METABOLIC CAPABILITY OF *Acinetobacter baumannii* BASED ON PHENOTYPE MICROARRAY ANALYSIS AND THE EFFECTS OF SELECTED SUBSTRATES ON ITS BIOFILM FORMING ABILITY

AHLAM HODAED ALSHEIKH

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

METABOLIC CAPABILITY OF *Acinetobacter baumannii* BASED ON PHENOTYPE MICROARRAY ANALYSIS AND THE EFFECTS OF SELECTED SUBSTRATES ON ITS BIOFILM FORMING ABILITY

AHLAM HODAED ALSHEIKH

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Ahlam Hodaed Alsheikh

Matric No: SGF160024

Name of Degree: Master of Biotechnology

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

METABOLIC CAPABILITY OF *Acinetobacter baumannii* BASED ON PHENOTYPE MICROARRAY ANALYSIS AND THE EFFECTS OF SELECTED SUBSTRATES ON ITS BIOFILM FORMING ABILITY

Field of Study: Microbial Biotechnology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature [redacted] Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name: Prof. Dr. Thong Kwai Lin

Designation

METABOLIC CAPABILITY OF *Acinetobacter baumannii* BASED ON PHENOTYPE MICROARRAY ANALYSIS AND THE EFFECTS OF SELECTED SUBSTRATES ON ITS BIOFILM FORMING ABILITY

ABSTRACT

Acinetobacter baumannii (A. baumannii) is one of the commonly reported nosocomial infectious agents causing high morbidity and mortality worldwide. The objective of the study was to determine the metabolic capability of *A. baumannii* by using the Phenotype Microarray technology and to determine the effects of selected substrates on the biofilm forming ability of *A. baumannii.* Two strains of *A. baumannii,* ACIBA-46 and ACIBA-47 which were previously isolated from the ventilator and hands of healthcare workers (HCWs), respectively, were tested. Kinetic data of utilization of various carbon, nitrogen, phosphorus and sulfur substrates which were previously generated, were converted into spreadsheet format using Biolog OmniLog Phenotype Microarray Software for further statistical analysis. The effects of selected substrates on the biofilm forming ability of these two strains were evaluated according to established protocols. The analyses of the carbon utilization data indicated that both ACIBA-46 and ACIBA-47 strains had a low carbon catabolic capability, utilizing only 48 of 190 (25%) carbon substrates tested. However, the HCWs strain *A. baumannii* ACIBA-47 appeared to utilize a higher number of carbon substrates (6 carbons) compared to the ventilator strain ACIBA-46. Both of the *A. baumannii* strains mainly used the two classes of carboxylic acids and amino acids as carbon sources, which include alanine, asparagine, serine, histidine, ornithine, arginine, pyruvic acid and succinic acid that are associated with energy metabolism. Interestingly, this study found some of nitrogen substrates inhibited the growth of ACIBA-46 strain such as uric acid and alloxan. Both ACIBA-46 and ACIBA-47 strains were able to form a moderate biofilm in rich medium (LB broth), but were non-biofilm formers in nutrient depleted medium (M9MM). When M9MM was supplemented with 22 substrates (tested

individually), there was a change in the biofilm forming ability for both strains. Substrates such as, L- arabinose, L- arginine and urea slightly increased the ability of forming biofilm for both strains. In addition, substrates such as L-histidine, L- leucine and thymidine were moderately increased the ability to form biofilm for both strains. However, substrates such as glycine and Tween 80 showed no effect on the biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains. The elucidation of the metabolic activity of *A. baumannii* will offer scientists insight into ways to manipulate such activity either to curtail the pathogens success or enhance its susceptibility to antibiotic agents.

Keywords: *A. baumannii*, phenotype microarray, metabolic capability, biofilm

KEUPAYAAN METABOLIK *Acinetobacter baumannii* BERDASARKAN ANALISIS FENOTIP MICROARRAY DAN KESAN SUBSTRAT TERPILIH PADA KEUPAYAAN MEMBENTUK BIOFILM

ABSTRAK

Acinetobacter baumannii (A. baumannii) merupakan agen utama jangkitan nosokomial yang menyebabkan bilangan kematian yang tinggi di seluruh dunia. Objektif kajian ini adalah untuk menentukan keupayaan metabolik A. baumannii dengan menggunakan teknologi "Phenotype Microarray" (PM) dan menentukan kesan substrat terpilih ke atas keupayaan pembentukan biofilm A. baumannii. Dua strain A. baumannii, ACIBA-46 dan ACIBA-47 yang sebelum ini diasingkan dari ventilator dan tangan pekerja kesihatan masing-masing telah diuji. Data kinetik penggunaan pelbagai substrat karbon, nitrogen, fosforus dan sulfur yang dihasilkan sebelum ini, telah ditukar kepada format spreadsheet menggunakan Biolog OmniLog Phenotype Microarray Software untuk analisis statistik selanjutnya. Kesan substrat terpilih pada keupayaan pembentukan biofilm kedua-dua strain dinilai berdasarkan protokol yang ditetapkan. Analisis data penggunaan karbon menunjukkan bahawa kedua-dua strain ACIBA-46 dan ACIBA-47 mempunyai keupayaan katabolik karbon rendah, dengan menggunakan hanya 48 daripada 190 (25%) substrat karbon yang diuji. Walau bagaimanapun, HCW strain A. baumannii ACIBA-47 kelihatan menggunakan jumlah substrat karbon yang lebih tinggi (6 karbon) berbanding dengan strain ventilator ACIBA-46. kedua-dua strain A. baumannii menggunakan dua kelas asid karboksilat dan asid amino sebagai sumber karbon, termasuk alanin, asparagin, serin, histidin, ornithin, arginin, asid piruvat dan asid succinic yang berkaitan dengan metabolisme tenaga. Menariknya, kajian ini mendapati beberapa substrat nitrogen menghambat pertumbuhan strain ACIBA-46 seperti asid urik dan alloxan. Kedua-dua strain ACIBA-46 dan ACIBA-47 dapat membentuk biofilm sederhana dalam medium kaya (LB broth), tetapi bukan pembentuk biofilm dalam medium berkhasiat nutren

(M9MM). Apabila M9MM ditambah dengan 22 substrat (diuji secara individu), terdapat perubahan keupayaan pembentukan biofilm bagi kedua-dua strain. Substrat seperti, L-arabinos, L-arginin dan urea sedikit meningkatkan keupayaan membentuk biofilm bagi kedua-dua strain. Di samping itu, substrat seperti L-histidin, L-leucin dan thimidin meningkat secara sederhana keupayaan untuk membentuk biofilm bagi kedua-dua strain. Walau bagaimanapun, substrat seperti glisin dan Tween 80 tidak memberi kesan ke atas keupayaan membentuk biofilm *A. baumannii* ACIBA-46 dan ACIBA-47 strain. Penjelasan aktiviti metabolik *A. baumannii* akan menawarkan para saintis wawasan cara untuk memanipulasi aktiviti tersebut sama ada untuk mengurangkan kejayaan patogen atau meningkatkan kerentanannya kepada agen antibiotik.

Kata kunci: *A. baumannii*, phenotype microarray, kapasiti metabolik, biofilm

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the Almighty God for giving me the opportunity, strength, and confidence to complete my research.

I pay my deep sense of gratitude to my supervisor, Professor Dr. Thong Kwai Lin for encouraging me, supervising my research and contributing in completing my thesis writing. Her valuable guidance and helpful suggestions shaped the present research as its shown.

My sincere appreciation to my co-supervisor, Dr. Chai Lay Ching for her helpful guidance throughout my research process. I would also like to extend my sincere thanks to Anis, Nowshin and Thenmoly, my lab-mates, who helped and supported me out.

Last but not least, I would like to pay a special appreciation to my parents for their blessings. Not forgetting my siblings for their endless support. Without their support none of this would have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
=	Equal to
≤	Less than or equal to
≥	More than or equal to
+ve	Positive
-ve	Negative
α	Alpha
β	Beta
γ	Gamma
$CaCl_2$	Calcium chloride
$MgSO_4$	Magnesium sulfate
A. baumannii	*Acinetobacter baumannii*
AUC	Area under the growth curves
CRAB	Carbapenem-resistant *A. baumannii*
g	Gram
h	Hour
ICU	Intensive care unit
L	Litter
LBA	Luria-Bertani agar

LBB	Luria-Bertani broth
M9MM	M9 minimal medium
MDR	Multidrug resistance / resistant
µl	Microliter
ml	Milliliter
mM	MilliMolar
OD	Optical density
PC	Phosphatidylcholine
PM	Phenotype Microarray
spp.	Species

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of study

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, coccobacillus, non-fermenting, catalase positive, non-motile, and multidrug resistant (MDR) bacterium (Howard et al., 2012; Roca et al., 2012). *A. baumannii* is one of the commonly reported nosocomial infection agents causing high morbidity and mortality worldwide (McConnell et al., 2013). *Acinetobacter* spp. is one of the most prevalent nosocomial pathogens in intensive care units (ICUs) from Malaysian hospitals. In addition, according to the International Surveillance Program reported between 2009-2011, *Acinetobacter* spp. was the eighth most common pathogen in the United State of America (USA) hospitals and the fifth in European and Mediterranean hospitals (Sader et al., 2014).

Infections caused by *A. baumannii*, particularly in patients in the ICUs, is a serious nosocomial problem. Sievert et al. (2013) highlighted that *A. baumannii* causes many diseases that include bacteremia, nosocomial pneumonia, respiratory tract, urinary tract bloodstream, surgical site, as well as wound contaminations. The bacterium is able to acquire antibiotic resistance genes and survive for a long time in the hospital environment and on the hands of healthcare workers (HCWs). These can lead to possible fomites patient transmission, which can also lead to the persistence of *A. baumannii* strains in hospitals (D'Agata et al., 2000; Webster et al., 2000).

There are limited studies focused on understanding the metabolic capability of *A. baumannii*. In one of such studies, Farrugia et al. (2013) demonstrated that *A. baumannii* isolated from community acquired has shown the ability to utilize 80 different types of carbon substrates out of 190 tested. In addition, *A. baumannii* showed the ability to utilize peptide nitrogen sources, but has reduced capability of utilizing most of the phosphorus and sulfur sources tested as reported in study by Peleg et al. (2012).

A. baumannii has the ability to adhere and form biofilm on both abiotic and biotic surfaces (Longo Vuotto & Donelli, 2014). This plays an important role in causing nosocomial infections, due to the surface colonization of hospital equipment and indwelling medical devices including central vein-related catheters (CVCs) and endotracheal tubes (Djeribi et al., 2012; Donlan, 2001; Trautner & Darouiche, 2004). The biofilm formation of *A. baumannii* is reported being controlled by various factors, which include presence of antibiotic resistance genes, availability of growth condition, cell density, temperature, pH and salinity of the medium (Gaddy & Actis, 2009; Pour et al., 2011).

Phenotype Microarray (PM) allows the concurrent tests of many bacterial phenotypes to be carried out in an automated method (Bochner, 2008). It involves the use of 96-well microtiter plates which are pre-coated with phosphorus, sulfur, nitrogen and carbon substrates, osmotic, ionic, pH, and sensitivity to chemical assays, which could be verified simultaneously (Bochner, 2008). Growing bacteria cells usually depend on the nutrient supplements that are given in every single well (Al Khaldi & Mossoba, 2004). Bochner et al. (2001) revealed that once respiration process was taken into account, redox reaction consequently changes the color of a tetrazolium dye, and this is recorded quickly using a CCD camera every 15 minutes. According to Bochner et al. (2001) changes in color, intensities can be quantified using kinetic charts with color codes, which allows for the assessment of biological variances between various organisms by making comparisons of the resultant profiles.

