involved in regulating motility. To test this, we compared swimming, swarming and twitching on 0.3%, 0.6% and 1% agar in the wild-type PAO1 and *PAO Δ mifR*, *PAO Δ mifS* and *PAO Δ mifSR* mutants harboring the pPcaT plasmid, in the presence of α -KG (30 mM) as the sole carbon source. The *mifSR*(pPcaT) mutants demonstrated no significant defect in the flagellum-mediated swimming and type-IV pili mediated twitching motilities in the presence of α -KG (Figure 3.6).

Figure 3.6: Swimming and twitching motility of *mifSR* mutants in the presence of α -KG. Swimming and twitching motility of the wild-type PAO1, PAO $\Delta mifS$ (pPcaT), PAO $\Delta mifR$ (pPcaT), PAO $\Delta mifSR$ (pPcaT) was assessed on media supplemented with α -KG (30 mM). The experiment was repeated multiple times with biological replicates.

Figure 3.7: α -KG specific swarming motility in *mifSR* mutants. Swarming motility of the wild-type PAO1, PAO1(pPcaT), PAO $\Delta mifS$ (pPcaT), PAO $\Delta mifR$ (pPcaT), PAO $\Delta mifSR$ (pPcaT) mutants and the pMifR and pMifSR complementing clones was assessed on media supplemented with α -KG or citrate (30 mM each). The experiment was repeated multiple times with biological replicates.

However, compared to *P. aeruginosa* wild-type PAO1, the ability of $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants to swarm was completely abolished in the presence of α -KG (Figure 3.7A). The swarming defect exhibited by these mutants could be restored to that of the wild-type PAO1 by expressing *mifR* and *mifSR* *in trans* in the $\Delta mifR$ and $\Delta mifSR$ mutants (Figure 3.7C). Furthermore, no difference in swarming motility was observed in the PAO1(pPcaT) control strain suggesting that the swarming motility defect in $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants is pPcaT-independent and MifSR-dependent.

In our previous study using rich media, such as LB, no role of MifSR TCS was established in regulating *P. aeruginosa* motility (Tatke *et al.*, 2015). The above data further supports the argument that the swarming defect is α -KG dependent. To further investigate, if the inability of *mifSR* mutants to swarm was α -KG-specific, we tested the ability of these mutants to swarm on other TCA cycle intermediates including succinate and citrate. Interestingly, *P. aeruginosa* wild-type PAO1 and the *mifSR* mutants with or without pPcaT exhibited identical swarming phenotypes in the presence of succinate or citrate (Figure 3.7B). Also, no difference in swimming and twitching motility was observed in presence of citrate or succinate as a carbon source. Together, these findings clearly argues for the requirement of a functional MifSR in modulating *P. aeruginosa* swarming motility exclusively in the presence of α -KG.

Swarming motility in *P. aeruginosa* is often regulated in response to rhamnolipid production (Kohler *et al.*, 2000, Caiazza *et al.*, 2005). Rhamnolipids are surface-active agents that help in reducing surface tension to drive flagellum-

mediated translocation over semi-solid surfaces (Kohler *et al.*, 2000). In addition, rhamnolipids have been recovered from CF patient sputa and are involved in inactivation of mammalian tracheal cells and erythrocyte destruction, indicating their importance as virulence factors (Hastie *et al.*, 1986, Kownatzki *et al.*, 1987, Jensen *et al.*, 2007). Lack of swarming in the $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants can be attributed to a difference in rhamnolipid production in the presence of α -KG. To test if the swarming defect in the *mifSR* mutants is concomitant with rhamnolipid production we performed the rhamnolipid plate assay with α -KG as a carbon source. No significant difference was observed in rhamnolipid production in the $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants as compared to the wild-type PAO1 with or without pPcaT in the presence of α -KG (Figure 3.8). Although interesting, this is not surprising as similar findings have been reported in *P. aeruginosa* where swarming phenotype was independent of rhamnolipid production (Yeung *et al.*, 2011, Guo *et al.*, 2014).

Swarming motility by itself is a complex surface associated behavior controlled by multiple physical factors (flagella, type IV pili and rhamnolipid) (Kohler *et al.*, 2000, Deziel *et al.*, 2003) and transcriptional regulatory networks (Yeung *et al.*, 2009). Additionally, swarming motility is also dependent on the availability of specific nutrients (Rashid & Kornberg, 2000, Harshey, 2003, Bains *et al.*, 2012). In *P. aeruginosa*, swarming motility is influenced by the presence of specific carbon and nitrogen sources (Kohler *et al.*, 2000, Bernier *et al.*, 2011). Swarming is augmented in response to nitrogen limitation, and in the presence of certain amino acid such as glutamate, histidine, proline and aspartate (Kohler *et al.*, 2000).

Interestingly α -KG is central to both these processes, it is a well-established signal for nitrogen starvation (Leigh & Dodsworth, 2007) and is also a precursor for the synthesis of amino acids glutamate, histidine and proline (Owen *et al.*, 2002). Together, our finding suggests a more intricate network of metabolic control exerted by α -KG on *P. aeruginosa* swarming motility that still needs to be further explored. Furthermore, in *P. aeruginosa* swarming motility and biofilm mode of growth are inversely regulated i.e., strains that are deficient in swarming motility produce more biofilm while strains that exhibit increased swarming motility produce less biofilms (Caiazza *et al.*, 2007). This is achieved by a complex interplay between several regulatory mechanisms including TCSs and the second messenger molecule cyclic-di-GMP (Goodman *et al.*, 2004, Caiazza *et al.*, 2007, Kuchma *et al.*, 2007, Merritt *et al.*, 2007, Merritt *et al.*, 2010). It has been shown that the intracellular levels of cyclic-di-GMP influence a wide array of bacterial behaviors, with the common trend being that an increase in the levels of cyclic-di-GMP encourages sessile behaviors, such as biofilm formation, while the degradation of cyclic-di-GMP favors motile behaviors, such as swarming. As the *mifSR* mutant exhibits a swarming defect and a hyperbiofilm phenotype, it will be of interest to examine if MifSR TCS plays a role in regulating the levels of cellular cyclic-di-GMP in the presence of α -KG. Regardless, our data is reminiscent of these findings that shows an inverse relationship between biofilm formation and swarming motility modulated through the MifSR signaling cascade. However, at this point, the precise transcriptional regulatory control exerted by MifS/MifR TCS proteins on biofilm formation and swarming motility in the presence of α -KG

remains to be elucidated. Our study shows that MifSR TCS positively regulates swarming motility and negatively regulates biofilm formation in an α -KG dependent manner.

Figure 3.8: Rhamnolipid production in the presence of α -KG. Rhamnolipid production of the wild-type PAO1 (Vector), PAO1 (pPcaT), PAO $\Delta mifS$ (pPcaT), PAO $\Delta mifR$ (pPcaT), PAO $\Delta mifSR$ (pPcaT) was assessed using the cetyltrimethylammonium bromide (CTAB) methylene blue agar plate (A) and the diameter of the blue halo around the colonies was measured as a mark of rhamnolipid production (B)

Pyocyanin production is negatively regulated by *mifSR*

One of the hallmarks of *P. aeruginosa* infections is the production of extracellular, redox-active phenazine compounds. Pyocyanin, a nitrogen-containing heterocyclic blue-green pigment is the major phenazine-derived compound produced by *P. aeruginosa* (Pierson & Pierson, 2010). Pyocyanin functions both as a crucial virulence factor (Lau *et al.*, 2004) and as a signaling molecule (Dietrich *et al.*, 2006). Furthermore, pyocyanin exhibits antibiotic properties that allow *P. aeruginosa* to gain dominance over other competing microorganisms, including bacteria and fungi, during infection (Baron & Rowe, 1981, Kerr *et al.*, 1999, Price-Whelan *et al.*, 2006). Importantly, both *in vitro* and *in vivo* studies have revealed that pyocyanin plays a pivotal role in establishing *P. aeruginosa* infections and is essential for its full virulence (Mahajan-Miklos *et al.*, 1999, Ran *et al.*, 2003, Lau *et al.*, 2004, Allen *et al.*, 2005, Cezairliyan *et al.*, 2013). In our earlier experiments when pPcaT was introduced in the $\Delta mifS$, $\Delta mifR$ and $\Delta mifSR$ mutants, a visual difference in the pigment production was observed in the presence of α -KG (Figure 3.9). In particular, there was a difference in the production of the blue-green color, a characteristic of pyocyanin (Frank & Demoss, 1959). This prompted us to ask if pyocyanin production is α -KG dependent and if it is regulated by the MifSR TCS. To test this, we compared the amount of pyocyanin made by $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants to that of wild-type PAO1 strain in the presence of TCA cycle intermediates including α -KG, citrate and succinate (30 mM each), as a carbon source. There was no significant difference in the amount of pyocyanin (ug/ml) produced in the presence

of citrate and/or succinate between the wild-type PAO1 and the *mifSR* mutant derivatives (Figure 3.9). However, as compared to the parent PAO1, the *mifSR*(pPcaT) mutants produced significantly (p -value < 0.0005) higher amount (a five-fold increase) of pyocyanin in the presence of α -KG (Figure 3.9). This phenotype was rescued by complementing the *mifR* and *mifSR* mutant strains with the wild-type *mifR* and *mifSR* genes (Figure 3.9). Also, the comparable amount of pyocyanin produced by the wild-type PAO1 and PAO1(pPcaT) suggests, that the presence of an extra copy of *pcaT* does not influence this phenotype. Furthermore, absorbance spectra analysis of the overnight cell free culture supernatants showed a characteristic peak of pyocyanin at 310-325 nm (Mavrodi *et al.*, 2001, Reszka *et al.*, 2004). The absorbance peak intensity of the $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants was significantly higher as compared to the wild-type PAO1 strain indicative of more pyocyanin production in the *mifSR* mutant derivatives (Figure 3.12). These findings strongly suggests an α -KG dependent regulation of *P. aeruginosa* pyocyanin production by the MifSR TCS proteins.

Pyocyanin production in *P. aeruginosa* involves a complex pathway consisting of two homologous operons *phzA1B1C1D1E1F1G1* (*phzA1*) and *phzA2B2C2D2E2F2G2* (*phzA2*) and two additional genes *phzM* and *phzS* encoding phenazine modifying enzymes (Mavrodi *et al.*, 2001). To determine if the difference in pyocyanin production in the presence of α -KG is a function of change in gene expression, qRT-PCR was performed to assess the transcript levels of *phzA1*, *phzA2*, *phzM* and *phzS* genes in the PAO1(pPcaT) and the *mifSR*(pPcaT) mutants. For the qRT-PCR analysis PAO1(pPcaT) was used as the reference

strain since it exhibited the same pyocyanin phenotype as that of the wild-type PAO1 strain without pPcaT. In the PAO1(pPcaT) strain, presence of α -KG appears to inhibit the expression of the *phz* genes namely, *phzA1*, *phzA2*, *phzM* and *phzS* (Figure 3.10). The above data suggests that α -KG is a repressor of pyocyanin synthesis in *P. aeruginosa*. In the *mifSR*(pPcaT) mutant derivatives the expression of *phzA1* was significantly induced in the presence of α -KG as compared to the wild-type PAO1(pPcaT). However, the second *phzA2* operon and, *phzS* and *phzM* genes did not show a significant increase in expression. Pyocyanin production is a characteristic of *P. aeruginosa* stationary growth phase. Here the cells were induced with α -KG for an hour, we suspect that prolong induction of the cells with α -KG might result in a differential expression of the other genes involved in pyocyanin synthesis in the *mifSR*(pPcaT) mutants. In *P. aeruginosa* PAO1, the expression of *phzA1* accounts for majority of phenazine production (Liang *et al.*, 2008), and our qRT-PCR findings suggests that the hyper-production of pyocyanin could be accounted for by an increased expression of *phzA1* in the *mifSR*(pPcaT) mutant strains. Together these findings indicate that MifSR TCS is negatively regulating *P. aeruginosa* pyocyanin production in the presence of α -KG.

Figure 3.9: Pyocyanin production in *mifSR* mutants in the presence of α -KG. Pyocyanin production was measured quantitatively from the overnight culture supernatants of the wild-type PAO1, PAO1(pPcaT), PAO1Δ*mifS*(pPcaT), PAO1Δ*mifR*(pPcaT), PAO1Δ*mifSR*(pPcaT) mutants and the pMifR and pMifSR complementing clones in M9 minimal media supplemented with α -KG, and/or succinate (30 mM each) , using the chloroform and HCL method, as described in materials and methods. Results shown are mean with standard deviation of three biological replicates. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001.