1.2 Purpose of study

There are limited studies on the metabolic requirement in the planktonic cells of *A. baumannii*. The types of substrates that favor growth of *A. baumannii* remain unknown. Hence, the study seek to determine the substrates that favor the selection and growth of

A. baumannii as well as to understand the metabolism of the organism based on these phenotypes.

1.3 Study objectives

The objectives of the study were:

i) To determine the metabolic capability of *Acinetobacter baumannii* by using Phenotype Microarray technology.

ii) To determine the effects of selected substrates on the biofilm forming ability of *A. baumannii*

CHAPTER 2: LITERATURE REVIEW

2.1 *Acinetobacter* Genus

In 1911, Martinus Willem Beigerinck, a microbiologist from the Netherland, discovered an aerobic, non-fermentative, and Gram-negative bacterium known as *Acinetobacter* (Nemec et al., 2009). *Acinetobacter* spp. is mainly found in soil, water and food in the humid and tropical environment (Anstey et al., 2002; Houang et al., 2001) and broadly present in hospitals and more specifically, in intensive care units (ICUs) (Joly-Guillou, 2005). *Acinetobacter* is considered a complex genus and historically, there has been a confusion as to whether there are multiple species (Wong et al., 2017). There are more than 50 species of *Acinetobacter,* most of them are nonpathogenic environmental organisms (Al Atrouni et al., 2016). However, the common pathogenic species are *A. baumannii*, followed by *A. calcoaceticus* and *A. lwoffii* (Dijkshoorn & van der Toorn, 1992). Nowadays, *Acinetobacter* infections have spread rapidly through hospitals IUCs worldwide. *Acinetobacter* spp. commonly cause nosocomial infections, predominantly aspiration pneumonia and catheter-associated bacteremia (Wong et al., 2017).

2.1.1 *Acinetobacter baumannii*

Acinetobacter baumannii (*A. baumannii*) is one of the most commonly nosocomial infection agents, causing a spectrum of diseases including wound infections, urinary tract infections, meningitis, septicemia and pneumonia (Antunes et al., 2014; Dijkshoorn et al., 2007). Recently, the US National Nosocomial Infection Surveillance System (NNIS) had highlighted a considerable increasing in *A. baumannii*- associated nosocomial pneumonia cases. Generally, these cases were attributed 5 - 10% of the ICU-acquired pneumonia in the US (Falagas et al., 2006; Kempf Rolain, 2012). In addtion, *A. baumannii*, owing to its infectious capability, ranks the tenth among the bacteria that caused bloodstream infections (Dijkshoorn et al., 2007; Peleg et al., 2008). Furthermore,

Gottig et al. (2014) had reported that *A. baumannii* is showing a steady increase in its resistance aginst a variety of antibiotics.

2.2 Epidemiology

A. baumannii is an emerging nosocomial pathogen in the community (Eveillard et al., 2013; Sengstock at al., 2010). Most of the nosocomial infections in the ICUs are caused by *A. baumannii* (Garnacho-Montero & Amaya-Villar, 2010). Contaminated surfaces and colonized or infected patients are the reservoirs of *A. baumannii* (El Shafie et al., 2004; Munoz-Price & Weinstein, 2008).

An international study on prevalence of infections in the ICUs from 75 countries showed that *A. baumannii* ranked the fifth as the most common nosocomial pathogen worldwide (Vincent et al., 2009). Besides, according to the global surveillance initiative conducted between 2009 and 2011 among hospitalized patients with pneumonia, *Acinetobacter* spp. ranked the eighth most prevalent in the hospitals of the United States of America and ranked the fifth in European and Mediterranean hospitals (Sader et al., 2014).

2.2.1 Clinical manifestations

Nosocomial pneumonia and bacteremia are the two common clinical manifestations of *A. baumannii.* In particular, endotracheal tubes create a perfect place for environmental transmission of *Acinetobacter*, as it binds to plastic surfaces, and consequently form biofilms on the tube surface (Gil-Perotin et al., 2012; Raad et al., 2011). Essentially, aspiration of *Acinetobacter* droplets directly into the patients' lungs where mechanical ventilation circumvents the ordinary host obstructions allows for tissue infection. However, there are other appearance of *A. baumannii* and these include urinary organ infections, which are associated with percutaneous tubes or urinary catheters, endocarditis, after-surgery and related to trauma wound infections or osteomyelitis, and

postsurgical or ventriculostomy-related meningitis (Carvalho et al., 2012; Joly-Guillou, 2005; Sievert et al., 2013).

Many community-acquired infections occur in humid, warm and tropical environments, particularly, in parts of Australia, Oceania, and Asia which include Taiwan, China, Malaysia and Thailand (Falagas et al., 2007; Peng et al., 2012). The community-onset infections are usually present in patients with severe pneumonia infection and, in rare cases, with cellulitis, meningitis, or principal bacteremia (Joly-Guillou, 2005). As Anstey et al. (2002) reported that regarding nosocomial cases, inappropriate initial antimicrobials were strongly correlated with increased mortality rate for community onset infections.

2.2.2 Transmission

In most instances, *Acinetobacter* spp. are transmitted to potential hosts via environmental surfaces, as well as the transient colonization or infection of the hands of HCWs (Spellberg & Bonomo, 2013). In addition, nosocomial bacteria can also be spread via aerosols, which are mainly from a colonized patient. For example, in one well-publicized case of bacterium spread, a healthcare worker was infected with pneumonia after inhaling *A. baumannii* aerosolized during the suctioning of the ventilated patient (Whitman et al., 2008). In another study, it was revealed that approximately 25% of air samples that were collected from the various patients' rooms had been contaminated with carbapenem-resistant *A. baumannii* (CRAB). All the hospitalized patients in these rooms were infected with CRAB (Munoz-Price et al., 2013; Spellberg & Bonomo, 2013). Since the ducts of air had not been colonized, it indicated that the patients were actually the sources of the airborne pathogens and bacteria (Munoz-Price et al., 2013; Spellberg & Bonomo, 2013).

2.3 Metabolism of *A. baumannii*

Brown et al. (2008) reported that human bodies are a rich source of nutrients such as amino acids, mucus sugars, lactic acid, and cholesterol for growth and metabolism as different pathogens utilize different carbon sources in the host colonization process. The epithelium of human intestines is covered with a layer of mucus, salts, water, as well as monomeric sugars (Hooper et al., 2002). Each of the bacterium is greatly served in cultivating a particular ecological niches, which is dependent on the availability of nutrients (Freter et al., 1983), such that the population sizes in the large intestine is dependent on nutrient availability and the concentration of the preferred nutrient in the host (Schinner, 2013).

Essentially, *Acinetobacter* species in most instances, are unique, particularly in their capabilities of using various carbon sources and metabolic pathways and thus can have survivability measures in diverse environments including biotic and abiotic surfaces (Howard et al., 2012). Buchan (1981) reported that *Acinetobacter* spp. can store phosphorus as a polyphosphate within the cellular granules, and these potentially occupy approximately 60% of the entire micro-organisms volume.

A. baumannii has the ability to utilize various carbon substrates (Howard et al., 2012). Farrugia et al. (2013) showed that *A. baumannii* was able to utilize 80 carbon sources, including carbohydrates, carboxylic acids, amino acids and miscellaneous compounds such as acetic acid, citric acid, lactic acid methyl ester, alanine, histidine and asparagine just to mention few. In addition, *A. baumannii* showed the ability to utilize peptide nitrogen sources, but reducing the capability of utilizing most of the phosphorus and sulfur sources tested as shown in study by Peleg et al. (2012).

Besides, *A. baumannii* can successfully colonize and invade the human endothelial and epithelial cells, and displays human serum resistance (Clemmer et al., 2011; Dorsey et al., 2003; Jawad et al., 1998). Notably, phospholipids are abundant in the human host as they are the building blocks of biological membranes which sereve as carbon and energy sources (Vance, 2008). In addition, *A. baumannii* shows the ability to obtain and utilize iron sources which is an important factor in its ability to survive in the environment and in the host (Zimbler et al., 2009).

2.3.1 Metabolism and virulence

There exists interdependency between virulence and metabolic activities of the pathogens. Essentially, this is attributed to the fact that the establishment of infections by these pathogens requires various metabolic functionalities, which consequently help the pathogens to adapt during infection process. Pathogens can sense nutrient, as well as other environmental changes once they enter the host, and they, in turn, control the virulence genes expression (Dorman et al., 2001; Dorman & Porter, 1998; Prosseda et al., 2002).

One of the fundamentals for the tenacity and perseverance of the bacteria in a human host is attributed to the utilization of available carbon sources. According to Brown et al. (2008), the human body rich with various nutrients. Pathogens consequently use various carbon sources such as lactic acid, cholesterol, and amino acids while colonizing the hosts. Phosphatidylcholine (PC) is abundant in the eukaryotic membranes and accounts for approximately 50 % of the entire range of phospholipids (Vance, 2008). The prevalence increases significantly to 80% in the lungs (Bernhard et al., 2001) and PC is a major phospholipid and impacts mucus wettability in the tracheobronchial excretions (Girod et al., 1992). There is an evidence that phosphatidylcholine often acts and serves as one of the nutrients source in instances of pathogen lung-infections by *Pseudomonas aeruginosa* (Sun et al., 2014). Since ventilation-associated pneumonia is one of the most prevalent of *A. baumannii* infections (McConnell et al., 2013), the high presence of PC

within the mucus raised the question whether it is a nutrient source for *A. baumannii* in human hosts. In addition, *A. baumannii* is known to target moist tissues including mucous membranes and exposed skin areas, which causing septicemia or death in the untreated cases (Sebeny et al., 2008).

2.4 Survival of *Acinetobacter baumannii*

A. baumannii undergoes morphological changes such as thickening of cell walls when it exposed to dry conditions (Fournier et al., 2006; Houang et al., 1998), which makes them more persistent once in contact with environmental surfaces. *A. baumannii* remains viable in hospital units even after years in solid surfaces, which underscores the challenge required in eliminating their environmental transmission once it has colonized nosocomial surfaces (Fournier et al., 2006; Houang et al., 1998). Also, Houang et al. (1998) reported that the survival ability of *A. baumannii* has a better rates in dry environment comparing to other strains that survive in wet environment. Moreover, Espinal et al. (2012) also reported the formation of biofilm increasing the survival rate of *A. baumannii* on the dry surfaces and could contribute to the persistence of the organism in the hospital environment, and increasing the probability of causing nosocomial infections. The survivability of *A. baumannii* is also associated with its antimicrobial drug and desiccation resistance (Jawad et al., 1998; Tomaras et al., 2003).

2.5 Biofilm forming ability

Biofilm is a structure when the cells of micro-organisms adhered to biotic and abiotic surfaces and enclosed in a self-produced polymeric matrix (Davey & O'toole, 2000; Gotz, 2002). Biofilm is the most important constraint to protect micro-organisms in dry environment as it enables them to survive and disperse. Biofilm is composed primarily of microbial cells and external polysaccharide (EPS) (Donlan, 2002). Synthesis of EPS in bacterial cell was determined by carbon availability, nutrient status of the growth medium and limitation of nitrogen, potassium, or phosphate (Sutherland, 2001).

Slow bacterial growth can enhance the production of EPS (Sutherland, 2001). There are five stages in biofilm formation process. The initial stage starts when the cells attach reversibly to surfaces with the support of extracellular organelles and proteins. Then, the EPS enables the irreversible attachment of the cells to the surfaces which subsequently replicates and grown into micro-colonies. After that, the community matures into a biofilm as the EPS accumulates and cells replicate. Finally, some cells detach from the biofilm and aggregates (Renner & Weibel, 2011).

Biofilm-forming microorganism are different from planktonic microorganisms based on their growth rates and antimicrobial resistance properties which restricts antibiotics transport through biofilm matrix. Therefore biofilm-forming microorganisms pose a major health problem in providing effective treatment for the disease (Donlan, 2002). Pathogenic microorganisms such as *A. baumannii* can form biofilms on equipment surfaces (Frank & Koff, 1990). The biofilm formation of *A. baumannii* is reported being controlled by various factors, which include presence of antibiotic resistance genes, availability of growth condition, and cell density (Gaddy & Actis, 2009). *A. baumannii* showed the ability to increase the biofilm forming under iron limitation condition compared to iron-rich conditions as reported in Tomaras et al. study (2003). In addition, factors such as different temperatures, pH and salinity of the medium affect the biofilm forming ability of *A. baumannii* (Pour et al., 2011). Longo et al. (2014) were reported that the ability of *A. baumannii* to adhere and form biofilm in both abiotic and biotic surfaces due to the surface colonization of hospital equipment and indwelling in the medical devices such as central vein-related catheters (CVCs), urinary catheters, as well as endotracheal tubes (Djeribi et al., 2012; Donlan, 2002; Trautner & Darouiche, 2004).

2.6 Phenotype Microarray

The phenotype microarray (PM) system is a highly quantified methodology from Biolog (Hayward, California), which enables screening of the variety of phenotypic characteristics of microorganism. The system enables in-vivo assessment functionality of the diverse pathways, including the cell nutritive paths for carbon, nitrogen, phosphorus, sulfur metabolic activities, regulating and controlling pH, biosynthetic pathways, osmotic and ionic evaluation of the sensitivities, as well as the sensitivity to the various chemical agents responsible for the disruption of a variety biological paths (Bochner, 2008).

The PM system contains twenty 96-well microtitre plates that are preloaded with sources of phosphorus, sulfur, nitrogen and carbon substrates, osmotic, ionic, and pH. The sensitivity or reaction to each of these chemical assays could be verified simultaneously (Bochner, 2008). The system capitalizes on the bacterial cell respiration and redox chemistry is used as a reporting mechanism in amplifying and quantifying the growth in the respective test substrates. According to Bochner (2008), the inoculants are consequently pipetted into the microtitre plate wells which contain various chemicals and nutrients required in creating a variety of culture conditions for the PM assay set. Growing cells reduce the chemical tetrazolium dye and consequently the intensity needed for the reaction process is subsequently transformed to redox signaling and recording in the microplated reader within omnilog unit (Biolog Hayward, CA). By comparing the growth observed in the plates helps to determine the changes in phenotypes in the various strains and environmental conditions.

Commercial test kits and biochemical test are commonly used for microbial identification. But these tests were both labor intensive and time consuming (Gilardi, 1978; Harding et al., 1990). Dooren de Long was the first person to attempt metabolic profiling in identifying a microbe based on the utilization of carbon sources (Greetham, 2014). In 2001, Bochner et al., described phenomics or the study of living cells by using

PM system in an equivalent 2-dimensional array. The technology permits researchers to perform numerous parallel assays simultaneously as this method only acquire a small volume of culturing.

PM technology is used to address specific issues that are related to organisms' metabolic functionality. Phenotype Microarray has been applied in assessing phenotypes of knockout mutants (Johnson et al., 2008; Perkins & Nicholson, 2008). PM technology has been used to study environmental changes such as antibiotic resistance, temperature or other stressors as bacterial species. (Bochner et al., 2010)

Figure 2.1: Biolog Phenotype Microarray analysis (Roberts, 2012). If the substrate is utilized, the tetrazolium dye in the well will be reduced and a violet color is founded (Figure 2.1, left). If the substrate is weakly catabolized or not catabolized, a less color or no color change is observed (Biolog Hayward, CA).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Bacterial strains

The bacterial strains used in the study were ACIBA46 and ACIBA47. ACIBA46 was previously isolated from a ventilator from ICU of a local teaching hospital in Kuala Lumpur in 2006 and it is a multidrug resistant (MDR) strain with resistance to 15 antibiotics (Kong et al., 2011b). ACIBA47 was previously isolated from the hands of HCWs in 2006 and is also a MDR strain with a similar antibiogram as the ACIBA-46 strain (Kong et al., 2011b). The bacterial strains were previously identified and described by Kong et al. (2011a, 2011b). The strains were retrieved from -20^{0}C glycerol stock and sub-cultured onto Luria-Bertani Agar (LBA) overnight at 37 ^{0}C for subsequent experimental work.

3.1.2 Chemicals, reagents and media

Appendix A shows the list of chemicals and reagents used in this study, while Appendix B lists all the media used for culturing of *A. baumannii*.

3.2 Methodology

3.2.1 Phenotype Microarray (PM) analysis

3.2.1.1 Phenotype Microarray (PM) Plates

Phenotype Microarray (PM) experiment was conducted in 2014 by a previous student. The data was exported using Biolog OmniLog® PM data collection Software and OmniLog DC Software. The PM analysis were involved the use of eight plates, each coated with different phosphorus, sulfur, nitrogen and carbon substrates. The plates that were used in this study were PM1-PM8 (Table 3.1). The inoculation and preparation of the solutions of PM plates was previously performed according to a standard Biolog PM procedure for *E. coli* and other Gram-negative bacteria (Biolog, Inc., US). *A. baumannii* strains were streaked from frozen glycerol stock and grown overnight on LB agar at 37^{0}C

for 18 h, sub-cultured by streaking again on LB agar and grown overnight. Two to three fresh colonies were suspended in 12.5 ml of Inoculation Fluid-0 (IF-0) GN Base, 2.35 ml sterile distilled water and 150 µl Dye Mix A. Suspension of 85% (transmittance) for usable concentration of inoculums, was achieved by using the Biolog Turbidimeter wells of PM1, PM2, PM3, PM4, PM5, PM6, PM7 and PM8, which were inoculated with 100 µl of the inoculum suspensions and incubated in the OmniLog reader at 37^0C for 48 h. All assays were performed in duplicates.

Table 3.1: List of Phenotype Microarray plates used for metabolic tests of *A. baumannii.*

Phenotype Microarray (PM) Plates	Sources
PM1	Carbon sources
PM2	Carbon sources
PM3	Nitrogen sources
PM4	Phosphorous and sulfur sources
PM5	Nutrient supplements
PM6	Peptide nitrogen sources
PM7	Peptide nitrogen sources
PM8	Peptide nitrogen sources

3.2.2 Statistical analysis of phenotype microarray data

The data was exported from the Biolog OmniLog PM Software. The area under the growth curves (AUC) based on these data was calculated by using the Microsoft Excel Software. For each well, the mean of AUC along with the mean slope of the time (0 h, 6 h, 12 h, 24 h and 48 h) was used to determine the threshold value scored for the growth of various Phenotype Microarray conditions. Mean values calculated by subtracting the 0 h value in negative control well for each data set. By using differences in the average

of AUC, it was possible to determine whether a particular substrate was utilized or not and check the rate of utilization. Once the reading in each well exceeded the standard average area's threshold value, it was scored as positive reaction. The data were checked for normalization and the result were grouped into three categories that are actively utilized, weakly utilized and not utilized, based on the average slope and average area. Statistical evaluations (mean, minimum, maximum and standard deviations) were calculated using the Microsoft Excel Software.

3.2.3 Validation of phenotype microarray data

Confirmation of the certain phenotype was performed in two types of nutrient depleted media which were M9 Minimal Medium (M9MM) and rich medium such as LB agar. From glycerol stocks and stab cultures *A. baumannii* strains were streaked on LB agar and was incubated at 37^0C for 24 h. Two to three fresh colonies of each strain of *A. baumannii* were suspended in 4 ml of LB broth and grown overnight. Then the culture were diluted to achieve a final concentration of 200 µl with 1:100 ratio prior to inoculation into M9MM. Substrates were selected for further confirmation based on the phenotype microarray result consistency between replicates as well as the availability of the substrates in laboratory. All the 22 substrates solutions used in this experiment were listed in Appendix C.

In 96-well micro-titer flat-bottom plates, wells were filled with 200 µl M9MM suspension, then supplemented with 20 mM of 22 substrates solutions (tested individually), except for two wells were filled with 200 µl LB broth culture and two wells were filled with 200 µl sterile M9MM alone served as controls. The 96-well micro-titer flat-bottom plates were incubated at 37 ^{0}C for 18 h. Finally, the optical density (OD) at 590 nm measured using micro-plate reader (Epoch, Biotek). The OD_{590} reading for each

substrate were acquired after deducting the control. For each tested substrate, two technical triplicates and two biological triplicates were performed.

3.2.4 Biofilm formation

To determine the bacterial ability to form biofilm, protocol described by Chelvam et al. (2015) was followed with slight modification. Briefly, the *A. baumannii* cells were grown on LB broth overnight. Then the overnight broth cultures were diluted to achieve a final concentration of 200 µl with 1:100 ratio prior inoculation into M9MM. In 96-well flat-bottom micro-titer plates, wells were filled with 200 µl M9MM suspension then supplemented with 20 mM of 22 substrates solutions (tested individually), that were selected based on the availability of the substrates in laboratory (Appendix C), except two wells were filled with 200 µl M9MM suspension, two wells were filled with 200 µl LB broth culture and two wells were filled with 200 µl sterile M9MM alone served as controls. The 96-well flat-bottom micro-titer plates were incubated at 37 ^{0}C for 24 h and 48 h. After incubation, the cultures were removed from the well by tapping then washed twice with 200 µl of 1X premixed phosphate-buffered saline (PBS) to remove non-adherent cells. The adherent cells were heat fixed for 30 min at 80^{o}C and subsequently stained with 200 µl crystal violet (0.5%) for 5 min. Following this, the wells were washed three times with 300 µl of sterile distilled water to remove the unbound cells. The micro-titer plate was air dried before adding 200 µl 80:20 ethanol-acetone. Finally, the OD_{590} had measured using micro-plate reader (Epoch, Biotek). The OD reading for each substrates were acquired after deducting the control.

The level of biofilm formation of the studied strains was scored as described in Chelvam et al. (2015) and Stepanovic et al. (2000). Briefly, the cut-off OD (OD_C) was defined as three standard deviations above of the mean OD of the negative control. The biofilm forming ability was scored as follows: $OD \leq OD_C$ = non-biofilm producer, OD_C

$< OD \leq (2x\ OD_C)$ = weak-biofilm producer, $(2x\ OD_C) < OD \leq (4x\ OD_C)$ = moderate-biofilm producer, $OD > (4x\ OD_C)$ = strong-biofilm producer. The experiment was performed in two biological replicates and repeated two times.

CHAPTER 4: RESULT

4.1 Phenotypic profile of *A. baumannii* strains

4.1.1 Carbon profile

The carbon profiling was based on the results of PM plates 1 and 2. These plates were pre-coated with eight types of carbon substrates. Based on the PM data analysis, the two strains ACIBA-46 and ACIBA-47 had shown 88.8% similarity in the carbon substrates utilization. Among the 190 carbon substrates tested, 48 substrates were utilized by both strains, while additional 6 carbon substrates were utilized by ACIBA-47 strain. This showed the ability of ACIBA-47 strain utilized a higher number of carbon sources compared to ACIBA-46 strain, specifically in carboxylic acids such as D-saccharic acid, citraconic acid, D,L-citramalic acid, and succinamic acid. The two strains utilized 25% (N= 48) to 28% (N= 54) of 190 carbon sources. Figure 4.1 shows the growth kinetics of ACIBA-46 and ACIBA-47 strains in carbon PM plate, while Figure 4.2 shows the list of carbon sources utilized.