Figure 3.10: Quantification of phenazine specific genes by qRT-PCR in the presence of pPcaT. Expression of the genes involved in phenazine biosynthesis, particularly, *phzA1* (*PA1899*), *phzA2* (*PA4210*), *phzM* (*PA4209*) and *phzS* (*PA4217*), were analyzed in the wildtype PAO1 with and without α -KG treatment (A). Additionally, effect of α -KG on the expression of *phz* genes were analyzed in the *mifSR* mutant derivatives relative to PAO1 (B). Bars above or below the line represents up- and down-regulation, respectively and the bars are standard errors. The *clpX* (*PA1802*) gene was used as the housekeeping control. Statistically significant difference between the wildtype PAO1 uninduced and induced cells as determined by Student's unpaired *t* test, ** *p*-value < 0.001 compared with expression in the wildtype PAO1 uninduced strain (A). Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** *p*-value < 0.001 compared with expression in PAO1 (B).

***mifSR* positively regulates pyoverdine production**

P. aeruginosa produces several siderophores that play a pivotal role in scavenging iron from the host, a vital process required for bacterial growth and infection (Bullen, 1981, Poole & McKay, 2003, Skaar, 2010). Amongst these, pyoverdine, a yellow-green fluorescent pigment is the primary siderophore produced by *P. aeruginosa* (Meyer, 2000, Lamont *et al.*, 2009). Pyoverdine is not only an efficient iron scavenger and transporter during *P. aeruginosa* infection and biofilm formation (Banin *et al.*, 2005, Visca *et al.*, 2007) but also, a signaling molecule established in regulating multiple virulence-associated traits (Beare *et al.*, 2003, Visca *et al.*, 2007). Indeed, several *in vivo* studies in animal and nematode models demonstrated the requirement of pyoverdine as an important virulence factor essential for successful *P. aeruginosa* infections (Meyer *et al.*, 1996, Takase *et al.*, 2000, Kirienko *et al.*, 2013). Considering the fact that *P. aeruginosa* MifSR TCS is involved in modulating *P. aeruginosa* virulence associated pigment production in the presence of α -KG, we investigated its role in pyoverdine production. To test this, we compared the amount of pyoverdine made by $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants to that of wild-type PAO1 strain in the presence of TCA cycle intermediates including α -KG and succinate (30 mM each), as a carbon source. As compared to the parent PAO1, the *mifSR*(pPcaT) mutants produced significantly (p -value < 0.005) less pyoverdine in the presence of α -KG (Figure 3.11). This phenotype was rescued by complementing the *mifR* and *mifSR* mutant strains with the wild-type *mifR* and *mifSR* genes (Figure 3.11). No difference in pyoverdine production was observed

when succinate was provided as the sole carbon source (Figure 3.11) suggesting an α -KG dependent regulation. Also, the comparable amount of pyoverdine produced by the wild-type PAO1 and PAO1(pPcaT) suggests, that the presence of an extra copy of *pcaT* does not influence this phenotype. These findings strongly suggests an α -KG dependent regulation of *P. aeruginosa* pyoverdine production by the MifSR TCS proteins.

Furthermore, absorbance spectra analysis of the overnight cell free culture supernatants showed a characteristic peak of pyoverdine at 400-405 nm (Vandenende *et al.*, 2004). The absorbance peak intensity of the $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants was lower as compared to the wild-type PAO1 strain indicative of less pyoverdine production in the *mifSR* mutant derivatives (Figure 3.12). Together the data suggest that MifSR TCS positively regulates pyoverdine production in the presence of α -KG, as the sole carbon source.

Figure 3.11: Pyoverdine production in *mifSR* mutants in the presence of α -KG. Pyoverdine levels of wild-type PAO1, PAO1(pPcaT), PAOΔ*mifS*(pPcaT), PAOΔ*mifR*(pPcaT), PAOΔ*mifSR* (pPcaT) mutants and the pMifR and pMifSR complementing clones in M9 minimal media supplemented with α -KG and/or succinate (30 mM each) was quantitated by measuring the absorbance A_{405} . Values are normalized with respect to culture density and A_{405}/A_{600} ratio expressed the pyoverdine levels. Each result represents the mean with standard deviation of three biological replicates. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001.

Figure 3.12: Pyocyanin and Pyoverdine absorbance spectra. Absorption spectra analysis of the overnight cell free culture supernatant show a characteristic peak of pyocyanin at 310–325 nm and pyoverdine at 400–405 nm.

Loss of *mifR* increases cell cytotoxicity

P. aeruginosa expresses and utilizes an arsenal of virulence mechanisms to gain competitive advantage over its host in-order to establish acute and chronic infections. Most of these mechanisms rely on numerous virulence factors and/or exoproteins, which are intentionally released by this pathogen via diverse and complex secretion machinery. Most of the secreted virulence factors are toxins and hydrolytic enzymes that induce cell cytotoxicity thus causing damage and annihilation of the host cells. Moreover, in a previous microarray gene expression study significant alteration in expression of genes encoding secreted virulence factors was observed in *mifR* mutant compared to its wild-type PAO1 (Petrova *et al.*, 2012). Since our data indicated that MifSR TCS proteins regulated the transport of extracellular α -KG, and modulate different virulence determinants, we examined the ability of *mifSR* mutant derivatives to infect and destroy A549 lung fibroblast cell cultures in the presence of α -KG.

Since all the virulence related phenotypes exhibited by the $\Delta mifS$ (pPcaT), $\Delta mifR$ (pPcaT), $\Delta mifSR$ (pPcaT) mutants were α -KG dependent, bacterial cells were grown overnight and sub-cultured in M9 minimal media with α -KG (30 mM) before infecting the respective cell cultures. Release of lactate dehydrogenase (LDH) was measured as a function of cell damage using colorimetric LDH activity assay (Hauser & Engel, 1999). The $\Delta mifR$ (pPcaT) and $\Delta mifSR$ (pPcaT) mutants exhibited two-fold increase (P -value < 0.005) in cytotoxicity compared to the wild-type PAO1 in the presence or absence *pcaT* (Figure 3.13A). Interestingly no difference in cytotoxicity was observed in case of $\Delta mifS$ (pPcaT) compared to the

parent strain PAO1. This could be the result of a potential crosstalk between MifR and other noncognate HKs that needs to be verified. Furthermore, introduction of the wild type *mifR* and *mifSR* genes into $\Delta mifR$ and $\Delta mifSR$ mutants restored cytotoxicity to the wild-type PAO1 levels. In-order to rule out the cytotoxicity seen is not specific to A549 lung fibroblast, HeLA and J774-Eclone cells were assessed. All three cell-lines exhibited the same phenotype (Figure 3.14) suggesting increased virulence of *mifR* and *mifSR* mutants leading to higher cytotoxicity. Subsequently, when α -KG was replaced with succinate as the carbon source, no discernable difference in cell cytotoxicity was observed between the *mifSR*(pPcaT) mutants and the parent PAO1 strains (Figure 3.13B). The data suggests that MifR independent of MifS, negatively regulates *P. aeruginosa* cell cytotoxicity.

Bacterial pathogens are one of the most portentous infiltrators of the host system, causing significant damage leading to cellular aberrations and injury. In the mammalian cells TCA cycle intermediates including α -KG are mainly found in cytoplasm and mitochondria. Cell injury, results in the efflux of intracellular metabolites leading to an increase in their extracellular concentrations. It is known that TCA cycle intermediates (C4, C5, and C6 dicarboxylates) are present at micromolar (μ M) concentrations in blood that increase with tissues damaged (He *et al.*, 2004, Hebert, 2004). Therefore it is possible that α -KG might act as a marker of the intensity and level of cellular damage that signals *P. aeruginosa* to downregulate its cytotoxic determinants via *mifR* regulatory circuit. This is one of the first report of α -KG mediated two-component signaling in regulating *P. aeruginosa* cell cytotoxicity.

Figure 3.13: Cytotoxicity effect of *P. aeruginosa* PAO1 and *mifSR* mutants on A549 lung fibroblast. Cell cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay post 24 h of infection. Bacteria grown in M9 media with α-KG (30 mM) were cocultured with A549 lung fibroblast cells with α-KG (A) and without α-KG (B), and the LDH release was monitored after 24 h of infection. The experiment was repeated three times and the data shown are the mean of three replicates with standard deviation. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001.

Figure 3.14: Cytotoxicity effect of *P. aeruginosa* PAO1 and *mifSR* mutants on HeLA and J774-Eclone cells. Cell cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay post 24 h of infection. Bacteria grown in M9 media with α -KG (30 mM) were cocultured with HeLA (A) and J774-Eclone cells (B), and the LDH release was monitored after 24 h of infection. The experiment was repeated three times and the data shown are the mean of three replicates with standard deviation. Statistically significant difference between the wild type and mutants as determined by one-way ANOVA with Bonferroni's post-hoc test, ** p -value < 0.001 .

MifSR TCS and α -KG dependent regulation of *P. aeruginosa* virulence.

To confirm that the expression of α -KG transporter PcaT itself in the *mifSR* mutants had no effect on *P. aeruginosa* α -KG-dependent virulence phenotypes, we performed pigment (pyocyanin and pyoverdine) production, absorbance spectra analysis and cell cytotoxicity assays with the wild type PAO1 and *mifSR* mutants in the presence of PA5530 (KgtP) (the previously reported α -KG transporter) (Tatke *et al.*, 2015). Similar to the *mifSR*(pPcaT) mutants, *mifSR*(pKgtP) mutants produced significantly (p -value < 0.0005) higher and lower amounts of pyocyanin and pyoverdine in the presence of α -KG, as compared to the parent PAO1 strain with and/or without pKgtP (Figure 3.15A and 3.15B). Furthermore, absorbance spectra analysis of the cell free culture supernatants showed the characteristic peak of pyocyanin at 310-325 nm and pyoverdine at 400-405 nm. The absorbance peak intensity of the $\Delta mifS$ (pKgtP), $\Delta mifR$ (pKgtP) and $\Delta mifSR$ (pKgtP) mutants was higher for pyocyanin and lower for pyoverdine as compared to the wild-type PAO1 strain (Figure 3.15C). In addition, in the presence of α -KG, similar to the *mifSR*(pPcaT) mutants, $\Delta mifR$ (pKgtP) and $\Delta mifSR$ (pKgtP) exhibited increased cell cytotoxicity, and no difference in cell-cytotoxicity was observed in the case of *mifS*(pKgtP) compared to the parent PAO1 strain (Figure 3.15D). The *mifSR*(pKgtP) results corroborates with our *mifSR*(pPcaT) data, suggesting that *mifSR* mutant exhibited phenotypes are transporter independent and α -KG-dependent.

Figure 3.15: *mifSR*(pKgtP) virulence assays in the presence of α -KG. Pyocyanin production (A), pyoverdine production (B), absorbance spectra (C) and cell cytotoxicity (D) of PAO1, PAO1(pKgtP), *mifS*(pKgtP), *mifR*(pKgtP), *mifSR*(pKgtP) mutants in the presence of α -KG (30 mM).

Conclusion

P. aeruginosa is one of the most formidable, opportunistic human pathogen infamous for its ability to cause several localized and systemic infections (Tummler *et al.*, 2014). Its ability to colonize different host niches is largely dependent on its metabolic flexibility and environmental adaptability dictated by genetic versatility (Mathee *et al.*, 2008). *P. aeruginosa* preferentially utilizes TCA cycle intermediates as carbon and energy source over other compounds (Collier *et al.*, 1996), and α -KG is a key intermediate of the TCA cycle (Krebs, 1940). α -KG plays a crucial role in regulating carbon-nitrogen metabolism (Doucette *et al.*, 2011) and importantly, α -KG is considered to be a signaling molecule in both prokaryotes (Ninfa & Jiang, 2005) and eukaryotes (Lancien *et al.*, 2000, He *et al.*, 2004). Furthermore, recent studies have reported preferential utilization of extracellular α -KG as a principle carbon source during infection by pathogenic bacteria (Guo *et al.*, 2012, Cai *et al.*, 2013). The data together suggests a profound role of α -KG in regulating bacterial physiology and metabolism. However, limited analysis of the physiological roles of α -KG in bacteria, including *P. aeruginosa*, has left its role in pathogenesis unclear. The present study provides a novel insight into the role of α -KG and MifSR TCS in regulating *P. aeruginosa* pathogenesis.

Our findings, suggests a complex regulatory cascade in modulating *P. aeruginosa* C₅-dicarboxylate, α -KG uptake involving a combined action of multiple α -KG transporters namely PcaT (PA0229) and KgtP (PA5530), the MifS/MifR TCS and the sigma factor RpoN (Figure 3.16). It appears that MifS senses the presence of α -KG and signals MifR. The activated-MifR along with the sigma factor RpoN

initiate the transcription of α -KG transporter genes *pcaT* and *kgtP*. Interestingly, our findings suggest that the MifSR TCS inversely regulates the expression of *pcaT* and *KgtP* genes (Figure 3). Furthermore, sequence analysis of *pcaT* promoter (P_{pcaT}) and our previous published P_{kgtP} findings (Tatke *et al.*, 2015) together indicates that the expression of both *pcaT* and *kgtP* genes is under several layers of transcriptional and post-transcriptional controls, suggesting a complex regulatory mechanism involved in modulating *P. aeruginosa* α -KG uptake. The presence of two α -KG transporters regulated in an opposite fashion in *P. aeruginosa* is of interest due to their possible function in responding to nutritional cues. Furthermore, it is also possible that the presence of two α -KG transporters is due to a difference in their substrate affinities and may indicate a role for each in changing gene expression based upon environmental α -KG concentrations. Future studies will determine if *P. aeruginosa* α -KG transporters are preferentially or hierarchically regulated depending on the carbon source.

In addition to the role of MifSR TCS in modulating extracellular α -KG transport, in this study we illustrate the interdependence of MifSR TCS and the C₅-dicarboxylate α -KG in regulating the expression of *P. aeruginosa*'s key virulence determinants. The results presented here show that MifSR TCS in an α -KG specific manner positively regulates swarming motility and pyoverdine production while negatively regulates biofilm formation and pyocyanin production. Interestingly, α -KG dependent cell cytotoxicity was found to be MifS-independent and MifR-dependent, suggesting a potential crosstalk between MifR and other noncognate HKs. Together, these findings strongly suggest a complex mechanism of MifSR

action involving multiple regulatory controls that are achieved by modulating expression of numerous genes in α -KG-dependent manner. Identifying the α -KG dependent members of MifSR regulon will significantly help in delineating the role of MifSR signaling pathway in *P. aeruginosa* pathogenesis.

Successful adaptation of *P. aeruginosa* to different niches depends extensively on its ability to regulate gene expression as specified by the environmental cues. Although a plethora of TCSs have been identified in *P. aeruginosa* (Winsor *et al.*, 2011) that warrants its adaptability, only a handful of TCS signaling molecules are known to date. Recent research progresses show that, TCA cycle metabolites in-addition to their canonical role in metabolism are increasingly been recognized as a bacterial TCS activating signals (Janausch *et al.*, 2002, Sevvana *et al.*, 2008, Zhou *et al.*, 2008, Brocker *et al.*, 2009, Cai *et al.*, 2013, Valentini & Lapouge, 2013, Tatke *et al.*, 2015). Our data indicates that the TCA cycle C₅-dicarboxylate α -KG is the signaling molecule activating *P. aeruginosa* MifSR TCS. This is one of the first reports to answer fundamental questions pertaining to α -KG utilization in *P. aeruginosa* and its role as a messenger in MifSR regulatory mechanism. Metabolic processes and virulence mechanisms are closely interconnected and are hallmarks of *P. aeruginosa* infections. Hopefully, this and future studies on this regulatory system and metabolism pathway will add significantly to the understanding of an intricate link between virulence and metabolism in pathogens that would provide a better stratagem to fight the often indomitable infections.

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CHAPTER 4

General Discussion

Overview

Pseudomonas aeruginosa is a metabolically versatile, Gram-negative γ -proteobacteria that is well known for its widespread spatiotemporal distribution (Ramos, 2004). Although, a primary inhabitant of soil and water, it has an extraordinary ability to survive and thrive in a myriad of natural and artificial environments (Walker *et al.*, 2004, Gellatly & Hancock, 2013). Importantly, *P. aeruginosa* is a dominant nosocomial pathogen and an epitome of opportunistic infections in humans (Bodey *et al.*, 1983, Lyczak *et al.*, 2000, de Bentzmann & Plesiat, 2011). It is the fourth most commonly-isolated nosocomial pathogen and accounts for 10 percent of all hospital-acquired infections (Gaynes *et al.*, 2005). *P. aeruginosa* is infamous for its ability to cause wide range of human infections that can vary from acute to chronic, local to systemic and self-limiting to life-threatening (Campa *et al.*, 1993, Sousa & Pereira, 2014, Tummler *et al.*, 2014). In particular, *P. aeruginosa* pose a high risk to individuals with impaired immunity and those suffering from the recessive genetic disorder, cystic fibrosis (Ratjen & Doring, 2003). In patients with CF, chronic and recurrent pulmonary infections by *P. aeruginosa* are a leading cause of morbidity and mortality (Sorde *et al.*, 2011, Winstanley *et al.*, 2016). *P. aeruginosa* produces multi-determinant virulence factors, both cell associated and secretory, which modulate its pathogenicity and ensures its success as a pathogen (Balasubramanian *et al.*, 2013). Despite extensive efforts in the development of new anti-pseudomonal drugs, therapeutic intervention in the treatment of *P. aeruginosa* infections has not been very effective, particularly due its extraordinary intrinsic and acquired resistance to a

repertoire of clinically significant antibiotics (Hancock & Speert, 2000, Falagas & Bliziotis, 2007, Lister *et al.*, 2009) . Thus, the pathogenesis of *P. aeruginosa* infections is multifactorial, and is manifested by its diverse metabolic capabilities, expression of virulence factors and antibiotic resistance.

Another key factor accounting for the success of *P. aeruginosa* as an invincible pathogen is its environmental and metabolic adaptability. This flexibility allows survival, growth and rapid colonization of different host milieus, leading to successful infection and disease progression (Silby *et al.*, 2011). In human host, since the environmental conditions are highly variable and erratic, pathogenic bacteria need a dedicated regulatory system that supports their adaptation to perpetual changes. To perform these adaptations, *P. aeruginosa* has evolved an intricate and dynamic array of signaling networks that in addition to modulating the basic housekeeping functions, coordinate the temporal expression of various genes in response to both inter- and intracellular signaling molecules and environmental cues (Rodrigue *et al.*, 2000, Ventre *et al.*, 2006, Gooderham & Hancock, 2009, Williams & Camara, 2009, Haussler, 2010, Jimenez *et al.*, 2012). Of the many signaling mechanisms elucidated in *P. aeruginosa*, two-component system (TCS) mediated signal perception play a pivotal role in sensing diverse environmental stimuli and elicit a pertinent and rapid adaptive response (Rodrigue *et al.*, 2000, Stock *et al.*, 2000, Alm *et al.*, 2006).

TCSs in *P. aeruginosa* have been shown to regulate many physiological aspects of its lifestyle, including global responses to stress (Wood *et al.*, 2006, Leech *et al.*, 2008), chemotaxis (Gooderham & Hancock, 2009), antibiotic

resistance (Gooderham & Hancock, 2009), carbon/nitrogen metabolism (Nishijyo *et al.*, 2001, Li & Lu, 2007), nutrient uptake (McPhee *et al.*, 2006, Valentini *et al.*, 2011, Tatke *et al.*, 2015), expression of virulence factors (Gooderham & Hancock, 2009, Mikkelsen *et al.*, 2011, Okkotsu *et al.*, 2014) and transition from free living planktonic to sessile biofilm mode of growth (Mikkelsen *et al.*, 2011). While a plethora of TCS signaling proteins (135) have been identified in *P. aeruginosa* only a few have been extensively characterized and the regulatory targets for many TCS systems are still unknown (Rodrigue *et al.*, 2000, Barakat *et al.*, 2009, Winsor *et al.*, 2011). Furthermore, in most of the cases, the environmental signals that influence TCS pathways remains obscure (Mascher *et al.*, 2006, Mitrophanov & Groisman, 2008). Though the environmental cues sensed by TCSs are poorly understood, they are largely accountable for determining the outcome of *P. aeruginosa* infections and pathogenesis. Also, in many instances TCSs are known to work in tandem with other signal transduction pathways, thus forming a complex network allowing *P. aeruginosa* to assimilate numerous external cues into a cohesive regulatory circuit that modulates a lifestyle continuum (Coggan & Wolfgang, 2012). Understanding the complexities of TCS signaling is the key to understanding *P. aeruginosa* pathogenesis.

Summary

MifSR TCS regulates *P. aeruginosa* α -KG transport and virulence

P. aeruginosa genome encodes one of the largest groups of TCS proteins identified in any sequenced bacterial species (Barakat *et al.*, 2009, Winsor *et al.*, 2011). Amongst these, only few TCSs have been completely characterized. This dissertation investigated the role of a previously uncharacterized TCS protein pair MifS and MifR in regulating *P. aeruginosa* pathogenesis. MifS is a sensor histidine kinase (HK) and MifR is the response regulator (RR). During the course of the dissertation, the RR MifR was reported to have a dual function, in regulating the maturation stage of biofilm formation (virulence) and in pyruvate fermentation (metabolism) (Petrova & Sauer, 2009, Petrova *et al.*, 2012). However, the mechanism by which MifR is activated in this process remains obscure and no relation with the histidine kinase MifS has been established. Our work identified a novel role of the MifS-MifR TCS proteins in regulating *P. aeruginosa* metabolism by exclusively modulating the utilization of a key tricarboxylic acid (TCA) cycle intermediate, α -Ketoglutarate (Chapter 2) (Tatke *et al.*, 2015). Specifically, we show that MifSR TCS regulates the uptake of extracellular α -KG by positively regulating the expression *P. aeruginosa* PA5530 (*kgtP*) gene encoding a putative α -KG permease (Chapter 2, Figure 2.11). Additionally, we show that the transport of extracellular α -KG in *P. aeruginosa* is a consequence of an orchestrated interplay between the MifSR TCS and the alternate sigma factor RpoN (σ^{54}) (Chapter 2, Figure 2.14). To date, this work is one of the first to establish the role of *P. aeruginosa* MifS/MifR TCS in regulates α -KG transport in association with the

sigma factor RpoN (Tatke *et al.*, 2015). In addition, we identified that besides KgtP, *P. aeruginosa* genome encodes a second α -KG permease PcaT (PA0229) whose expression is independent of MifSR TCS, suggesting an additional layer of regulation involved in *P. aeruginosa* α -KG uptake (Chapter 3). Lastly, our phenotypic assay revealed the α -KG specific exclusivity of MifSR TCS in modulating *P. aeruginosa* virulence mechanism (Chapter 3). We show that MifSR TCS in the presence of α -KG as a sole carbon source regulates the expression of *P. aeruginosa* biofilm formation, swarming motility, pigment production (pyocyanin and pyoverdine) and cell cytotoxicity (Chapter 3 and Figure 4.1). Simultaneous regulation of multiple mechanisms involved in *P. aeruginosa* pathogenesis suggests a complex mechanism of MifSR action. To date, to our knowledge, this is one of the first report of α -KG mediated two-component signaling in regulating *P. aeruginosa* metabolism and virulence. Together, this work established the role of α -KG as a signaling molecule and that MifSR TCS system is a master regulator of α -KG transport in *P. aeruginosa*. Here we propose a model for α -KG dependent MifSR TCS signaling in *P. aeruginosa* (Figure 4.2).

Figure 4.1: Overview of *P. aeruginosa* virulence phenotypes differentially regulated by MifSR TCS in the presence of α -KG.

Figure 4.2: Proposed model for α -KG dependent MifSR TCS signaling in *P. aeruginosa*. Sensor MifS senses the extracellular α -KG as an environmental cue to undergo phosphorylation. The phosphate is then transferred to the response regulator MifR. The phosphorylated MifR together with the alternate sigma factor RpoN activates the expression of α -KG specific transporter gene *kgtP*. KgtP then enables the influx of extracellular α -KG to meet the metabolic demands of the cells. In addition, MifSR TCS in the presence of α -KG as a carbon source differentially regulates *P. aeruginosa* key virulence determinants including biofilm formation, swarming motility, cell cytotoxicity and the production of pyocyanin and pyoverdine.

Future Directions

Two-component signal perception mechanisms that rely on phosphotransfer from histidine kinase (HK) to the response regulator (RR) is one of the predominant strategy used by bacteria for coupling environmental signal to adaptive responses. Bacterial TCS signal transduction mechanisms have always been a subject of extensive research because of the versatility of its components (HK and RR) to probe the environmental conditions and decide how best to respond by orchestrating the expression of several desired genes. In the recent years, with the advent of new biochemical and molecular biology techniques, substantial advances have been made in understanding the molecular mechanisms regulated by TCS signaling pathways. While the work in this dissertation using high-throughput phenotypic microarray studies and molecular and biochemical techniques have helped significantly to get an insight into a novel role of α -KG mediated signaling via the MifS and MifR TCS proteins in *P. aeruginosa*, there remains many unanswered questions.