The 54 carbon sources were grouped into carbohydrates (4), carboxylic acid (31), fatty acid (3), amino acids (13), amine (1) and ester (2) (Table 4.1). The two major classes of compounds utilized by *A. baumannii* strains as carbon sources were carboxylic acids and amino acids. The common carbon sources utilized by both strains including L-arabinose, D-ribose, D-xylose, D-ribono-1,4-lactone, succinic acid, L-lactic acid, D,L-malic acid, acetic acid, α-ketoglutaric acid, α-ketobutyric acid, α-hydroxybutyric acid, citric acid, fumaric acid, bromosuccinic acid, propionic acid, tricarballylic acid, D-malic acid, L-malic acid, pyruvic acid, γ-amino-N-butyric acid, butyric acid, caproic acid, 4-hydroxybenzoic acid, β-hydroxybutyric acid, α-keto-valeric acid, malonic acid, quinic acid, D-ribono-1,4-lactone, sebacic acid, sorbic acid, D,L-carnitine, Tween 20, Tween 40, Tween 80, L-aspartic acid, L-proline, D-alanine, L-glutamic acid, L-asparagine, L-

glutamine, L-alanine, L-arginine, L-histidine, hydroxy-L-proline, L-phenylalanine, L-pyroglutamic acid, D-lactic acid, methyl ester and methylpyruvate.

Based on PM data analysis, the two strains ACIBA-46 and ACIBA-47 had shown similar utilization rate in the selected carbon substrates (L- arabinose, L aspartic acid, D-xylose, α – ketoglutaric acid, α - hydroxyl butyric acid, D- malic acid, Caproic acid and malonic acid). *A. baumannii* ACIBA-46 and ACIBA-47 strains showed the ability to rapidly utilize L- arabinose, L aspartic acid and D- xylose, however, slowly utilized α – ketoglutaric acid, α - hydroxyl butyric acid, D- malic acid, Caproic acid and malonic acid substrates (Table 4.2).

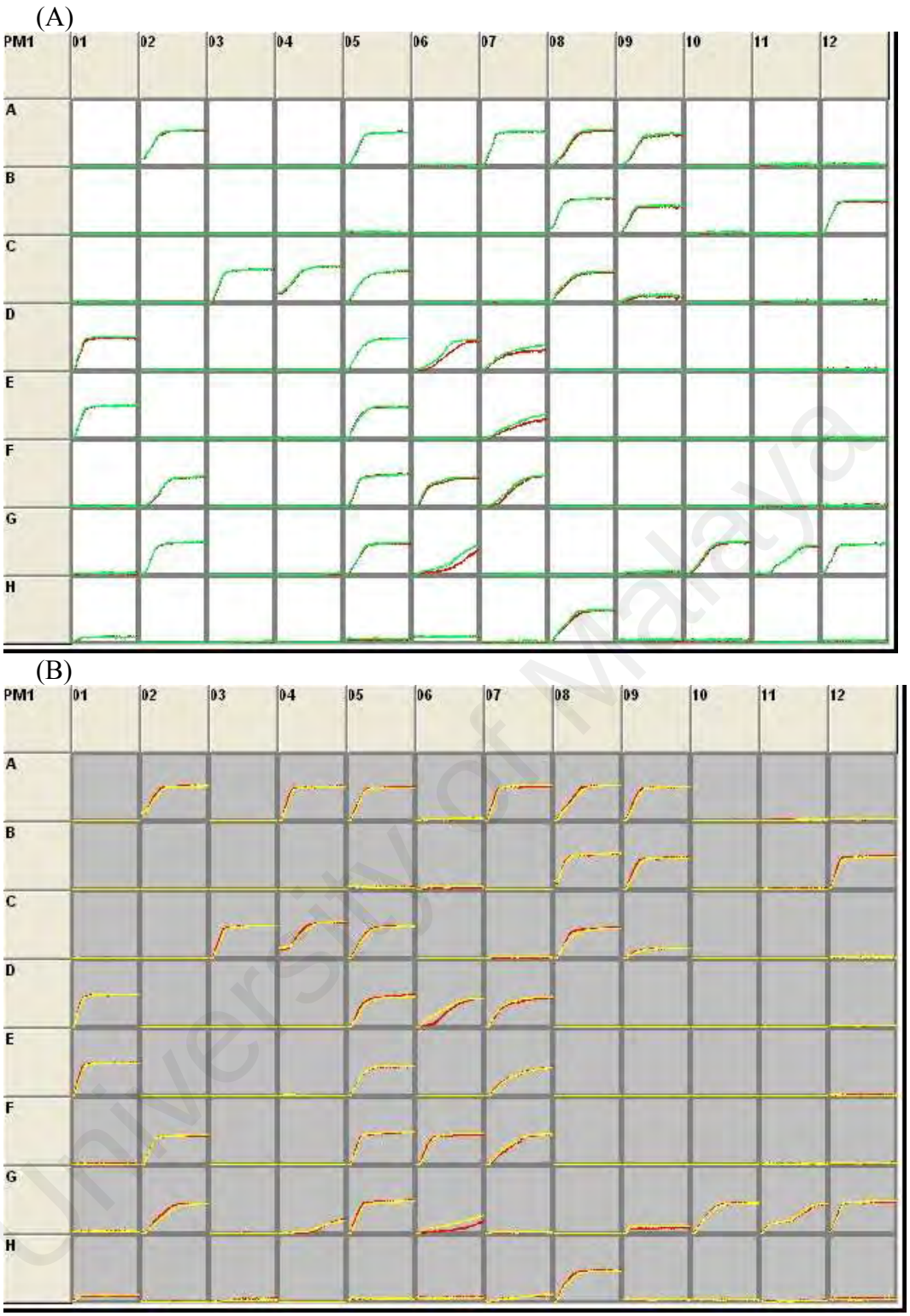

Figure 4.1: Representative growth kinetics of ACIBA-46 (A) and ACIBA-47 (B) strains in PM1 plate (carbon). The negative control well (A1) (absence of substrate) shows no growth in the replicates, while other wells (presence of substrates) such as A2, A5, F2, G12 and H10 well show positive growth in the replicates for each well in both strains. The two different colours green and red in (A), yellow and red in (B) represent the replicates for each well.

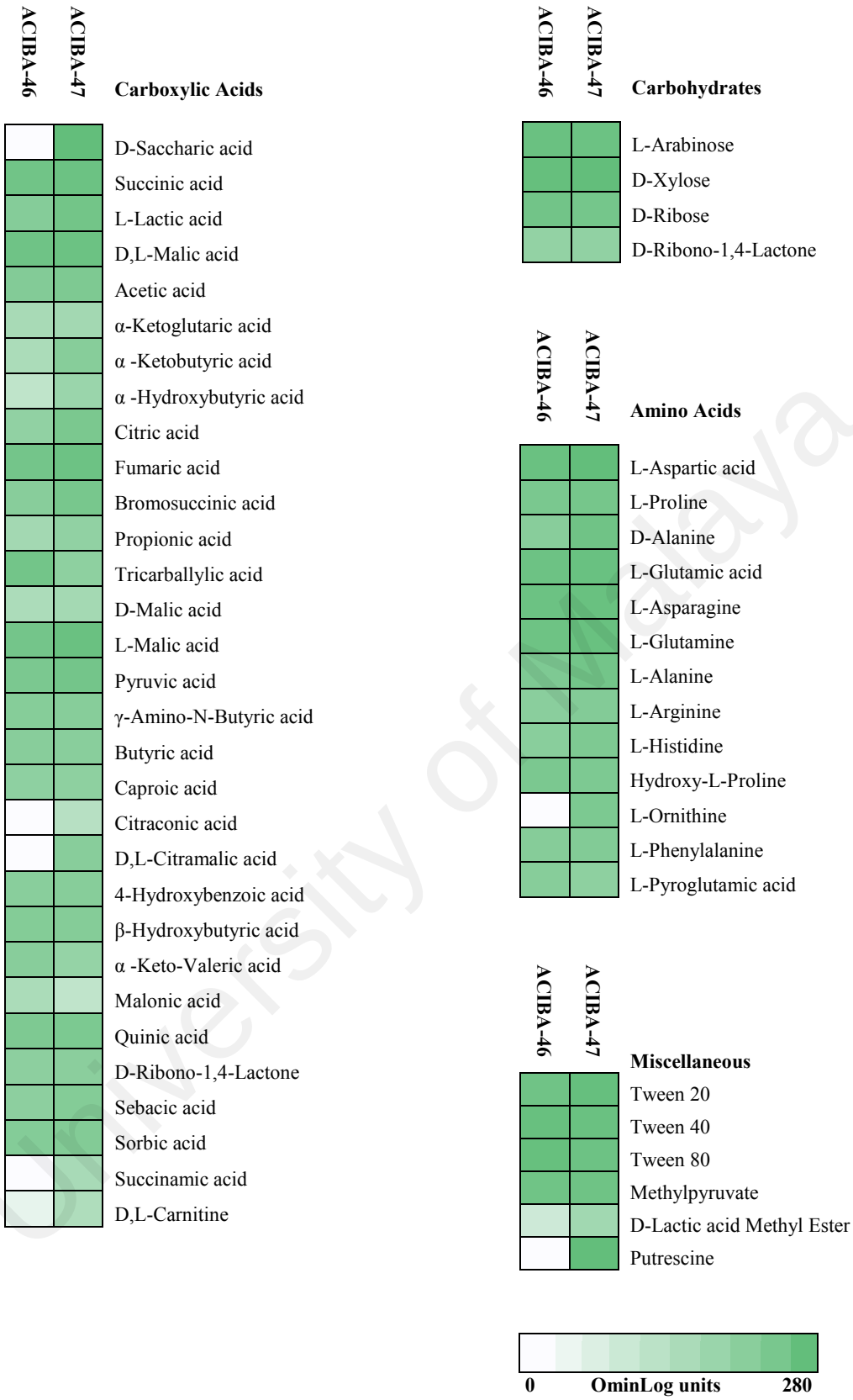

Figure 4.2: Carbon sources utilized by *A. baumannii* ACIBA-46 and ACIBA-47 strains. The data are determined using Biolog Phenotype Microarray plates PM1 and PM2. Phenotypes are arranged from strongest (dark green) to weakest (white) were considered negative.

Table 4.1: Catabolism of carbon substrates by ACIBA-46 and ACIBA-47 strains.

Carbon Sources	Carbon substrate catabolized by both strains	Carbon substrate catabolized by only ACIBA-46	Carbon substrate catabolized by only ACIBA-47
Carbohydrates (4 out of 68)	L-Arabinose D-Ribose D-Xylose D-Ribono-1,4-Lactone	—	—
Carboxylic acids (31 out of 59)	Succinic acid L-Lactic acid D,L-Malic acid Acetic acid α-Ketoglutaric acid α-Ketobutyric acid α-Hydroxybutyric acid Citric acid Fumaric acid Bromosuccinic acid Propionic acid Tricarballylic acid D-Malic acid L-Malic acid Pyruvic acid γ-Amino-N-Butyric acid Butyric acid Caproic acid 4-Hydroxybenzoic acid β-Hydroxybutyric acid α-Keto-Valeric acid Malonic acid Quinic acid D-Ribono-1,4-Lactone Sebacic acid Sorbic acid D,L-Carnitine	—	D-Saccharic acid Citraconic acid D,L-Citramalic acid Succinamic acid
Fatty acids (3 out of 3)	Tween 20 Tween 40 Tween 80	—	—
Amino acids (13 out of 30)	L-Aspartic acid L-Proline D-Alanine L-Glutamic acid L-Asparagine L-Glutamine L-Alanine L-Arginine L-Histidine Hydroxy-L-Proline L-Phenylalanine L-Pyroglutamic acid	—	L-Ornithine

Table 4.1, continued.

Carbon Sources	Carbon substrate catabolized by both strains	Carbon substrate catabolized by only ACIBA-46	Carbon substrate catabolized by only ACIBA-47
Amine (1 out of 5)	—	—	Putrescine
Esters (2 out of 2)	Methylpyruvate D-Lactic acid Methyl Ester	—	—
Total carbon	**48**	**0**	**6**

Table 4.2: The rate of utilization of selected carbon substrates for *A. baumannii* ACIBA-46 (A) and ACIBA-47 (B) strains.

(A)

Time	L-arabinose	L aspartic acid	D- xylose	α – ketoglutaric acid	α - hydroxyl butyric acid	D- malic acid	Caproic acid	Malonic acid
0 h	57	0	54	0	0	4	0	0
6 h	114	107	147	9	10	9	34	0
12 h	221	246	245	36	42	15	116	63
24 h	268	260	264	143	96	118	210	162
48 h	276	263	278	232	176	224	218	169
Interpretation	Rapidly utilized	Rapidly utilized	Rapidly utilized	Slowly utilized	Slowly utilized	Slowly utilized	Slowly utilized	Slowly utilized

(B)

Time	L-arabinose	L aspartic acid	D- xylose	α – ketoglutaric acid	α - hydroxyl butyric acid	D- malic acid	Caproic acid	Malonic acid
0 h	59	0	58	0	0	5	0	0
6 h	124	154	170	35	42	68	54	4
12 h	215	251	252	57	107	81	145	32
24 h	255	256	259	148	161	115	199	109
48 h	264	263	271	216	261	221	209	155
Interpretation	Rapidly utilized	Rapidly utilized	Rapidly utilized	Slowly utilized	Slowly utilized	Slowly utilized	Slowly utilized	Slowly utilized

4.1.2 Nitrogen profile

The nitrogen, phosphorus, sulfur and nutrient supplements utilization data (PM plates 3 - 8) for ACIBA-47strain was not available and therefore only; the data for nitrogen, phosphorus, sulfur and nutrient supplements for ACIBA-46 strain was analyzed in this study.

Three hundred and eighty nitrogen substrates were tested in this study. However, there were growth on the (PM plates 3, 6, 7 and 8) negative control wells (absence of nitrogen substrates). This could be a false positive or it could be due to carry over of nutrients from the enrichment media in the capsule or biofilm or slime layer on the surface of the bacterial cells. Thus, the nitrogen data in this study could not be interpreted. However, surprisingly, 7 wells, namely D10, E12, F6, G3, G4, G6 and G10 (ethylenediamine, N-acetyl-D-galactosamine, guanine, uric acid, alloxan, parabanic acid and D,L-α-amino-caprylic acid) showed no growth (Figure 4.3). If there was cross-contamination, these wells would have been positive as well like the negative control well. However, since these wells showed no growth, this suggest that these tested nitrogen could be inhibiting the growth of *A. baumannii* ACIBA-46 strain.

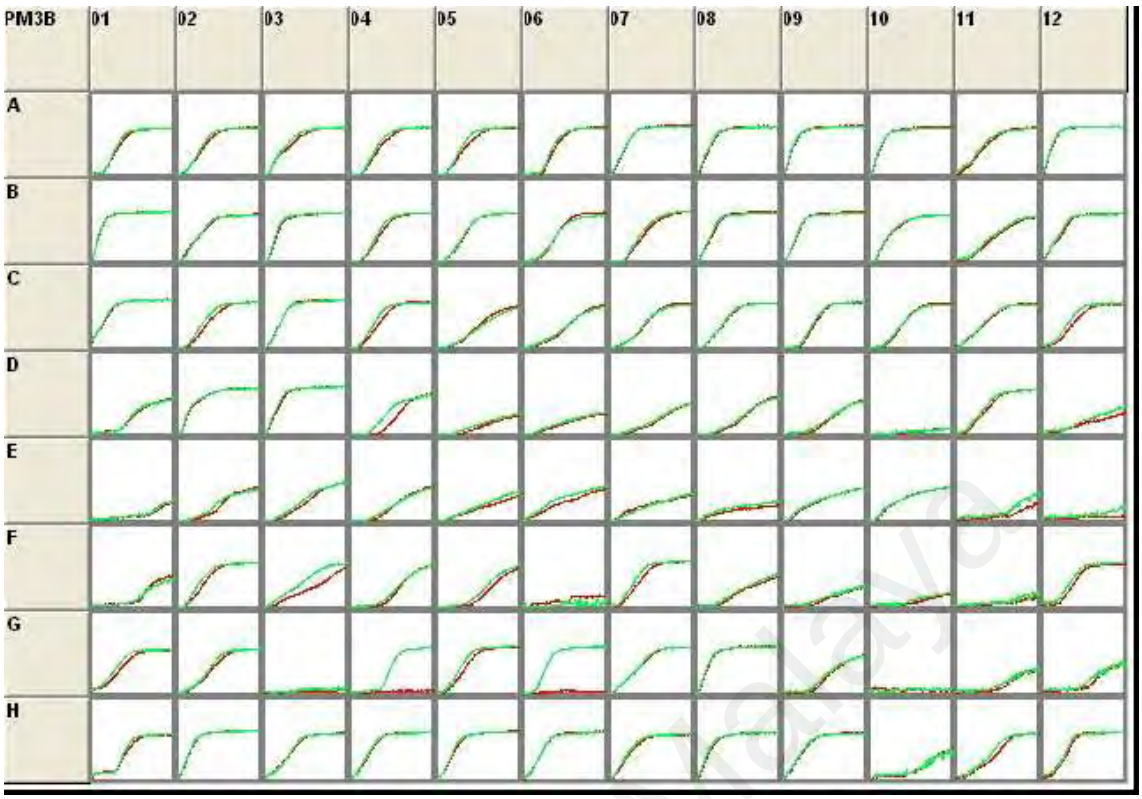

Figure 4.3: Representative growth kinetics of ACIBA-46 in PM3-B plate (Nitrogen). The negative control well (A1) (absence of substrate) shows positive growth in the replicates, while other wells (presence of substrates) such as D10, E12, F6, G3 and G10 well show no growth in the replicates for each well. The two different colours (green and red) represent the replicates for each well.

4.1.3 Phosphorus, sulfur and nutrient supplements profile

Ninety-five phosphorus and sulfur substrates as well as 95 nutrient supplement substrates were tested in this study. However, there were a positive reaction (PM plates 4 and 5) in all negative control wells (absence of substrates). This could be a false positive or it could be due to contamination or that *A. baumannii* probably carry a capsule that contain nutrient sources. Thus, the phosphorus, sulfur and nutrient supplement data in this study could not be interpreted.

4.2 Validation of Phenotype Microarray data

In order to independently confirm the PM data, the ability of *A. baumannii* ACIBA-46 and ACIBA-47 to grow on minimal medium with presence of 22 carbon, nitrogen, phosphorus and sulfur substrates were tested. The list of 22 substrates is shown in Figure 4.4. The OD_{590} was measured using micro-plate reader (Epoch, Biotek).

Turbidity with cellular replication was observed in all the substrates tested. The results for carbon substrates citric acid, L- arabinose, L- arginine, Tween 80, fumaric acid, D,L-malic acid, pyruvic acid, L- histidine and succinic acid were shown to be catabolized (Figure 4.4) and this was concordant with the phenotype microarray data for both strains ACIBA-46 and ACIBA-47, as all these carbon substrates were shown to be actively utilized in the PM plates.

However, L- ornithine carbon substrate which was catabolized by both strains was contradictory with the PM data for ACIBA-46 strain (was not able to utilize it). Farrugia et al. (2013) reported that *A. baumannii* was able to actively utilize L- ornithine in the PM plate, and based on the validation test, this substrate was catabolized.

The nitrogen, phosphorous and sulfur substrates tested for ACIBA-46 strain showed different abilities of utilization. However, these substrates could not be validated and interpreted correctly since there was growth on the negative control. Nevertheless, based on the bench work to retest 12 nitrogen, phosphorous and sulfur substrates (Figure 4.4), L- leucine and glutathione were strongly catabolized while other substrates such L-threonine, L- lysine and L- cysteine were weakly or not utilized.

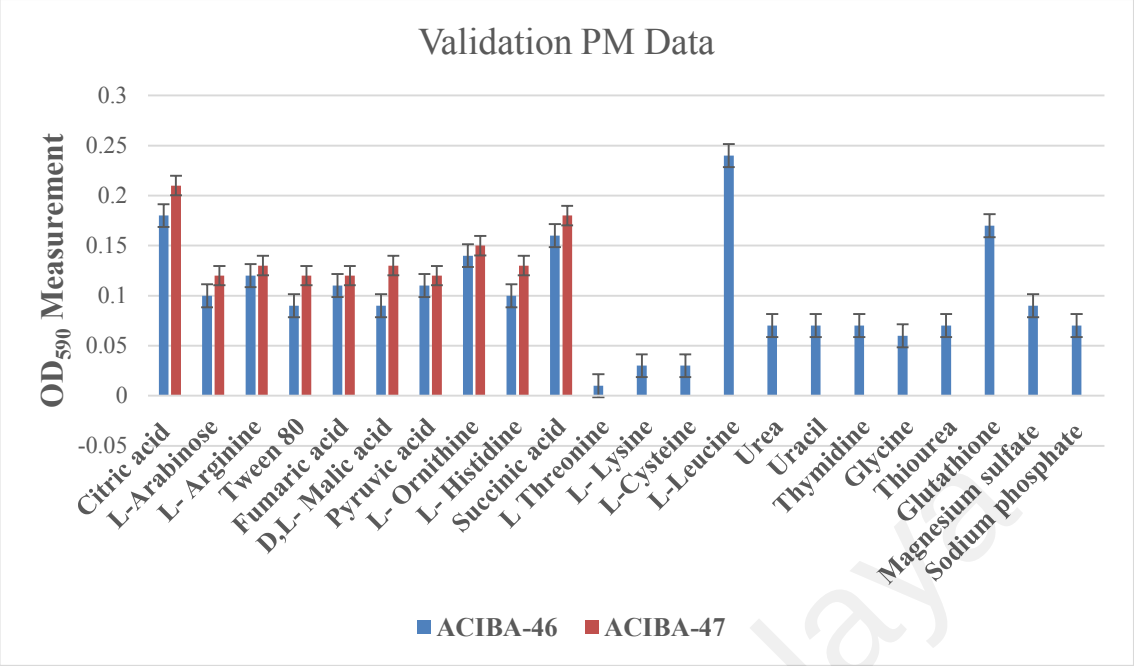

Figure 4.4: Validation of PM data using selected carbon, nitrogen, phosphorous and sulfur substrates. The figure summarizes the result of the validation experiment of PM data after deducting the negative control value.