Outlined below are some ideas and avenues for further research that are based on the results published in this dissertation and/or are based on my preliminary data mentioned in this section.

- Determine the α -KG dependent MifR regulon
- Elucidate the role of RpoN (σ^{54}) and TCS signaling in regulating *P. aeruginosa* TCA cycle intermediate transport and pathogenesis

(Each future research direction is strengthen with a brief discussion)

Determine the α -KG dependent MifR regulon

P. aeruginosa response regulator MifR is a part of the signal transduction MifS/MifR TCS. MifR on average is 60-65% similar to the known *P. aeruginosa* NtrC superfamily of TCS response regulators including DctD, CbrB, AauR, NtrC, AlgB, FleR and PilR, suggesting similar functions (Winsor *et al.*, 2011). These transcriptional regulators are known to regulate a myriad of functions in *P. aeruginosa* including carbon assimilation, nitrogen metabolism, nutrient uptake, motility, antibiotic resistance (Ritchings *et al.*, 1995, Arora *et al.*, 1997, Gooderham & Hancock, 2009) and alginate synthesis (Wozniak & Ohman, 1991, Ma *et al.*, 1998, Leech *et al.*, 2008). Our phenotypic assays under nutrient rich conditions indicated no role of MifR in regulating *P. aeruginosa* antibiotic resistance (Figure 4.3) or other virulence related characteristics (Figure 4.4) (Tatke *et al.*, 2015). Therefore, we performed a high throughput phenotypic microarray (BioLOG) with *mifR* deletion mutant and the wild-type PAO1 strains where numerous metabolic and chemical sensitivity phenotypes were tested simultaneously (Chapter 2, Figure 2.5). Our phenotypic microarray data indicated the role of RR MifR in regulating *P. aeruginosa* metabolism by modulating the utilization of C₅-dicarboxylate α -KG, a key TCA cycle intermediate as a carbon and energy source. Subsequently, our growth profile, genetic complementation and qRT-PCR expression studies showed that MifR in concert with the sigma factor RpoN (σ^{54}) regulate extracellular α -KG transport by modulating the expression of *P. aeruginosa* α -KG-specific permease PA5530 (KgtP) (Chapter 2) (Tatke *et al.*, 2015).

Figure 4.3: Effect of *mifSR* deletion on *P. aeruginosa* antibiotic resistance. Antibiotic resistance profile of the parent PAO1 and *mifSR* deletion mutants to four major classes of antibiotics. Representative data from three different biological replicate trials are shown.

Figure 4.4: Effect of *mifSR* deletion on *P. aeruginosa* virulence. Overview of *P. aeruginosa* virulence phenotypes under nutrient rich conditions.

In *P. aeruginosa*, TCA cycle intermediates, including α -KG is known to trigger carbon catabolite repression (CCR), a mechanism by which cells facilitate hierarchical uptake and utilization of carbon substrates for efficient growth (Wolff et al., 1991, Collier 1996). Subsequent studies have showed that, CCR in *Pseudomonas* Spp. is mediated by the catabolite repression control (Crc) protein, a translational repressor (Rojo 2010). Our *in silico* analysis of the *kgtP* promoter region revealed the presence of a CA-rich motif with a signature sequence (AAc/uAAc/uAA) for the Crc protein (Chapter 2, Figure 2.13) (Tatke et al 2015). It is now well established that the expression of *crc* gene encoding the Crc protein is modulated by RpoN (σ^{54}) dependent small non-coding RNA CrcZ and the CbrA/CbrB TCS (Sonnleitner 2009). Also, our analyses indicates that the *kgtP* promoter is under a small non-coding antisense RNA (asRNA) regulation (Chapter 2, Figure 2.13) (Tatke et al., 2015). This suggests additional layers of regulation superimposed on the need for MifR in regulating *P. aeruginosa* α -KG transport. Subsequently, we also identified a second chromosomally encoded α -KG permease in *P. aeruginosa* namely, PA0229 (PcaT) (Chapter 3). Interestingly, our qRT-PCR expression data and complementation studies suggests that the expression of *pcaT* gene is independent of the MifS-MifR TCS signaling cascade. (Chapter 3, Figure 3.3). Furthermore, *in silico* analysis of the *pcaT* promoter region revealed the presence of a weak RpoN (σ^{54}) binding site, the signature sequence (AAc/uAAc/uAA) for the translational repressor Crc protein and four putative GGA trinucleotide motifs, that are the characteristic binding sites for the post-transcriptional control RsmA family of proteins (Chapter 3, Figure 3.3). Presence,

of shared common motifs (RpoN (σ^{54}) and Crc binding sites) in the *kgtP* and *pcaT* promoter regions indicates an overlapping regulatory control. Though the role of asRNA, CrcZ, Crc and RsmA in *P. aeruginosa* α -KG transport and utilization is currently unknown, it suggest a more profound and complex mechanism of MifR controlled α -KG transport. This is one of the first study to report the role of a TCS response regulator in regulating bacterial α -KG assimilation.

Furthermore, efficient utilization and catabolism of extracellular nutrients is a primary requirement for all pathogenic bacteria to manifest their full virulence potential during the course of infection. Metabolic processes and virulence mechanisms are closely interconnected and are hallmarks of *P. aeruginosa* infections. Therefore, it is not surprising that the regulation of many virulence determinates is controlled by nutrient availability. In *P. aeruginosa*, specific carbon and nitrogen sources have been known to influence several clinically relevant virulence phenotypes such as motility (Kohler *et al.*, 2000), pigment production (Palmer *et al.*, 2007), biofilm development (Klausen *et al.*, 2003(a), Shrout *et al.*, 2006) and quorum sensing (Dandekar *et al.*, 2012). In this study we illustrated the interdependence of RR MifR and the C₅-dicarboxylate α -KG in regulating the expression of *P. aeruginosa*'s key virulence determinants (Chapter 3). We show that MifR in the presence of α -KG as the sole carbon source positively regulates swarming motility and pyoverdine production while negatively regulates biofilm formation, pyocyanin production and cell cytotoxicity (Chapter 3 and Figure 4.1). Furthermore, a previous microarray transcriptome study of the parent PAO1 and *mifR* deficient mutant cultivated under biofilm-specific condition exhibited

significant alteration in the expression of genes encoding transporter proteins, secretion factors, type II and type III secretion systems (Petrova *et al.*, 2012). Hyper pyocyanin production and increased cell cytotoxicity of the *mifSR* mutants suggested an overall increase in the concentration of extracellular proteins. Using the Bradford assay (Bradford, 1976) we quantitated the amount of total proteins present in the culture supernatant of the *mifSR* mutants grown overnight in the presence of α -KG and/or succinate (30 mM each), as a sole carbon source. The results obtained were intriguing as the concentrations of extracellular proteins in the culture supernatant were significant lower in the *miSR* mutants as compared to the wild-type PAO1 strains in the presence of α -KG (Figure 4.5). In addition, when cells were grown in the presence of succinate no difference was observed in the extracellular protein concentration in *mifSR* mutants compared to the wild-type PAO1 (Figure 4.5). Together, this data indicates that MifR in the presence of α -KG affects numerous transport and virulence associated mechanisms in *P. aeruginosa*. However, at this point, the transcription regulatory network and the gene expression pattern regulated by MifR that manifest these phenotypic effects are unclear.

There are numerous previously described signaling pathways in *P. aeruginosa* that independently or in association are capable of positively regulating swarming motility and negatively regulating biofilm formation and pyocyanin production. These pathways include the cell density dependent quorum sensing (Las, Rhl and PQS) signaling system, GacS/GacA two-component system and cyclic-di-GMP signaling (Goodman *et al.*, 2004, Caiazza *et al.*, 2007, Kuchma *et*

al., 2007, Merritt *et al.*, 2007, Merritt *et al.*, 2010). In a recent study, Petrova and co-workers reported that, *mifR* overexpression in the *P. aeruginosa* parent PAO1 strain positively affects cyclic-di-GMP levels in a biofilm-specific manner (Petrova *et al.*, 2012). At this point it is not clear if any of these pathways are regulated by MifR in the presence of α -KG, either directly or indirectly. Future work focused on elucidating the α -KG dependent members of MifR regulon using the high throughput RNA-seq approach will provide a new perspective to decode and better understand the correlations between *P. aeruginosa* metabolism and virulence. These studies will also substantially impact our understanding of the profound role played by the metabolite α -KG as a messenger in regulating molecular pathogenesis of the intractable pathogen *P. aeruginosa*. I have finished the required RNA-Seq experiments using the wild-type PAO1 and PAO Δ *mifR* mutant derivatives expressing *pcaT* *in trans* in the presence and absence of α -KG. The RNA-seq was performed using the Next-generation sequencing Illumina Platform and the RAW FASTQ data has been generated. Currently, I am analyzing and validating the RNA-seq data using CLC Genomic Workbench, Python programming and qRT-PCR expression studies. Future work focused on validation of RNA-seq data using phenotypic assays will help to better understand the role of RR MifR and the metabolite α -KG in regulating *P. aeruginosa* physiology.

Figure 4.5: Effect of *mifSR* deletion on extracellular protein concentration.

The extracellular proteins were quantitated using the Bradford assay from the culture supernatant of *mifSR* mutants grown over night in the presence of α-KG and succinate (30 mM each), as the sole carbon source.

Elucidate the role of RpoN (σ^{54}) and TCS signaling in regulating *P. aeruginosa* TCA cycle intermediate transport and pathogenesis

Success of *P. aeruginosa* as a recalcitrant pathogen can be largely attributed to the repertoire of transcriptional regulators encoded by its genome. These regulators form an intricate and dynamic networks to exquisitely tune the expression of genes responsible for phenotypic adaptation and virulence during infection. *P. aeruginosa* has one of the largest genome amongst sequenced bacteria and transcriptional regulators encompass about 10% of the genome (Galan-Vasquez *et al.*, 2011). Amongst transcriptional regulators, sigma (σ) factors are the indispensable global regulators of gene expression. They confer promoter recognition specificity to the RNA polymerase core enzyme (Borukhov & Severinov, 2002, Campbell *et al.*, 2002, Murakami & Darst, 2003) in order to orchestrate the process of transcription initiation (Burgess *et al.*, 1969), a key step in gene regulation (Browning & Busby, 2004). To date, the regulatory machinery predicted in *P. aeruginosa* prototypic strain PAO1 comprises of approximately 550 transcriptional regulators and 26 putative sigma factors (one RpoN (σ^{54}), eight RpoD (σ^{70}), and 17 of extra cytoplasmic function (ECF) family (Potvin *et al.*, 2008, Perez-Rueda *et al.*, 2009, Galan-Vasquez *et al.*, 2011). Amongst these of special interest is the alternate sigma factor RpoN (σ^{54}) which is a global regulator involved in *P. aeruginosa* nitrogen metabolism, motility, carbon assimilation, nutrient transport, quorum sensing and virulence (Potvin *et al.*, 2008).

Our comparative growth curve analyses of *P. aeruginosa* wild-type PAO1 and the PAO Δ rpoN mutant strains in the presence of TCA cycle C₄, C₅ and C₆ di-

and tricarboxylic acid intermediates exhibited intriguing results (Figure 4.6). The PAO Δ *rpoN* mutant failed to grow in the presence of all the TCA cycle intermediates including succinate (C₄), α -KG (C₅) and citrate (C₆) as the sole carbon source (Figure 4.6). Furthermore, the growth defect exhibited by PAO Δ *rpoN* could be restored to the parent strain PAO1 levels by introducing the wild-type *rpoN* in the PAO Δ *rpoN* mutant (Figure 4.6). This data strongly hints at the role of RpoN (σ^{54}) as the master regulator of TCA cycle C₄, C₅ and C₆ di- and tricarboxylic acid intermediate transport in *P. aeruginosa*. A unique feature of the RpoN (σ^{54}) dependent transcription activation of genes is the requirement of an additional transcriptional regulatory protein called the RpoN (σ^{54}) binding protein that aids in transcription initiation (Thony & Hennecke, 1989, Potvin *et al.*, 2008). In *P. aeruginosa*, to date, 22 RpoN (σ^{54}) binding proteins have been predicted of which nine (close to 41%) belong to the signal transduction TCS. In the extensively studied *Rhizobium meliloti* and *Rhizobium leguminosarum*, RpoN (σ^{54}) together with the RR DctD, a part of the DctB/DctD TCS pair, is reported to regulate the transport of C₄-dicarboxylates succinate, fumarate and malate (Ronson *et al.*, 1987, Watson, 1990, Janausch *et al.*, 2002).

In *P. aeruginosa*, three TCS protein pairs namely, DctB/DctD, MifS/MifR and PA1336/PA1335 have been identified to be homologous to the *Rhizobium* RpoN dependent C₄-dicarboxylate transport regulatory DctB/DctD TCS (Tatke *et al.*, 2015). The response regulator proteins of these TCS pairs namely, DctD, MifR and PA1335 share 67%, 69% and 68% identity to the *R. meliloti* RR DctD (Winsor *et al.*, 2011), suggesting a similar function. During the course of the dissertation,

Valentini *et al.* showed that, *P. aeruginosa* DctD in concert with RpoN (σ^{54}) regulates the transport of C₄-dicarboxylate succinate, fumarate and malate by initiating the transcription of *dctA* and *dctPQM* genes encoding the C₄-dicarboxylate transporters (Valentini *et al.*, 2011). Also, we recently reported a key finding that the assimilation of extracellular C₅-dicarboxylate α -KG requires a functional RpoN (σ^{54}) to work in tandem with the RR MifR in order to activate the expression of *P. aeruginosa* α -KG permease encoding *kgtP* (PA5530) gene (Chapter 2) (Tatke *et al.*, 2015). Interestingly, *P. aeruginosa* RRs DctD and MifR are functionally exclusive with the only shared feature being the requirement of RpoN (Valentini *et al.*, 2011, Tatke *et al.*, 2015). This suggests that *P. aeruginosa* has a dedicated sensory-response mechanism to facilitate the assimilation of specific class of TCA cycle intermediates (C₄, C₅ and C₆), respectively. While the role of *P. aeruginosa* RpoN in the transport of C₄ (succinate, fumarate, malate) (Valentini *et al.*, 2011) and C₅ di-carboxylates (α -KG) (Tatke *et al.*, 2015) is reported, its role in the transport of C₆-tricarboxylate (citrate) is unclear. Also, the role of PA1336/PA1335 TCS in *P. aeruginosa* is unknown.

Our sequence analysis of *P. aeruginosa* PAO1 genome revealed the presence of two ORFs PA5476 (*citA*) and PA5468 encoding the putative C₆-tricarboxylate citrate transport proteins (Winsor *et al.*, 2011). The promoter for *citA* and PA5468 genes have the putative -12/-24 RpoN (σ^{54}) binding signature sequences (Thony & Hennecke, 1989, Winsor *et al.*, 2011). Furthermore, the RR PA1335 has the conserved domains found among NtrC family of regulators, an N-terminal regulatory, a central RpoN (σ^{54}) activation and a C-terminal DNA binding

domains (Winsor *et al.*, 2011). Since PA1335 exhibits high identity to RpoN (σ^{54}) dependent transcriptional regulators, it is possible that the RR PA1335, part of the PA1336/1335 TCS in concert with RpoN facilitate extracellular citrate transport by regulating the expression of *citA* and *PA5468* genes. Future studies aimed at characterizing the role of RpoN and PA1336/PA1335 TCS in citrate transport will provide a vital missing link in our understanding of the fundamental aspects of *P. aeruginosa* di- and tri carboxylate transport (Figure 4.7).

It is worth mentioning that *P. aeruginosa* utilizes TCA cycle intermediates as the preferred carbon, nitrogen and energy source (Wolff *et al.*, 1991, Collier *et al.*, 1996). The involvement of RpoN (σ^{54}) in regulating the uptake of TCA cycle intermediates strongly suggests its role as a protagonist that decides the fate of carbon and energy utilization patterns in *P. aeruginosa*. Recently, amongst all sigma factors, RpoN has been recognized as a central player having the largest impact on *P. aeruginosa* global gene expression that ensures its pathogenic success (Schulz *et al.*, 2015). Future studies using comparative transcriptome profiling approaches should elucidate the function-specific molecular mechanisms manifested by RpoN in concert with DctB/DctD, MifS/MifR and PA1336/PA1335 TCS regulatory proteins. Furthermore, it would be interesting to determine if RpoN and the DctB/DctD, MifS/MifR and PA1336/PA1335 function together in any capacity to regulate *P. aeruginosa* virulence in a nutrient source specific manner. These studies will significantly contribute to a greater understanding of *P. aeruginosa*'s complex metabolic networks and will provide a reliable scaffold for elucidating the transcriptional network of this portentous pathogen.

Figure 4.6: Growth curve analysis in the presence of TCA cycle intermediates. Growth curves of *P. aeruginosa* wild-type PAO1, PAOΔrpoN mutant and PAOΔrpoN::rpoN complementing strain was tested in M9 minimal media supplemented with TCA cycle intermediates α-KG (A), succinate (B) and citrate (C) at 30 mM each, as the sole carbon source. Cells were cultured for 18 h at 37°C and their growth was monitored by measuring the absorbance at 600 nm.

Figure 4.7: *P. aeruginosa* RpoN and TCS mediated TCA cycle intermediate transport. Model representing *P. aeruginosa* RpoN and TCS response regulator mediated transport mechanism of TCA cycle C₄ (yellow), C₅ (blue) and C₆ (purple) di- and tri-carboxylates. The response regulators are represented in orange. Green and red arrows represent positive regulation

Concluding Remarks

Due to the rapid emergence of antibiotic resistance in bacterial pathogens, researchers are relentless search for new targets for drug discovery. Although, TCSs have been identified in lower eukaryotes such as yeast and plants, no reports on the presence of TCS in mammalian cells have been reported (Gotoh *et al.*, 2010). This has made TCS proteins an attractive drug target (Schreiber *et al.*, 2009, Gotoh *et al.*, 2010, Bem *et al.*, 2015). Furthermore, the metabolite α -KG itself has been considered to have significant therapeutic potential in mammals (Grzesiak *et al.*, 2016, Zdzisinska *et al.*, 2016). Our results indicates that α -KG is a repressor of *P. aeruginosa* acute (pyocyanin and cell cytotoxicity) and chronic infection (biofilm formation) phenotypes. Understanding the physiological effects of α -KG on *P. aeruginosa* and its effect manifested through MifR transcriptional control will help significantly in development of new therapeutic options.

APPENDICES

Appendix 1

70th Anniversary Collection of the Society for General Microbiology: Journal of Medical Microbiology

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Abstract

In the last 70 years, we have seen a radical change in our perception and understanding of the microbial world. During this period, we learned from Woese and Fox there exists a third kingdom called “Archea” based on the phylogenetic studies of the 16S ribosomal RNA that revolutionized microbiology (Woese & Fox, 1977, Woese *et al.*, 1978). Further, we were forced to reckon with the fact that Koch and Pasteur’s way of growing cells in test-tubes or flasks planktonically does not necessarily translate to the real-life scenario of bacterial life style, where they prefer to live and function as a closely knit microbial community called biofilm. Thanks are due to Costerton, who led the crusade on the concept of biofilms and expanded its scope of inquiry, which forced scientists and clinicians worldwide to rethink how we evaluate and apply the data. Then progressively, disbelief turned into belief, and now it is universally accepted that the micro-organisms hobnob with the members of their community to communicate and coordinate their behavior, especially in regards to growth patterns and virulence traits via signaling molecules. Just when we thought that we were losing the battle against bacteria, antimicrobials were discovered. We then witnessed the rise and fall of antibiotics and development of antibiotic resistance. Due to space and choice limitation, we will focus on the three areas that caused this major paradigm shift (i) antimicrobial resistance (AMR), (ii) biofilm and (iii) quorum sensing (QS), and how the Journal of Medical Microbiology played a major role in advancing the shift.

Antimicrobial resistance (AMR)

It is notable that this 70th anniversary collection for the journal coincides with the 70th anniversary of the Nobel Prize to Florey and Chain for their work on penicillin, a β -lactam antibiotic. It was first used to treat a patient in 1942, which revolutionized medicine and ushered in the age of antibiotics. Yet, resistance mechanisms that could inactivate penicillin, the β -lactamases, were already present among bacteria (Abraham & Chain, 1988). As has been uncovered over the years, the origins of most drug-specific antibiotic resistance mechanisms are genetically encoded functions that predate the introduction of the antibiotics. These mechanisms have been derived from the antibiotic-producing organisms, from the pathogens themselves or from commensals or environmental bacteria. Selective pressure by antibiotics has led to horizontal transmission of these resistance genes and further optimization (evolution) of the mechanisms (Davies, 1994, Davies & Davies, 2010).

The Journal of Medical Microbiology has published many insightful reviews on AMR, and important laboratory and epidemiological studies of the prevalence and spread of specific resistance mechanisms. A 1973 paper (Anderson *et al.*, 1973) presented a study of antibiotic-resistance transfer in the intestines of human volunteers, finding that Resistance-factors (R-factors) were successfully transferred when treated with antibiotics, and not in the absence of treatment. A later review of plasmid-mediated, horizontally-transmitted, enzymatic resistance to ampicillin and trimethoprim was the subject of an Oakley Lecture (Amyes, 1989). This lecture acknowledged the growing numbers of resistance determinants to

each antibiotic, but emphasized evolutionary patterns showing that the enzymes fell into a small number of groups. The metallo- β -lactamases, which can hydrolyze the carbapenems, were flagged more than 20 years ago as an emerging medical challenge (Payne, 1993). That challenge was realized recently with the advent of the NDM-1 metallo- β -lactamase. A recent review uses the rapid global spread of the NDM-1 β -lactamase as an example – and a warning – of the looming public health problem of antibiotic resistance that may only be controlled through international cooperation (Johnson & Woodford, 2013).

It is not only transmissible factors that are responsible for AMR. Especially in Gram-negative pathogens, intrinsic impermeability of the cell envelope and efflux mechanisms also plays a role. Mutational changes in these factors can lead to decreased susceptibility. *Pseudomonas aeruginosa* is a particularly problematic pathogen because it utilizes a variety of these mechanisms to put up a formidable barrier, limiting greatly the number of classes of chemotherapeutics that can overcome those mechanisms, to β -lactams, aminoglycosides, fluoroquinolones and colistin. A recent review in the Journal of Medical Microbiology describes this phenomenon (Strateva & Yordanov, 2009). In addition to permeability barriers and efflux, certain antibiotics, such as fluoroquinolones, can select mutations in the genes encoding their molecular targets. For the commonly used systemic monotherapeutic agents, resistance does not occur via single mutations in the target as they are generally not single proteins but are, rather, the products of multiple genes or a pathway (Silver, 2007). It is clear that new antimicrobial therapies are needed to bolster the diminishing antibacterial armamentarium, but

this is a difficult undertaking (Silver, 2011). AMR has become a real and proximate threat that requires constant monitoring - and the Journal of Medical Microbiology remains at the forefront of the undertaking.

Biofilm

The notion that microorganisms, in nature, exist as a sessile, systematized, multicellular communities was first described in the late 17th century by Van Leeuwenhoek. He examined plaque samples from his own teeth and found them to be a complex aggregation of micro-organisms, which he then referred to as “animalcules”. Even though the biofilm mode of bacterial existence was known for several centuries, the concept was not promulgated until the late 20th century. The late 1980s and 1990s saw an increased recognition of the fact that bacteria in their natural habitat survive and function as organized communities, encapsulated within a polymeric matrix - a crusade led by Costerton, the pioneer and father of biofilm research (Costerton *et al.*, 1987, Lappin-Scott *et al.*, 2014).

In the years that followed, *P. aeruginosa* emerged as the model organism to glean information on the various aspects of biofilm mode of bacterial lifestyle. Our appreciation for this lifestyle was facilitated by the advent of non-destructive investigation techniques, especially the use of live-monitor systems with confocal laser scanning microscopy. This provided an unprecedented ability to see (and almost feel) the beautiful architectural formations held together by an extracellular matrix. This also led us to question many aspects of biofilm that were accepted as a fact, especially where repetition has led to persuasion – the illusory truth effect. At that point, it was well established that *P. aeruginosa* secretes alginate, a

complex exopolysaccharide made of guluronic and mannuronic acid that provides selective advantage for its survival in lungs of patients with cystic fibrosis (Song *et al.*, 2003). The scientific community was persuaded that alginate was the matrix in *P. aeruginosa* biofilms. The Journal of Medical Microbiology was bold enough to publish the paper by Stapper *et al* that questioned the “illusion of truth” (Stapper *et al.*, 2004). Notwithstanding the importance of alginate in infection, this paper alluded to the potential contribution of other polysaccharides, which turned out to be Psl and Pel polysaccharides (Ma *et al.*, 2012, Jennings *et al.*, 2015).