4.3 Biofilm formation

The biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains in LB broth, M9MM and M9MM supplemented with 22 selected substrates based on the availability in laboratory (Appendix C) was determined using Crystal Violet assay. Overall, there were variations in the effects of the substrates on the biofilm forming capability for both strains. Based on the interpretation scheme adopted from Stepanovic et al. (2000), both ACIBA-46 and ACIBA-47 strains were able to form a moderate biofilm in rich medium (LB broth) ($OD_{590} > 0.82$), but were not able to form biofilm in medium depleted nutrient (M9MM) ($OD_{590} < 0.41$). When M9MM was supplemented with 22 substrates (tested individually), there was a difference in the biofilm forming ability for ACIBA-46 (ventilator) and ACIBA-47 (hands of HCWs) strains. Substrates such as L-arabinose, L- arginine, fumaric acid, D, L- malic acid, pyruvic acid, L- threonine, L-cysteine, urea, uracil and magnesium sulfate slightly increased the ability to form biofilm for both strains, meanwhile L- ornithine, L- histidine, L- leucine and thymidine moderately increased the ability of forming biofilm for both strains (Table 4.3 & 4.4).

Some substrates showed different effect on the biofilm forming capability by ACIBA-46 and ACIBA-47 strains. For example, citric acid showed the ability to effect moderately on the biofilm forming ability of ACIBA-46 strain, while showed weak effects on ACIBA-47 strain. Moreover, substrates such as sodium phosphate showed ability to effect on ACIBA-47 strain weakly, but showed no effect on ACIBA-46 strain. In contrast, substrate such as Tween 80, succinic acid, glycine, Thiourea, glutathione and L- lysine showed no effect on both strains (Tables 4.3, 4.4).

Table 4.3: The effects of substrates on biofilm formation ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains.

Strains	
ACIBA-46	**ACIBA-47**
1. Effects of supplemented substrates on the biofilm formation ability	
(a). Substrates slightly increased the ability to form biofilm (weak biofilm producers)	
L- arabinose L- arginine Fumaric acid D, L- malic acid Pyruvic acid L- threonine L- cysteine Urea Uracil Magnesium sulfate	L- arabinose L- arginine Fumaric acid D, L- malic acid Pyruvic acid L- threonine L- cysteine Urea Uracil Magnesium sulfate Sodium phosphate Citric acid
(b). Substrates moderately increased the ability to form biofilm (moderate biofilm producers)	
L- ornithine L- histidine L- leucine Thymidine Citric acid	L- ornithine L- histidine L- leucine Thymidine
2. Substrates do not effect on biofilm formation ability	
Tween 80 Succinic acid Glycine Thiourea Glutathione L-lysine Sodium phosphate	Tween 80 Succinic acid Glycine Thiourea Glutathione L-lysine

Table 4.4: Summary of effects of substrates on biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains.

Strains	**ACIBA-46**		**ACIBA-47**	
Media/ substrates	**Mean OD_{590}**	**Biofilm production**	**Mean OD_{590}**	**Biofilm production**
LB broth	0.83	Moderate	1	Moderate
M9MM	0.32	Non-biofilm producer	0.32	Non-biofilm producer
M9MM + Citric acid	0.9	Moderate	0.72	Weak
M9MM + L- Arabinose	0.46	Weak	0.42	Weak
M9MM + L- Arginine	0.43	Weak	0.5	Weak
M9MM + Tween 80	0.16	Non-biofilm producer	0.13	Non-biofilm producer
M9MM + Fumaric acid	0.59	Weak	0.44	Weak
M9MM + D,L- Malic acid	0.55	Weak	0.59	Weak
M9MM + Pyruvic acid	0.68	Weak	0.67	Weak
M9MM + L- Ornithine	1.44	Moderate	1.37	Moderate
M9MM + L- Histidine	1	Moderate	1	Moderate
M9MM + Succinic acid	0.25	Non-biofilm producer	0.21	Non-biofilm producer
M9MM + L- Threonine	0.55	Weak	0.43	Weak
M9MM + L- cysteine	0.51	Weak	0.6	Weak
M9MM + L-Leucine	1.28	Moderate	1.33	Moderate
M9MM + Urea	0.63	Weak	0.6	Weak
M9MM + Uracil	0.48	Weak	0.55	Weak
M9MM + Thymidine	1.32	Moderate	1.19	Moderate

Table 4.4, continued.

Strains	**ACIBA-46**		**ACIBA-47**	
Media/ Substrates	**Mean OD_{590}**	**Biofilm production**	**Mean OD_{590}**	**Biofilm production**
M9MM + Glycine	0.39	Non-biofilm producer	0.3	Non-biofilm producer
M9MM + Thiourea	0.35	Non-biofilm producer	0.32	Non-biofilm producer
M9MM + Glutathione	0.18	Non-biofilm producer	0.15	Non-biofilm producer
M9MM + L- Lysine	0.38	Non-biofilm producer	0.33	Non-biofilm producer
M9MM + Magnesium sulfate	0.44	Weak	0.51	Weak
M9MM + Sodium phosphate	0.33	Non-biofilm producer	0.44	Weak

The level of biofilm formation of *A. baumannii* strains was scored as described in Chelvam et al. (2015) and Stepanovic et al. (2000). Briefly, the cut-off OD (OD_C) was defined as three standard deviations above of the mean OD of the negative control. The ODc=0.41 in this study (Table 4.5), and the biofilm forming ability was scored as follow: $OD \leq 0.41$ = non-biofilm producer, $0.41 < OD \leq 0.82$ = weak-biofilm producer, $0.82 < OD \leq 1.64$ = moderate-biofilm producer, $OD > 1.64$ = strong-biofilm producer. The experiment was performed in two biological replicates and repeated two times. Appendix D shows the result of biofilm forming experiment.

Table.4.5: Calculation of the cut-off OD (ODc) of *A. baumannii* ACIBA-46 and ACIBA-47 strains based on the negative control reads.

Strains	OD_1	OD_2	OD_3	OD_4	Mean OD	Standard Deviation (SD)	3SD	ODc	2ODc	4ODc
ACIBA-46	0.36	0.33	0.28	0.32	0.32	0.03	0.09	0.41	0.82	1.64
ACIBA-47	0.36	0.32	0.28	0.32	0.32	0.03	0.09	0.41	0.82	1.64

CHAPTER 5: DISCUSSION

In this study, it was surprising that *A. baumannii* ACIBA-46 and ACIBA-47 strains showed 88.8% similarity in carbon metabolic profiles (Table 4.1), as they were isolated from different sources, ventilator and hands of HCWs, respectively. Out of 190 substrates tested, 48 (25%) were catabolized by both strains tested. Amino acids and carboxylic acids were the two major classes of compounds utilized by *A. baumannii* strains as carbon sources, However, only 4 (out of 68) carbohydrates tested which were L-arabinose, D-ribose, D-xylose and D-ribono-1,4-lactone that serve as energy sources were catabolized. The inability to use certain carbohydrates could be due to the inability to transport these molecules into the cytoplasm and/or different metabolic routes to synthesize sugars (Cook & Fewson, 1973; Knight et al., 1995).

The types of carbon substrates utilized by *A. baumanii* ACIBA-46 and ACIBA-47 strains include ribose and arabinose, which serve as energy sources for growth and are found in the mucus layer in humans and provide the nutrients to this pathogen to invade the host cell (Fabich et al., 2008). In addition, both strains were able to utilize Tween 20, Tween 40 molecules which contain fatty acid and long aliphatic chains (palmitate, oleate, laurate) (Mara et al., 2012). Also, both strains were able to utilize Tween 80, which can provide oleic acid for cell growth and lipase production (Li et al., 2005). Amino acids including alanine, asparagine, histidine, ornithine, serine, and arginine were utilized by both strains. Succinic acid and pyruvic acid which are associated with energy metabolism were also catabolized actively (Lin, 1977). In a separate independent assay, tween 80, arabinose, ornithine, histidine, succinic acid and pyruvic acid were shown to be utilized by both strains. However, lactic acid and L-serine were not further tested in this study due to the unavailability of these substrates in the laboratory.

In a study by Farrugia et al. (2013), the carbon catabolism capability of the Australian clinical (blood) *A. baumannii* strains tested were higher compared to local environmental strains tested in this study (Table 5.1). *A. baumannii* ACIBA-46 and ACIBA-47 strains have lower nutrient requirement for growth compared to *A. baumannii* strain in Farrugia et al.'s study, as ACIBA-46 and ACIBA-47 strains showed the ability to grow in nutrient poor environment. The four strains of *A. baumannii* tested in Farrugia et al study were reported to be able to utilize all the carbon substrates which utilized by ACIBA-46 and ACIBA-47 strains (54 carbon). Moreover, they were able to utilize a higher number of carbon substrates (80 carbon).

Table 5.1: Comparison of carbon substrates utilized by the tested strains in this study and the strains tested in Farrugia et al. study (2013).

Environmentally isolated strains (from this study)		**Clinically (blood) isolated strains (Farrugia et al., 2013)**			
ACIBA-46	ACIBA-47	D1279779	ACICU	AYE	ATCC 17978
54 carbon substrates (28%) out of 190 tested		80 carbon substrates (43%) out of 190 tested			

The data for nitrogen, phosphorus, sulfur and nutrient supplements (PM plates 3 - 8) showed a growth in all negative control wells (absence of substrates). This could be a false positive or it could be due to carry over of nutrients from the enrichment media in the capsule or biofilm or slime layer on the surface of the bacterial cells. Thus, the nitrogen, phosphorus, sulfur and nutrient supplement data in this study could not be interpreted in such a way that all these positive wells are indicative of catabolic positive for these substrates tested. However, surprisingly, there were certain nitrogen substrates ethylenediamine, N-acetyl-D-galactosamine, guanine, uric acid, alloxan, parabanic acid and D,L-α-amino-caprylic acid which not support any growth. This result suggests that

these certain nitrogen could be inhibiting the growth of *A. baumannii* ACIBA-46 strain. However, due to the lack of these substrates in laboratory, the result could not be validate. Further studies are required to examine these particular nitrogen substrates to find the reasons causing inhibition.

Peleg et al. (2012) reported that the clinical strain of *A. baumannii* ATCC 19606^T was able to utilize peptide nitrogen sources (PM 6-8), more efficiently compare to the three other species tested, *A. nosocomial*, *A. calcoaceticus*, and *A. pittii*, but has reduced capability of utilizing most of the phosphorous and sulfur sources tested. However, *A. baumanni* ACIBA-46 strain in this study showed a positive growth in all the phosphorous and sulfur wells including the negative control wells. Therefore, it could not be interpreted as in such a way that all these positive wells are indicative of catabolic positive for these substrates tested.

The ability of *A. baumannii* to adhere and form biofilm plays an important role in causing nosocomial infections due to its increased resistance to the antibiotics (Donlan, 2002; Longo Vuotto & Donelli, 2014). The biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains in LB broth, M9MM and M9MM supplemented with 22 substrates was determined using crystal violet assay. The biofilm formation of *A. baumannii* is controlled by various factors including the presence of antibiotic resistance genes, growth availability condition, and cell density (Gaddy & Actis, 2009). Moreover, Stepanovic et al. (2003) reported that the nutrient composition is one of the factors that may effect on microorganisms ability to form biofilms. When bacterial cells are incubated for prolong period, these cells could undergo starvation-induced stress which makes the formation of biofilm in some bacteria as a defense mechanism. Previous studies showed that starvation could stimulate biofilm forming in *E coli* and *L. monocytogenes* (Dewanti & Wong, 1995; Helloin et al., 2003). In contrast, *Citrobacter* spp. showed a low biofilm forming in poor nutrient environment (Allan et al., 2002). Similar to *Citrobacter* spp., *A.*

baumannii ACIBA-46 and ACIBA-47 strains were able to form a moderate biofilm in rich nutrient medium (LB broth), but would not form biofilm in low nutrient medium (M9MM) (absence of substrates). Starvation affects the characteristics of bacterial surface such as hydrophobicity, charge and irreversible attachment which are main factors in biofilm formation (Brown et al., 1977).