Micro-organisms coexist in a complex society that includes bacteria, fungi, archaea and viruses, forming the multifaceted polymicrobial or mixed species biofilm communities. This provides the communities a competitive advantage such as AMR, metabolic cooperation, quorum sensing (QS), and many other synergies. It is only in the last few years that we have begun to fathom the complex nature of the mixed-species biofilm phenotype and its physiological role during infection. Adam *et al* presented a study on mixed fungal-bacterial biofilms using *Candida albicans* and *Staphylococcus epidermidis*, organisms that are frequently implicated in catheter-associated infections (Adam *et al.*, 2002). Their findings highlighted the coexistence of different microbial species, and their interdependence in the biofilm formation and AMR. In a follow-up study, the same group focused on the composition of *Candida*'s polymeric matrix, which contains carbohydrates, proteins, hexosamine, phosphorous and uronic acid, and its importance in promoting AMR (Al-Fattani & Douglas, 2006). Their work provided a critical insight into the complex biofilm phenotype. Following a series of articles in

the journal, Wilson published an insightful review that highlighted the antimicrobial sensitivity in oral biofilms (Wilson, 1996). This review summarized studies pertaining to the susceptibility of oral bacteria to antimicrobials, based on the type of biofilm models used. The author also emphasized the need for research in studying the resistance profile of oral biofilm communities, which can be crucial in developing an efficient therapeutic regimen. The Journal of Medical Microbiology has been instrumental in advancing biofilm research by publishing these studies, first of their kind to focus on the mixed-species biofilm and its contribution to AMR.

More importantly, the recognition of mixed biofilms in nature was the precursor to the microbiome studies that were facilitated by metagenomic analyses. Now, one could look at the potential relationship between groups of organisms within a single niche that makes a bacterial social network (Fernandez *et al.*, 2015). This was clearly facilitated by metagenomic analysis heralded by polymerase chain reaction (PCR) that has become a breastwork in all laboratories, replacing traditional techniques. This has revolutionized how we view ourselves, realizing that the number of bacteria exceeds the number of human cells by 10:1. The ease with which DNA is being sequenced and the rapid growth of DNA databases, and the development of eubacterial-specific, fungal-specific and species-specific primers, will enable rapid detection of micro-organisms that will become a mainstay in clinical laboratories. Although we borrowed the knowledge of soil microbiologists who developed bacterial-specific primers over 20 years ago, we have to thank the human microbiome project (HMP) launched in 1990, which truly contributed to the refinement of many bacterial-specific DNA primers (Jaric *et*

al., 2013). The Journal of Medical Microbiology contributed to this revolution by publishing two articles, one that focuses on fungal-specific (Makimura *et al.*, 1994) and the other on bacterial-specific (Harris & Hartley, 2003) analyses. The latter compared molecular diagnostics with standard culture techniques and offers a broad-range 16S rDNA PCR optimized to obtain the highest level of sensitivity for the detection of bacteria in clinical specimens. The future of rapid detection and diagnosis of infection truly rests on adopting molecular tools as part of routine clinical work.

The HMP studies have contributed significantly to our knowledge of the human microbiome (Turnbaugh *et al.*, 2007). It is now known that the human gastrointestinal tract alone harbors a diverse array of microbes that are critical to host nutrition, regulation of intestinal angiogenesis, and development of the immune response. Alterations in the composition of the intestinal microbiota have been associated with various disease states (Larson & Welch, 1993, Masseret *et al.*, 2001). A leading example of this is *Clostridium difficile*-associated diarrhea (CDAD), an infection caused by the Gram-positive *C. difficile*. CDAD occurs primarily in patients whose colonic microbiotas have been functionally altered by antibiotic therapy (Kelly & LaMont, 2008). *C. difficile* was identified in 1935 in a stool sample from a healthy neonate (Hall & O'Toole, 1935). However, it was not until 1976, just over four decades after its identification, that it was successfully cultured using the reinforced clostridial medium (Hafiz & Oakley, 1976). The Journal of Medical Microbiology took the lead in publishing the findings at a time when *C. difficile* was still considered a human commensal and was not associated

with any disease. It was later in 1978 that *C. difficile* was first reported to be a human pathogen (Larson *et al.*, 1978). In the recent years CDAD has become rampant, with morbidity and mortality increased over the decades past (Kelly & LaMont, 2008, Britton & Young, 2014). Colonic dysbiosis due to the use of antibiotics favors *C. difficile* germination and growth, as it is resistant to most of the antibiotics (Knecht *et al.*, 2014, Theriot *et al.*, 2014). Following the HMP, many studies have targeted alternative therapeutic regimens against *C. difficile*, including fecal transplantation. The Journal of Medical Microbiology has always been in the vanguard even before the HMP published data pertaining to the fecal microbial load (Stephen & Cummings, 1980) and the effect of *C. difficile* infection on fecal microbiome (Hopkins & Macfarlane, 2002).

Quorum sensing (QS)

In the 1960s and 70s, scientists decried the notion that bacteria have the ability to communicate. In the 1990s this notion was christened as QS, by which bacteria can sense bacterial numbers (cell density), integrate and process the environmental cues, and coordinately modify their behavior by expressing target genes (Nealson & Hastings, 1979, Greenberg, 2003). Now, ample evidence exists for inter-species, inter-genera and inter-kingdom communications using largely diffusible small molecules called quoromones or autoinducers (Williams, 2007). In Gram negative, these molecules are largely made of small chemicals, whereas Gram positive tend to use oligopeptides. These signaling molecules interact with cognate receptors. It became clear that bacteria employ QS to coordinate the expression of virulence factors critical for initiation and establishment of infection.

It was also shown that the QS molecules themselves acted as virulence factors by being potent stimulators of multiple eukaryotic cells (Smith & Iglewski, 2003). Subsequently, a flurry of activities began in this area with everyone looking to link their pet system to QS. A significant publication in Science using the *P. aeruginosa* PAO1, which had seen many passages and its isogenic QS mutants, argued the complete dependence on QS for biofilm formation (Davies *et al.*, 1998). Once again, the Journal of Medical Microbiology led the way in debunking this story. In 2007, Schaber and colleagues were able to isolate QS-deficient clinical isolates and demonstrate their ability to form biofilms, although the quality of the biofilm varies (Schaber *et al.*, 2007). This seriously questioned whether QS was critical for biofilm formation and infection. In fact, recent work by Greenberg's team showed that within a colonizing community, one could have social cheaters who can coexist and that QS plays a role in policing this cooperation (Wang *et al.*, 2015).

With the emergence of AMR it seems natural that research turn towards finding anti-QS molecules as an alternative therapy. With the first report of furanones as anti-QS molecules (Manefield *et al.*, 1999), there was a slew of articles that focused on everything from natural products to synthetic compounds. Once again, the opportunistic pathogen *P. aeruginosa* became the protagonist in the search for anti-QS molecules, but investigations also included Gram-positives such as *Staphylococcus* spp. (Nostro *et al.*, 2007). Some researchers randomly looked at herbs, including ancient remedies such as ginseng (Schneper *et al.*, 2011), and others took an ethnobotanic approach by looking at natural products

that are used by local communities for medicinal value (Adonizio *et al.*, 2006). The latter identified various medicinal plants that had anti-QS activities which attenuated bacterial pathogenic virulence factor production, and could rescue *Caenorhabditis elegans* from *P. aeruginosa*-induced death (Adonizio *et al.*, 2008). However, with over 15 years of searching, an anti-QS panacea has not emerged. This is, in part, due to the ability of the QS and the anti-QS molecules to elicit an immune response that can lead to inflammation.

Though the focus has largely been on looking at the infecting organism for drug targets, we tend to forget that the host contributes to the bacterial shenanigans. It was well known that in the lungs of cystic fibrosis patients, *P. aeruginosa* lives in a novel biofilm lifestyle as aggregates (Sriramulu *et al.*, 2005) surrounded by “frustrated” phagocytes (Høiby *et al.*, 1993), and accumulate mutations (Oliver *et al.*, 2000). However, it was in the late 1990s that the role of neutrophils in the critical phenotypic conversion contributing to poor prognosis for the patient was demonstrated (Mathee *et al.*, 1999). This study suggested anti-oxidants and anti-inflammatory agents as potential therapeutic measures. In 2009, a paper by Parks and colleagues not only explored the role of neutrophil in the quality and quantity of biofilm, but also proposed neutrophil necrosis as a potential drug target (Parks *et al.*, 2009).

In this 70 year journey, the Journal of Medical Microbiology has been one of the torch bearers in advancing scientific research by critically reviewing and publishing articles related to the diverse medical microbiology field. The journal has never shied away from thinking outside the box, allowing it to embrace new

viewpoints. This will substantially benefit basic research and will help in solving the mysteries of the microbial world, whether they be antibiotic resistance, biofilm formation, QS or the more advanced microbiome studies.

Appendix 2

Rapid Detection of Mucoid *Pseudomonas aeruginosa* using Microextraction of Volatiles

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* Equal Contribution (Being Submitted)

Abstract

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium with remarkable abilities to adapt to environmental challenges. A primary pathogen responsible for all Gram negative infections in clinical settings, it is associated with lung decline and the untimely demise of people with cystic fibrosis (CF). One of the hallmarks of *P. aeruginosa* chronic infection in CF patients is the presence of mucoid isolates that spells poor prognosis. Here we report a rapid detection technique for *P. aeruginosa* based on capillary microextraction of volatiles (CMV) using the newly invented dynamic headspace sampling device. The method is composed of a one min dynamic sampling followed by 17 min GC-MS analysis time. The simple, sensitive and rapid method identified 2-aminoacetophenone and 1-undecene as biomarkers for the presence of *P. aeruginosa*. The comparison results between the mucoid and non-mucoid strains show potential use of 1-undecene for differentiation of acute and chronic infection of *P. aeruginosa* using the non-invasive CMV-based breath analyzer.

Introduction

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen implicated in causing systemic infections in immunocompromised and immunosuppressed individuals (Bodey *et al.*, 1983, Ramos, 2004). Its metabolic versatility and adaptability has significantly contributed to its ubiquity (Ramos, 2004). It has increasingly been recognized as a major cause of morbidity and mortality in many clinical settings accounting for 1 in every 10 hospital-acquired infections (Abraham & Chain) (Giamarellou, 2000). From 1975 to 2003, the percent of HAI pneumonias caused by *P. aeruginosa* nearly doubled in the US, from 9.6 to 18.1% (Gaynes & Edwards, 2005, Talbot *et al.*, 2006). Unfortunately, the prognosis for these patients is often poor. Beyond high infection rate, *P. aeruginosa* also contributes to the highest case fatality rate of all Gram-negative infections (Aliaga *et al.*, 2002). It is the primary pathogen responsible for lung decline and mortality in patients with cystic fibrosis (CF) (Doggett, 1969, Lyczak *et al.*, 2002). In the CF lungs, transition from acute to chronic infections is marked by the development of resistance to antibiotics (Workentine *et al.*, 2013), and conversion to a mucoid phenotype (Penketh *et al.*, 1983, Govan & Nelson, 1992). The mucoid phenotype is due to constitutive production of alginate (Alg), a component of extracellular polymeric (EPS) matrix (Flemming *et al.*, 2007). Mucoidy protects the bacteria from opsonization, phagocytosis, reactive oxygen species and antibiotics (Mathee *et al.*, 1999, Pritt *et al.*, 2007). Thus, early detection of *P. aeruginosa* infections plays a major role in the treatment stratagem and clinical outcome.

For early and precise detection of *P. aeruginosa*, numerous culture, biochemical, Polymerase Chain Reaction (PCR) and serology based approaches have been developed (Qin *et al.*, 2003, Xu *et al.*, 2004, Tramper-Stranders *et al.*, 2005, Tramper-Stranders *et al.*, 2006, Daines *et al.*, 2014). In particular, during the last decade, there has been a substantial upsurge in PCR-based approaches for early detection of *P. aeruginosa* in the respiratory samples of CF patients (Deschaght *et al.*, 2011). However, in spite of the fact that PCR amplifications have been successfully used as a routine technique for identification of viruses in clinical samples (Niesters, 2002), their use in bacterial diagnostics including *P. aeruginosa*, is still limited. This is mostly due to the questionable specificity and sensitivity of these techniques. To date, *P. aeruginosa* detection and identification greatly relies on culture-based approaches which are time consuming, influenced by testing conditions and subject to physician interpretation which may lead to misinterpretation and misidentification (Deschaght *et al.*, 2011). This together strongly suggests the need for development of new diagnostic tools that will help pave the way for a more efficient and specific *P. aeruginosa* detection techniques.