In addition, structures of cell surface such as fimbria required for initial attachment (Neidhardt, 1996) and biofilm establishment (Pratt & Kolter, 1998) are also affected by availability of carbon and nitrogen sources (Xie et al., 1997). In this study, the different carbon, nitrogen, phosphorus and sulfur substrates tested showed increased effects on the biofilm forming capability for both strains. When M9MM was supplemented with 22 substrates (tested individually), there was a change in the biofilm forming ability for both strains. Substrates such as, L- arabinose, L- arginine, fumaric acid, D, L- malic acid, Pyruvic acid and urea slightly increased the ability of forming biofilm for both strains (weak biofilm producers). In addition, substrates such as L-ornithine, L-histidine, L- leucine and thymidine moderately increased the ability to form biofilm for both strains (moderate biofilm producers). However, substrate such as Tween 80, glycine, Thiourea and L-lysine showed no effect on the biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains.

Citric acid substrate effects moderately on increasing the biofilm forming capability of ACIBA-46 strain, while showed slightly increased on the biofilm forming capability (weak biofilm producers) on ACIBA-47 strain. Moreover, substrates such as sodium phosphate showed ability to effect on ACIBA-47 strain weakly, but showed no effect on ACIBA-46 strain (Tables 4.3 & 4.4). These substrates showed different effect on the biofilm forming capability for ACIBA-46 and ACIBA-47 strains as they isolated from different environment, ventilator and hands of HCWs, respectively.

There are some limitations found in this study which are the lack of nitrogen, phosphorus, sulfur and nutrient supplements sources data (PM plates 3-8) of *A. baumannii* ACIBA-47strain. Due to lack of resources (research funding) it was not possible to repeat the PM experiments for the nitrogen, phosphorous, sulfur and nutrient supplements data. In addition, it was difficult to determine the exact nutrients used due to the nature of the Biolog plate contents. Besides, the concentrations of the nutrients in some cases could be quite low or unavailable for the microorganism tested. However, the elucidation of the metabolic activity of *A. baumannii* will offer scientists insight into ways to manipulate such activity either to curtail the pathogens success or enhance its susceptibility to antibiotic agents.

CHAPTER 6: CONCLUSION

In this study, two selected strains of *Acinetobacter baumannii,* ACIBA-46 (isolated from ventilator) and ACIBA-47 (isolated from hands of HCWs) were studied to determine their metabolic capability using the Phenotype Microarray technology. This study showed that 88.8% similarity in the carbon substrates utilized by the planktonic cells of *A. baumannii* ACIBA-46 and ACIBA-47 strains. Between two strains, ACIBA-47 strain showed the ability to utilize a higher number of carbon sources (6 carbon) compared to ACIBA-46 strain. The study showed that carboxylic acids and amino acids were the two major classes of compounds utilized by *A. baumannii* strains as carbon sources. Interestingly, this study found some of nitrogen substrates that inhibited the growth of *A. baumannii* ACIBA-46 strain. However, it is still not known why these nitrogen substrates caused such inhibition.

The effects of the 22 selected substrates on the biofilm forming ability of *A. baumannii* ACIBA-46 and ACIBA-47 strains were studied. The M9 minimal medium (M9MM) supplemented with 22 sole substrates tested showed increase in the biofilm forming ability of ACIBA-46 and ACIBA-47 strains. Different substrates tested such as citric acid showed various effects on the biofilm forming ability for both strains of *A. baumannii*.

REFERENCES

Al Atrouni, A., Joly-Guillou, M. L., Hamze, M., & Kempf, M. (2016). Reservoirs of non-baumannii *Acinetobacter* species. *Frontiers in Microbiology*, *7*(1), 49-58.

Al-Khaldi, S. F., & Mossoba, M. M. (2004). Gene and bacterial identification using high-throughput technologies. *Nutrition*, *20*(1), 32-38.

Allan, V. J., Callow, M. E., Macaskie, L. E., & Paterson-Beedle, M. (2002). Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* sp. *Microbiology*, *148*(1), 277-288.

Anstey, N. M., Currie, B. J., Hassell, M., Palmer, D., Dwyer, B., & Seifert, H. (2002). Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with carriage in the throat in at-risk groups. *Journal of Clinical Microbiology*, *40*(2), 685-686.

Antunes, L., Visca, P., & Towner, K. J. (2014). *Acinetobacter baumannii*: Evolution of a global pathogen. *Pathogens and Disease*, *71*(3), 292-301.

Bernhard, W., Hoffmann, S., Dombrowsky, H., Rau, G. A., Kamlage, A., Kappler, M., ... Poets, C. F. (2001). Phosphatidylcholine molecular species in lung surfactant: Composition in relation to respiratory rate and lung development. *American Journal of Respiratory Cell and Molecular Biology*, *25*(6), 725-731.

Bochner, B. R. (2008). Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews*, *33*(1), 191-205.

Bochner, B. R., Gadzinski, P., & Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Research*, *11*(7), 1246-1255.

Bochner, B., Gomez, V., Ziman, M., Yang, S., & Brown, S. D. (2010). Phenotype microarray profiling of *Zymomonas mobilis* ZM4. *Applied Biochemistry and Biotechnology*, *161*(1-8), 116-123.

Brown, C. M., Ellwood, D. C., & Hunter, J. R. (1977). Growth of bacteria at surfaces: Influence of nutrient limitation. *FEMS Microbiology Letters*, *1*(3), 163-166.

Brown, S. A., Palmer, K. L., & Whiteley, M. (2008). Revisiting the host as a growth medium. *Nature Reviews Microbiology*, *6*(9), 657-666.

Buchan, L. (1981). The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal. *Water Science Articles 7*(1), 1-7.

Carvalho, V. C. D., Oliveira, P. R. D. D., Dal-Paz, K., Paula, A. P. D., Félix, C. D. S., & Lima, A. L. L. M. (2012). Gram-negative osteomyelitis: Clinical and microbiological profile. *Brazilian Journal of Infectious Diseases*, *16*(1), 63-67.

Chelvam, K. K., Yap, K. P., Chai, L. C., & Thong, K. L. (2015). Variable responses to carbon utilization between planktonic and biofilm cells of a human carrier strain of *Salmonella enterica* serovar Typhi. *PLoS ONE*, *10*(5), e0126207.

Clemmer, K. M., Bonomo, R. A., & Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology*, *157*(9), 2534-2544.

Cook, A. M., & Fewson, C. A. (1973). Role of cabohydrates in the metabolism of *Acinetobacter calcoaceticus*. *Biochimica et Biophysica Acta*, *320*(1), 214-216.

D'agata, E. M., Thayer, V., & Schaffner, W. (2000). An outbreak of *Acinetobacter baumannii*: the importance of cross-transmission. *Infection Control & Hospital Epidemiology*, *21*(9), 588-591.

Davey, M. E., & O'toole, G. A. (2000). Microbial biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, *64*(4), 847-867.

Davis, K. A., Moran, K. A., McAllister, C. K., & Gray, P. J. (2005). Multidrug-resistant *Acinetobacter* extremity infections in soldiers. *Emerging Infectious Diseases*, *11*(8), 1218.

Dewanti, R., & Wong, A. C. (1995). Influence of culture conditions on biofilm formation by *Escherichia coli* O157: H7. *International Journal of Food Microbiology*, *26*(2), 147-164.

Dijkshoorn, L., & van der Toorn, J. (1992). *Acinetobacter* species: Which do we mean? *Clinical Infectious Diseases*, *15*(4), 748-749.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: Multidrug-resistant *Acinetobacter baumannii*. *Nature Reviews Microbiology*, *5*(12), 939-951.

Djeribi, R., Bouchloukh, W., Jouenne, T., & Menaa, B. (2012). Characterization of bacterial biofilms formed on urinary catheters. *American Journal of Infection Control*, *40*(9), 854-859.

Donlan R.M. (2001). Biofilms and device-associated infections. *Emerging Infectious Diseases.* 7, 277-281.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881.

Dorman, C. J., & Porter, M. E. (1998). The *Shigella* virulence gene regulatory cascade: A paradigm of bacterial gene control mechanisms. *Molecular Microbiology*, *29*(3), 677-684.

Dorman, C. J., McKenna, S., & Beloin, C. (2001). Regulation of virulence gene expression in *Shigella flexneri*, a facultative intracellular pathogen. *International Journal of Medical Microbiology*, *291*(2), 89-96.

Dorsey, C. W., Beglin, M. S., & Actis, L. A. (2003). Detection and analysis of iron uptake components expressed by *Acinetobacter baumannii* clinical isolates. *Journal of Clinical Microbiology*, *41*(9), 4188-4193.

El Shafie, S. S., Alishaq, M., & Garcia, M. L. (2004). Investigation of an outbreak of multidrug-resistant *Acinetobacter baumannii* in trauma intensive care unit. *Journal of Hospital Infection*, *56*(2), 101-105.

Espinal, P., Marti, S., & Vila, J. (2012). Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. *Journal of Hospital Infection*, *80*(1), 56-60.

Eveillard, M., Kempf, M., Belmonte, O., Pailhoriès, H., & Joly-Guillou, M. L. (2013). Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections. *International Journal of Infectious Diseases*, *17*(10), 802-805.

Fabich, A. J., Jones, S. A., Chowdhury, F. Z., Cernosek, A., Anderson, A., Smalley, D., ... Leatham, M. P. (2008). Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. *Infection and Immunity*, *76*(3), 1143-1152.

Falagas, M. E., Bliziotis, I. A., & Siempos, I. I. (2006). Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Critical Care*, *10*(2), R48.

Falagas, M. E., Karveli, E. A., Kelesidis, I., & Kelesidis, T. (2007). Community-acquired *Acinetobacter* infections. *European Journal of Clinical Microbiology & Infectious Diseases*, *26*(12), 857-868.

Farrugia, D. N., Elbourne, L. D. H., Hassan, K. A., Eijkelkamp, B. A., Tetu, S. G., Brown, M. H., … Paulsen, I. T. (2013). The complete genome and phenome of a community-acquired *Acinetobacter baumannii. PLoS ONE, 8*(3), 586-599.

Fournier, P. E., Richet, H., & Weinstein, R. A. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clinical Infectious Diseases*, *42*(5), 692-699.

Frank, J. F., & Koffi, R. A. (1990). Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection*, *53*(7), 550-554.

Freter, R., Brickner, H., Botney, M., Cleven, D., & Aranki, A. (1983). Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. *Infection and Immunity*, *39*(2), 676-685.

Gaddy J.A., Actis L.A. (2009). Regulation of *Acinetobacter baumannii* biofilm formation. *Future Microbiology*. *4*, 273-278.

Garnacho-Montero, J., & Amaya-Villar, R. (2010). Multiresistant *Acinetobacter baumannii* infections: epidemiology and management. *Current Opinion in Infectious Diseases*, *23*(4), 332-339.

Gilardi, G. L. (1978). Identification of miscellaneous glucose-nonfermenting Gram-negative bacilli. In G.L. Gilardi (Ed.), *Glucose-nonfermenting Gram-negative bacteria in clinical microbiology* (p. 45). West Palm Beach, Florida: CRC Press.

Gil-Perotin, S., Ramirez, P., Marti, V., Sahuquillo, J. M., Gonzalez, E., Calleja, I., ... Bonastre, J. (2012). Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: A state of concept. *Critical Care*, *16*(3), R93.

Girod, S., Galabert, C., Lecuire, A., Zahm, J. M., & Puchelle, E. (1992). Phospholipid composition and surface-active properties of tracheobronchial secretions from patients with cystic fibrosis and chronic obstructive pulmonary diseases. *Pediatric Pulmonology*, *13*(1), 22-27.

Göttig, S., Gruber, T. M., Higgins, P. G., Wachsmuth, M., Seifert, H., & Kempf, V. A. (2014). Detection of pan drug-resistant *Acinetobacter baumannii* in Germany. *Journal of Antimicrobial Chemotherapy*, *69*(9), 2578-2579.

Götz, F. (2002). Staphylococcus and biofilms. *Molecular Microbiology*, *43*(6), 1367-1378.

Greetham, D. (2014). Phenotype microarray technology and its application in industrial biotechnology. *Biotechnology Letters*, *36*(6), 1153-1160.

Harding, G. B., Klein, E., Pass, T., Wright, R., & Million, C. (1990). Endotoxin and bacterial contamination of dialysis center water and dialysate; a cross sectional survey. *The International Journal of Artificial Organs*, *13*(1), 39-43.

Helloin, E., Jänsch, L., & Phan-Thanh, L. (2003). Carbon starvation survival of *Listeria monocytogenes* in planktonic state and in biofilm: a proteomic study. *Proteomics*, *3*(10), 2052-2064.

Hooper, L. V., Midtvedt, T., & Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Review of Nutrition*, *22*(1), 283-307.

Houang, E. T., Chu, Y. W., Leung, C. M., Chu, K. Y., Berlau, J., Ng, K. C., & Cheng, A. F. B. (2001). Epidemiology and Infection Control Implications of *Acinetobacter* spp. in Hong Kong. *Journal of Clinical Microbiology*, *39*(1), 228-234.

Houang, E. T., Sormunen, R. T., Lai, L., Chan, C. Y., & Leong, A. S. (1998). Effect of desiccation on the ultrastructural appearances of *Acinetobacter baumannii* and *Acinetobacter lwoffii*. *Journal of Clinical Pathology*, *51*(10), 786-788.

Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence, 3*(3), 243-250.

Jawad, A., Seifert, H., Snelling, A. M., Heritage, J., & Hawkey, P. M. (1998). Survival of *Acinetobacter baumannii* on dry surfaces: Comparison of outbreak and sporadic isolates. *Journal of Clinical Microbiology*, *36*(7), 1938-1941.

Johnson, D. A., Tetu, S. G., Phillippy, K., Chen, J., Ren, Q., & Paulsen, I. T. (2008). High-throughput phenotypic characterization of *Pseudomonas aeruginosa* membrane transport genes. *PLoS Genetics*, *4*(10), e1000211.

Joly-Guillou, M. L. (2005). Clinical impact and pathogenicity of *Acinetobacter*. *Clinical Microbiology and Infection*, *11*(11), 868-873.

Kempf, M., & Rolain, J. M. (2012). Emergence of resistance to carbapenems in *Acinetobacter baumannii* in Europe: Clinical impact and therapeutic options. *International Journal of Antimicrobial Agents*, *39*(2), 105-114.

Knight, G. C., Seviour, R. J., Soddell, J. A., McDonnell, S., & Bayly, R. C. (1995). Metabolic variation among strains of *Acinetobacter* isolated from activated sludge. *Water Research*, *29*(9), 2081-2084.

Kong, B. H., Hanifah, Y. A., Yusof, M.Y., & Thong, K. L. (2011a). Application of amplified ribosomal DNA restriction analysis in identification of *Acinetobacter baumannii* from a tertiary teaching hospital, Malaysia. *Tropical Biomedicine, 28*(3), 563-568.

Kong, B. H., Hanifah, Y. A., Yusof, M.Y., & Thong, K. L. (2011b). Antimicrobial susceptibility profiling and genomic diversity of multidrug-resistant *Acinetobacter baumannii* isolates from a teaching hospital in Malaysia. *Japanese Journal of Infectious Diseases, 64*(4), 337-340.

Li, C. Y., Chen, S. J., Cheng, C. Y., & Chen, T. L. (2005). Production of *Acinetobacter radioresistens* lipase with repeated fed-batch culture. *Biochemical Engineering Journal*, *25*(3), 195-199.

Lin, E. C. C. (1977). Glycerol utilization and its regulation in mammals. *Annual Review of Biochemistry*, *46*(1), 765-795.

Longo, F., Vuotto, C., & Donelli, G. (2014). Biofilm formation in *Acinetobacter baumannii*. *New Microbiology*, *37*(2), 119-127.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M. C., Maida, I., ... Van den Barselaar, M. (2012). Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Research in Microbiology*, *163*(3), 161-172.

McConnell, M. J., Actis, L., & Pachón, J. (2013). *Acinetobacter baumannii*: Human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiology Reviews, 37*(2), 130-55.

Munoz-Price, L. S., & Weinstein, R. A. (2008). *Acinetobacter* infection. *New England Journal of Medicine*, *358*(12), 1271-1281.

Munoz-Price, L. S., Fajardo-Aquino, Y., Arheart, K. L., Cleary, T., DePascale, D., Pizano, L., ... Doi, Y. (2013). Aerosolization of *Acinetobacter baumannii* in a trauma ICU. *Critical Care Medicine*, *41*(8), 1915-1918.

Neidhardt, F. C. (Ed.). (1996). *Escherichia coli* and *Salmonella: Cellular and molecular biology.* Washington, DC: ASM Press

Nemec, A., Musilek, M., Maixnerova, M., De Baere, T., van der Reijden, T. J., Vaneechoutte, M., & Dijkshoorn, L. (2009). *Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov., haemolytic organisms isolated from humans. *International Journal of Systematic and Evolutionary Microbiology*, *59*(1), 118-124.

Peleg, A. Y., de Breij, A., Adams, M. D., Cerqueira, G. M., Mocali, S., Galardini, M., ... & Seifert, H. (2012). The success of *Acinetobacter* species, genetic, metabolic and virulence attributes. *PLoS ONE*, *7*(10), 469-480.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). *Acinetobacter baumannii*: Emergence of a successful pathogen. *Clinical Microbiology Reviews*, *21*(3), 538-582.

Peng, C., Zong, Z., & Fan, H. (2012). *Acinetobacter baumannii* isolates associated with community-acquired pneumonia in West China. *Clinical Microbiology and Infection*, *18*(12). 491-496.

Perkins, A. E., & Nicholson, W. L. (2008). Uncovering new metabolic capabilities of *Bacillus subtilis* using phenotype profiling of rifampin-resistant *rpoB* mutants. *Journal of Bacteriology*, *190*(3), 807-814.

Pour, N. K., Dusane, D. H., Dhakephalkar, P. K., Zamin, F. R., Zinjarde, S. S., & Chopade, B. A. (2011). Biofilm formation by *Acinetobacter baumannii* strains isolated from urinary tract infection and urinary catheters. *FEMS Immunology & Medical Microbiology*, *62*(3), 328-338.

Prosseda, G., Falconi, M., Nicoletti, M., Casalino, M., Micheli, G., & Colonna, B. (2002). Histone-like proteins and the *Shigella* invasivity regulon. *Research in Microbiology*, *153*(7), 461-468.

Pratt, L. A., & Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology*, *30*(2), 285-293.

Raad, I. I., Mohamed, J. A., Reitzel, R. A., Jiang, Y., Dvorak, T. L., Ghannoum, M. A., ... Chaftari, A. M. (2011). The prevention of biofilm colonization by multidrug-resistant pathogens that cause ventilator-associated pneumonia with antimicrobial-coated endotracheal tubes. *Biomaterials*, *32*(11), 2689-2694.

Renner, L. D., & Weibel, D. B. (2011). Physicochemical regulation of biofilm formation. *Material Research Society*, *36*(5), 347-355.

Roberts, J. P. (2012). Appreciating the Art of Assay Development. *Genetic Engineering & Biotechnology News*, *32*(7), 22-26.

Roca, I., Espinal, P., Vila-Farrés, X., & Vila J. (2012). The *Acinetobacter baumannii* oxymoron: Commensal hospital dweller turned pan-drug-resistant menace. *Frontiers in Microbiology, 23*(3), 1-30.

Sader, H. S., Farrell, D. J., Flamm, R. K., & Jones, R. N. (2014). Antimicrobial susceptibility of Gram-negative organisms isolated from patients hospitalized in intensive care units in United States and European hospitals (2009–2011). *Diagnostic Microbiology and Infectious Disease*, *78*(4), 443-448.

Schinner, S. A. C. (2013). *Gluconeogenesis and colonization of the mouse intestine by E. coli EDL933 and E. coli nissle 1917* (Master thesis, University of Rhode Island). Retrieved December 3, 2017, from http://digitalcommons.uri.edu/theses/141

Sebeny, P. J., Riddle, M. S., & Petersen, K. (2008). *Acinetobacter baumannii* skin and soft-tissue infection associated with war trauma. *Clinical Infectious Diseases*, *47*(4), 444-449.

Sengstock, D. M., Thyagarajan, R., Apalara, J., Mira, A., Chopra, T., & Kaye, K. S. (2010). Multidrug-resistant *Acinetobacter baumannii*: An emerging pathogen among older adults in community hospitals and nursing homes. *Clinical Infectious Diseases*, *50*(12), 1611-1616.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., ... Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. *Infection Control & Hospital Epidemiology*, *34*(1), 1-14.

Spellberg, B., & Bonomo, R. A. (2013). "Airborne assault": A new dimension in *Acinetobacter baumannii* transmission. *Critical Care Medicine*, *41*(8). 1094-1097.

Stepanović, S., Djukić, N., Opavski, N., & Djukić, S. (2003). Significance of inoculum size in biofilm formation by staphylococci. *The New Microbiologica*, *26*(1), 129-132.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, *40*(2), 175-179.

Sun, Z., Kang, Y., Norris, M. H., Troyer, R. M., Son, M. S., Schweizer, H. P., ... Hoang, T. T. (2014). Blocking phosphatidylcholine utilization in *Pseudomonas aeruginosa*, via mutagenesis of fatty acid, glycerol and choline degradation pathways, confirms the importance of this nutrient source in vivo. *PLoS ONE*, *9*(7), 103-115.

Sutherland, I. W. (2001). Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology*, *147*(1), 3-9.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: Involvement of a novel chaperone-usher pili assembly system. *Microbiology*, *149*(12), 3473-3484.

Trautner B.W., Darouiche R.O. (2004). Role of biofilm in catheter-associated urinary tract infection. *American Journal of Infection Control,* 32, 177-183.

Vance, D. E. (2008). Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. *Current Opinion in Lipidology*, *19*(3), 229-234.

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., ... Reinhart, K. (2009). *Jama Network*, *302*(21), 2323-2329.

Webster, C., Towner, K. J., & Humphreys, H. (2000). Survival of *Acinetobacter* on three clinically related inanimate surfaces. *Infection Control and Hospital Epidemiology*, *21*(4), 246-246.

Whitman, T. J., Qasba, S. S., Timpone, J. G., Babel, B. S., Kasper, M. R., English, J. F., ... Eshoo, M. W. (2008). Occupational transmission of *Acinetobacter baumannii* from a United States serviceman wounded in Iraq to a health care worker. *Clinical Infectious Diseases*, *47*(4), 439-443.

Wong, D., Nielsen, T. B., Bonomo, R. A., Pantapalangkoor, P., Luna, B., & Spellberg, B. (2017). Clinical and pathophysiological overview of *Acinetobacter* infections: A century of challenges. *Clinical Microbiology Reviews*, 30(1), 409-447.

Xie, H., Cai, S., & Lamont, R. J. (1997). Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. *Infection and Immunity*, *65*(6), 2265-2271.

Zimbler, D. L., Penwell, W. F., Gaddy, J. A., Menke, S. M., Tomaras, A. P., Connerly, P. L., & Actis, L. A. (2009). Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*. *Biometals*, *22*(1), 23-32.