Breath analysis is a potential revolution in disease diagnostics since the collection of exhaled breath is a safe, non-invasive, easy and simple procedure, and each individual contains information regarding their own internal state (Cepelak & Dodig, 2007, Hunter & Dweik, 2008). Urea breath test (UBT) has been effectively used to detect *Helicobacter pylori* infections with a success rate of 90% (Gisbert & Pajares, 2004). Breath analyses have also been widely tested in experimental studies to identify biomarkers that can be correlated to various

diseases (Dent *et al.*, 2013, Koutsokera *et al.*, 2013, Sarbach *et al.*, 2013). Bulk matrix of breath is largely composed of nitrogen, oxygen, carbon dioxide, water, and inert gases. However, the breath condensate contains trace amounts (parts per billion (ppb) to parts per trillion (ppt)) of inorganic gases, volatile organic compounds (VOCs), and other normally nonvolatile substances that can serve as biomarkers (Miekisch *et al.*, 2004). For example, nitric oxide (NO) gas was reported with an increased concentration in patients with airway inflammation such as asthma and bronchiectasis (Kharitonov *et al.*, 1997); and the odor of acetone in the breath was a characteristic feature of diabetic coma (Crofford *et al.*, 1977).

P. aeruginosa has been reported to produce a “grape-like” odor. The compound responsible for this peculiar odor has been identified as 2-aminoacetophenone (2-AA) by Mann in 1966 (Mann, 1966). Importantly, 2-AA was not detected in any other respiratory pathogens and thus was used as a biomarker for *P. aeruginosa* infection and/or colonization in the lung (Groenewold *et al.*, 2011). Although specific for *P. aeruginosa* infections, 2-AA has also been identified in certain foods including corn, dairy, honey products and wine (Karagul-Yuceer *et al.*, 2002, Fan *et al.*, 2007, Schmarr *et al.*, 2007, Scott-Thomas *et al.*, 2010). Breath sample analysis of uninfected individuals shortly after eating these foods may give a false positive which could limit the diagnostic procedures (Scott-Thomas *et al.*, 2010). In addition to 2-AA, spontaneously expectorated sputum containing *P. aeruginosa* show the presence of 2-nonanone, 2,4-dimethyl-1-heptene, 1-heptene, isopentanol, and limonene (Savelev *et al.*, 2011); and *P. aeruginosa*

stationary growth culture releases dimethyl sulfide, 1-undecene, and 2-nonanone (Zscheppank *et al.*, 2014, Briard *et al.*, 2016).

In this study, a novel, fast, dynamic headspace sampling device, capillary microextraction of volatiles (CMV), was used to capture volatile compounds released by *P. aeruginosa* on standard culture plates. The analytical method consists of sampling the headspace above the culture plates for 1 min using the CMV device followed by inserting it into a thermal probe that is connected to a GC split/splitless (S/SL) injection port, followed by ~17 min chromatographic program of GC-MS analysis after thermal desorption of the absorbed analytes from CMV. The CMV tube (Figure A2.1) performs similarly to commercial sorbent tubes in terms of extraction efficiency, sensitivity, breakthrough and recovery. However, it does not require the complicated thermal desorption accessory normally associated with sorbent tubes. Due to its extremely high surface area and phase volume, small volume sampling (~ 1 L) results in sub-ng absolute detection limits for the analytes of interest, making the CMV technique capable of detecting ppb concentrations of VOCs in air. In this study, we report the presence of the biomarkers 1-undecene and 2-AA among others from the *P. aeruginosa*. Moreover, we report that the presence of 1-undecene can be used for the differentiation between mucoid and nonmucoid strains of *P. aeruginosa*. Compare to previously described method, this is one of the first reporting of an inexpensive, fast and ultra-sensitive technique for the detection of *P. aeruginosa* that is accessible to a moderately equipped analytical laboratory requiring only GC-MS instrumentation (Table A2.1).

Table A2.1. Comparison of extraction and analytical parameters between this work and previous reports of biomarker detection from pathogens.

	(Savelev <i>et al.</i> , 2011)	(Zscheppank <i>et al.</i> , 2014)	This Report	(Scott-Thomas <i>et al.</i> , 2010)
Extraction Method	SPME (DVB-Carboxen-PDMS), closed system	NT, closed system	CMV, semi-closed system	SPME (Carbowax/PEG), closed system
Setup	Easy - Moderate	Moderate	Easy	
Extraction Time	50 min	12 min	1 min	5 min
Extraction Temperature	37 °C		RT	
Injection Port	Split/Splitless		TDU	PTV-1079
Mode	Pulsed Splitless Mode		Split/splitless	Split (50:1)
Detector	EI, 70 eV, full-scan			
Column	ZB-Wax, 30 m x 0.25 mm x 0.25 μ m	Optima 1, 30 m x 0.32 μ m x 1 μ m	DB-5ms, 8 m x 0.25 mm x 0.25 μ m	ZB-Wax, 30 m x 0.25 mm x 0.25 μ m
Initial temp	40 °C, 5 min	35 °C	40 °C, 1 min	60 °C, 2 min
Ramp	5 °C/min to 100 °C	1 °C/min to 53 °C, 3 min	15 °C/min to 115 °C, 1 min	10 °C/min to 250 °C, 2 min
Ramp	5 °C/min to 150 °C, 2 min	15 °C/min to 120 °C	25 °C/min to 280 °C, 3 min	
Ramp	5 °C/min to 230 °C	5 °C/min to 160 °C		
Ramp		2.5 °C/min to 180 °C		
Ramp		2 °C/min to 200 °C		
Run Time	44 min	51 min	16.6 min	21 min
He	1 mL/min	1.5 mL/min	1 mL/min	1.2 mL/min

Figure A2.1. Capillary Microextraction of Volatiles (CMV) device. (a) A 2 cm CMV device was shown in scale. (b) Detailed schematic of a CMV device where vinyl-terminated sol-gel PDMS glass fiber filters were packed in glass capillary tubing.

Materials and Methods

Bacteria strains, media and growth conditions

Bacterial strains used in this study are listed in Table A2.2. Luria-Bertani (LB) (Fisher Scientific) broth was used for routine cultivation of bacterial cultures and was supplemented with 1.5% agar when needed, unless specified otherwise. All cultures were grown overnight in LB broth with shaking at 200 rpm, at 37°C for *Pseudomonas aeruginosa* (Mucoid/Non-mucoid), *Staphylococcus aureus*, *Escherichia coli*, 26°C for *Serratia marcescens*, *Chromobacterium violaceum* and 30°C for *Bacillus* strains. Briefly, overnight cultures were diluted to obtain equal optical densities. Culture densities were normalized to a final A_{600} of 0.05-0.08 before inoculating on LB agar plates by swabbing the surface with adjusted culture suspensions. Plates were allowed to dry before incubating overnight for 12 to 16 h at 37°C, 30°C and/or 26°C depending on the culture. In addition, mixed culture suspension of *P. aeruginosa* prototypic PAO1, *E. coli* (DH5 α), *S. marcescens*, *B. cereus* and *S. aureus* in different combination were used. For mixed bacterial cultures, the plates were incubated overnight at 30°C. In total, 280 culture plates were sampled, and each strain was analyzed at least three times. All the sampling and analyses were accomplished using blind study method. The culture plates containing all the bacteria, including the negative controls, were labeled with reference IDs to minimize any bias during the headspace sampling. The LB agar plate with no culture was used as a negative control.

Dynamic headspace sampling using CMV devices

The CMV devices were recently developed and reported as an efficient way to detect trace amounts of explosives (Fan & Almirall, 2014). Fabrication of the CMV devices has been described elsewhere (Fan & Almirall, 2014). These 2-cm length open-ended sampling devices allow for fast dynamic headspace sampling. The CMV devices were conditioned in an oven at 250 °C for an hour prior to use, cooled to room temperature and connected to a hand-held air monitoring vacuum pump (Escort Elf Air Sampling Pump, Zefon International Inc., Ocala, FL) that provides a flow through the CMV device at the rate of 1.5 liters per min (LPM). For the culture plates sampling, the LB agar culture plates were popped open with a small opening where the CMV devices were simply inserted into the plates for 1 min dynamic sampling at a flow rate of 1.5 L/min (Figure A2.2). The CMV sampling device capable of sub-nanogram detection of VOCs, when coupled to GC-MS (Fan & Almirall, 2014, Tarifa & Almirall, 2015) (Air Chemistry, Inc, Miami, FL).

Sample analysis using GC-MS

After 1 min sampling, the CMV device was disconnected from the tubing and inserted into a probe for thermal desorption into an Agilent Technologies 7890A GC S/SL injector that was connected to thermal separation probe adaptor from Agilent Technologies Inc., Santa Clara, CA. The injector was set to 180 °C. The Agilent Technologies 7890A GC system was equipped with a 5975C inert XL mass spectrometer detector (MSD). The GC column used in this research was a DB-5ms Ultra Inert, 8 m × 0.25 mm × 0.25 µm column. For the initial study, the GC oven temperature ramp started at 40 °C, was held for 1 min and ramped at 15

°C/min to 200 °C and held for 1 min, ramped again at 15 °C/min to 240 °C, held for 6.5 min and ramped at 25 °C/min to 270 °C, ramped at 5 °C/min to a final temperature of 280 °C and held for 4 min. The total analyses time was 29.33 min that provided with a high resolution for detection of various volatile organic compounds in the headspace over the culture plates. Once biomarkers were identified for *P. aeruginosa* strains, a fast GC oven temperature program was used to reduce the analyses time. The oven temperature started at 40 °C for 1 min, and ramped to 115 °C at 15 °C/min and held for 1 min, and ramped to final temperature of 280 °C at 25 °C/min and held for 3 min. The total analyses time was reduced to 16.6 min.

Results

Identification of VOCs in the Headspace of *P. aeruginosa*

The VOCs present in the prototypic *P. aeruginosa* strain PAO1 were differentiated from other bacteria, *E. coli* DH5 α and *C. violaceum*, in this particular study and characteristic VOCs were found in the *P. aeruginosa* strains using the 1-min. headspace sampling method. After 24 hours of incubation, the headspace of the plates were sampled with the CMV for 1 min. 1-Undecene and 2-aminoacetophenone (2-AA) were detected in *P. aeruginosa*, and indoles were detected in *E. coli* (Figure A2.3). After 48 hours of incubation at room temperature, limonene was also detected in both the *P. aeruginosa* and *C. violaceum* plates; thus, limonene wasn't chosen as a biomarker along with 1-undecene and 2-AA in this research.

Differentiation of *P. aeruginosa* from Other Bacterial Species

The 1-min. headspace sampling using CMV resulted in the differentiation of the 199 plates containing *P. aeruginosa* strains from the 66 plates containing other bacteria using blind sampling studies. The compounds 1-undecene and 2-AA were found only in the headspace of the *P. aeruginosa* strains (see Table A2.3) and these results are consistent with previous workers who also reported finding these compounds as biomarkers of *P. aeruginosa*. Almost every plate containing the *P. aeruginosa* strain resulted in the detection of one or both of these biomarkers. Additionally, none of the plates containing the other bacteria resulted in the presence of either or both of the biomarkers. A confusion matrix was built (Table A2.3) in which the theoretical biomarker present is equivalent to *P. aeruginosa*

plate and the theoretical biomarker absence is equivalent to a plate containing other bacteria. Based on this confusion matrix, two observations can be made. First, where the true negative rate equals to 1.00 indicates that the two biomarkers chosen are not found in the other bacterial strains tested in this study. Second, the false negative rate of 0.12 indicates that a few *P. aeruginosa* strains do not emit either 1-undecene or 2-AA. Muroid strains may be retaining the VOCs better than the non-muroid strains and therefore the biomarkers are more difficult to detect.

Detection of 2-AA and 1-undecene is specific for *P. aeruginosa*

To determine if this detection method is specific and sensitive, several mixtures of species were made (Table A2.4). The headspace of each mixture was also sampled and the chromatograms were overlaid (Figure A2.4). As predicted, the two compounds, 1-undecene and 2-AA were detected in Group 1 (*P. aeruginosa*, *S. marcescens*, and *S. aureus*) and 4 (*P. aeruginosa* alone), and indole was found in Group 2 (*E. coli*, *S. marcescens*, and *B. cereus* UW85), 3 (*E. coli*, *S. marcescens*, and *S. aureus*) and 5 (*E. coli* alone). Thus, the test method for 1 min fast dynamic sampling followed by analysis in GC-MS is suitable even when a complex mixture is present.

Differentiation between muroid and non-muroid *P. aeruginosa* strains

P. aeruginosa strains often transition from acute to chronic infections in patients with CF. The transition is marked by development of resistance to antibiotics and conversion to muroid phenotype. The muroid phenotype is due to overproduction of alginate (Figure A2.5). The muroid strains are referred to as Alg⁺. Thus we hypothesized that the transition will lead to concomitant change in

odor the profile. To test this hypothesis, eight non-mucoid and 10 mucoid strains were tested (Figure A2.6).

For the eight mucoid strains analyzed, 1-undecene was detected in all 83 plates (100% detection rate); while 2-AA was found in 80 plates (96.4% detection rate) (Figure A2.6). The only strain that didn't have 2-AA in headspace is strain CDN118, which is a pan-resistant bacterimic isolate from Nigeria. The absence of 2-AA in this single strain needs further study.

The 2-AA biomarker was detected in 85 of 116 (73.3%) of the mucoid strains (Figure 2A.6). The plates that failed to have detectable 2-AA in the headspace included strain ZW77 (3 plates), PAO1 (3 plates), and PDO300 (17 plates). The 1-undecene was detected in all of the non-mucoid (Alg^-) strains whereas only 41.3 % of the mucoid isolates presented 1-undecene in the headspace (Figure 2A.6). The mucoid strains may not produce appreciable amounts of 1-undecene. The presence of 1-undecene in some of the plates could result from the presence of non-mucoid strains in the plates due to their ability to transition during the incubation process. In fact, the Alg^+ phenotype of PDO300 is unstable and up to 80% of the isolates can revert to non-mucoid phenotype (Sautter *et al.*, 2012). To test this hypothesis, volatiles from three stable mucoid strains PA2192 (12 plates), FRD39 (13 plates), were compared with the Alg^- PAO1 (6 plates) strains. As expected, only 2-AA was found in all 31 plates. The stable mucoid strains were differentiated from the non-mucoid strains by only the presence of these two biomarkers using the CMV headspace test described.

Figure A2.2. The dynamic headspace sampling process using CMV device. (a) A CMV device is connected to the handheld pump for 1 min sampling. (b) A CMV device was inserted into the small opening of a culture plate.

Figure A2.3. Chromatograph of headspace samples. The chromatographs from 1 min dynamic sampling of LB plates with no bacteria, with *E. coli*, *P. aeruginosa*, and *C. violaceum* were overlaid. 1-undecene and 2-AA were only found in the headspace of *P. aeruginosa*, whereas indole was detected in the headspace of *E. coli*.

Figure A2.4. Overlaid chromatograms for plates contain multiple bacteria strains. The two biomarkers used in the study, 2-AA and 1-undecene, were only found in the mixtures which contained *P. aeruginosa* strain.

Figure A2.5. Non-mucoid (Alg⁻) and mucoid (Alg⁺) *P. aeruginosa*. The prototypic *P. aeruginosa* such as PAO1 are non-mucoid. The conversion seen during chronic infection, especially in patients with cystic fibrosis is due to a mutation in *mucA* encoding an anti-sigma factor. The mucoid phenotype reverts back to non-mucoidy in the absence of selection due to second site suppressors strain (Sautter *et al.*, 2012).

Figure A2.6. The detection frequency of 1-undecene and 2-aminoacetophenone (2-AA) in mucoid and non-mucoid *P. aeruginosa* strains. 1-Undecene was 100% detectable in non-mucoid *P. aeruginosa* strains, while only 43% detectable in mucoid strains. The significant drop in detection frequency led to the hypothesis of using 1-undecene as a biomarker for separation of mucoid and non-mucoid strains.

Table A2.2. Bacterial strains used in this study

Strain ID	Strain Background	Relevant characteristics	Source
<i>P. aeruginosa</i> strains			
PAO1	PAO1	Alg ⁻ ; Prototypic wild type, a wound isolate	(Stover <i>et al.</i> , 2000)
PDO300	PAO1	Alg ⁺ ; PAO <i>mucA22</i> , constitutively mucoid	(Mathee <i>et al.</i> , 1999)
PKM315	PAO1	Alg ⁻ ; PAOΔ <i>ampR</i>	(Balasubramanian <i>et al.</i> , 2012)
PKM816	PDO300	Alg ⁻ ; PDO300 revertant, non-mucoid; PAO <i>lagO96 mucA22</i>	<i>sap26</i> ; Mathee Lab
PKM831	PDO300	Alg ⁻ ; PDO300 revertant, non-mucoid; PAO <i>mucP451 mucA22</i>	<i>sap21</i> ; Mathee Lab
PKM900	PAO1	Alg ⁻ ; PAOΔ <i>mifR</i> ; in-frame deletion of <i>mifS</i>	(Tatke <i>et al.</i> , 2015)
PKM901	PAO1	Alg ⁻ ; PAOΔ <i>mifS</i> ; In-frame deletion of <i>mifS</i>	(Tatke <i>et al.</i> , 2015)
PKM902	PAO1	Alg ⁻ ; PAOΔ <i>mifSR</i> ; in-frame deletion of <i>mifS</i> and <i>mifR</i>	(Tatke <i>et al.</i> , 2015)
CDN118		Alg ⁻ ; Panresistant clinical isolate from Nigeria	C.D. Nkwonta
PA2192	2192	Alg ⁺ ; Mucoid cystic fibrosis (CF) clinical isolate	(Pier <i>et al.</i> , 1983)
FRD1	FRD1	Alg ⁺ ; Mucoid CF clinical isolate	(Ohman & Chakrabarty, 1981)
FRD39	FRD1	Alg ⁺ ; Mucoid isolate	(Ohman & Chakrabarty, 1981)
ZW77		Alg ⁺ ; <i>poxB15</i> , CF throat swab, London, UK, 1997	(Kong <i>et al.</i> , 2005)
ZW98		Alg ⁻ ; CF throat swab, The Hague, Netherlands, 1997	(Kong <i>et al.</i> , 2005)
PT22		Alg ⁻ ; Water, Mühlheim, Germany, 1992	(Kong <i>et al.</i> , 2005)
641 HD		Alg ⁻ ; <i>poxB22</i> ; Water, Mühlheim, Germany, 1992	(Kong <i>et al.</i> , 2005)
PKM122	PAO1	Alg ⁺ ; PAO <i>mucA22</i> ; mucoid strain isolated from PAO1 biofilm after PMN exposure	(Mathee <i>et al.</i> , 1999)

PKM123	PAO1	Alg ⁺ ; PAO <i>mucA</i> 22; mucoid strain isolated from PAO1 biofilm after PMN exposure	(Mathee <i>et al.</i> , 1999)
Bacillus strains			
ATCC 35866	<i>B. thuringiensis</i>	NRRL B4488 [HD73]	ATCC
ATCC 23857	<i>B. subtilis</i>	<i>ind- tyr</i> ⁺	ATCC
ATCC 14581	<i>B. megaterium</i>		ATCC
ATCC 53522	<i>B. cereus</i> UW85		ATCC
Other bacterial strains			
CV026	<i>Chromobacterium violaceum</i>	Violaceum negative mutant	(Pier <i>et al.</i> , 1983)
DH5α	<i>Escherichia coli</i>	F ⁻ Φ80 <i>lacZ</i> ΔM15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ mk ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	New England Biolabs
ATCC 13880	<i>Serratia marcescens</i>	Isolated from pond water	ATCC
KM1000	<i>Staphylococcus aureus</i>	Wild type	Mathee Lab

Table A2.3. Confusion matrix built upon the blind study.

Confusion Matrix		Theoretical	
		Biomarker Present	Biomarker not Present
Experimental	Biomarker Present	176 (TP=0.88)	0 (FP=0)
	Biomarker not Present	23 (FN=0.12)	66 (TN=1.00)
TP: True Positive Rate; FP: False Positive Rate; FN: False Negative Rate; TN: True Negative Rate			

Table A2.4. Mixture of bacterial cultures

	Strain Background	Strain IDs
Mixture 1	<i>P. aeruginosa</i>	PAO1
	<i>S. marcescens</i>	ATCC 13880
	<i>S. aureus</i>	KM1000
Mixture 2	<i>E. coli</i>	DH5α
	<i>S. marcescens</i>	ATCC 13880
	<i>B. cereus</i> UW85	ATCC 53522
Mixture 3	<i>E. coli</i>	DH5α
	<i>S. marcescens</i>	ATCC 13880
	<i>S. aureus</i>	KM1000
Mixture 4	<i>P. aeruginosa</i>	PAO1
Mixture 5	<i>E. coli</i>	DH5α

Discussion

The rapid sampling technique using CMV devices coupled to GC-MS for sample analysis is an effective method to differentiate *P. aeruginosa* strains from the other bacterial pathogens. The two biomarkers used in this study, 2-AA and 1-undecene, are only found in the headspace of *P. aeruginosa* strains, in comparison to the headspace of several other pathogen strains. No significant levels of biomarkers were found for the other bacterial pathogens except that indole was found in the headspace of DH5 α (*E. coli*). Beyond this, when multiple bacteria were mixed together, the method doesn't show any interference and is specific in identifying the two biomarkers only from the mixtures that contained the *P. aeruginosa* strains.

In addition, to differentiate *P. aeruginosa* strains from the other bacterial pathogens, the method could also be used for separation of *P. aeruginosa* mucoid and non-mucoid strains. In the analysis of 8 mucoid strains, the 1-undecene detection rate dropped significantly from 100% to 41.3%, when compared to the analysis of 10 non-mucoid strains. These results suggest that either the mucoid strains do not produce 1-undecene in the headspace as a biomarker or the mucoidy prevents the release (absorbs) the 1-undecene. The headspace analysis of the three stable mucoid strains shows that 2-AA was the only biomarker detected in the headspace. Previously, solid phase microextraction (SPME) has been used for studying the headspace of *P. aeruginosa* (Scott-Thomas *et al.*, 2010, Savelev *et al.*, 2011). Because of the static sampling nature of the SPME device and its limited surface area and phase volume, the static sampling SPME

methods required longer analysis times. In addition, the SPME glass fiber is very fragile and thus not suitable for field applications. In this study, the newly developed method is rugged, dynamic, fast, and has comparable or better sensitivity as SPME. However, in the analysis of about 300 culture plates, a relatively low true positive rate (88%) was found which resulted from three out of eight mucoid strains. As discussed above, the mucoid strains may have the polysaccharide cover to prevent the release of biomarkers into the headspace; thus, culturing the plates under a condition to prevent conversion from mucoid to non-mucoid might be useful for successful detection that can lead to a higher true positive rate. Additionally, both stable and unstable mucoid strains should be analyzed. Further experiments should also include quantitative analysis that addresses the number of bacteria on the culture plate versus the headspace biomarker signals. These studies may provide further insights into the concentration of biomarker compounds that can differentiate the mucoid and nonmucoid strains of *P. aeruginosa*.

Conclusions

The newly reported CMV device for dynamic headspace sampling of VOCs over bacteria culture plates provides for a simple and inexpensive extraction setup. The 1 min sampling time significantly increases the sampling throughput and the direct introduction of the CMV into a port of a GC-MS followed by thermal desorption produces a VOC profile in ~ 17 min for each plate. 1-undecene and 2-AA were identified as the biomarkers for *P. aeruginosa* in these studies. In further screening test, 265 plates were sampled and no false positive was generated with a total number of 66 other bacteria (not containing *P. aeruginosa*) plates analyzed. In the analysis of non-mucoid strains, the clinical isolate CDN118 did not produce 2-AA in the headspace, but 1-undecene was found in all plates; thus, 1-undecene can be used as the primary biomarker while 2-AA as a secondary marker for confirmation. In addition, 1-undecene might be used as the biomarker to differentiate mucoid and non-mucoid *P. aeruginosa* isolates. The method was also successfully applied to the culture plates with multiple bacteria (mixtures). The detection results showed the method is sensitive and effective even with a relative complex matrix. In conclusion, the simple and fast sampling method developed in this research is effective in screening bacteria culture plates to identify *P. aeruginosa* strains. Additional developmental research may lead to rapid, non-invasive detection of *P. aeruginosa* in the breath of patients that are susceptible to *P. aeruginosa* infection such as potential cystic fibrosis patients.

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2014	NIH MBRS RISE Biomedical Research Initiative (BRI) Student Summer Research Award, Florida International University
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Major Professor	Department	Project Title
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Dr. Almirall	Chemistry & Biochemistry	Rapid Detection of Muroid <i>Pseudomonas aeruginosa</i> using Microextraction of Volatiles
Dr. Liuzzi	Dietetics & Nutrition	Developing <i>Caenorhabditis elegans</i> assay

PUBLICATIONS

O. Weeks, G. Tatke, K. Mathee, C. Espinal (2016). Report: 6th International Conference on Global Health. http://ghc.fiu.edu/?page_id=3491

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