DePaul University
UNIVERSITY LIBRARIES

DePaul University
Digital Commons@DePaul

College of Liberal Arts & Social Sciences Theses and Dissertations

College of Liberal Arts and Social Sciences

11-2011

Effect of iron and sodium chloride on biofilm development of stenotrophomonas maltophilia

Rudy F. Martinez
DePaul University, rmarti46@mail.depaul.edu

Follow this and additional works at: https://via.library.depaul.edu/etd

Recommended Citation

Martinez, Rudy F., "Effect of iron and sodium chloride on biofilm development of stenotrophomonas maltophilia" (2011). *College of Liberal Arts & Social Sciences Theses and Dissertations*. 102.
https://via.library.depaul.edu/etd/102

This Thesis is brought to you for free and open access by the College of Liberal Arts and Social Sciences at Digital Commons@DePaul. It has been accepted for inclusion in College of Liberal Arts & Social Sciences Theses and Dissertations by an authorized administrator of Digital Commons@DePaul. For more information, please contact digitalservices@depaul.edu.

Effect of Iron and Sodium Chloride on Biofilm Development of

Stenotrophomonas maltophilia

A Thesis Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

By

Rudy F. Martinez

November 2011

Department of Biological Sciences

College of Liberal Arts and Sciences

DePaul University

Chicago, Illinois

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Joanna Brooke for accepting me into her lab and for all of her support both inside and outside of the lab. Her passion for teaching and helping students achieve their potential is evident in her guidance. Thank you, Dr. Brooke, for your encouragement and for motivating me to finish and complete my degree.

I would also like to thank the rest of my committee, Dr. John Dean and Dr. Stanley Cohn, for their patience and continual support. The successful completion of my degree would not have been possible without them. I had the additional benefit of serving as teaching assistants for both of them, so it was a pleasure working for them as well.

I also need to thank all the members of the biology department at DePaul who helped me in many ways. I especially thank Dr. Margaret Silliker. She is an excellent role model and a genuinely caring and encouraging graduate advisor.

Finally, I would like to thank my family, especially my parents Rodolfo and Clementina Martinez. I would not be where I am today if it were not for them. Thank you, Mom and Pop, for all of your love and support. From an early age, they helped me realize that I can accomplish my goals and dreams. I also would like to recognize my brothers and sisters; I hope that I continue to serve as a role model to you all.

TABLE OF CONTENTS

Page

ABSTRACT……. 1

INTRODUCTION……... 2

Background and Significance of Respiratory Pathogens: *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* …….3

Biofilm Background ……6

Antimicrobial Resistance and Biofilms…….…….7

Stages of Biofilm Development……...… 8

Factors Affecting Biofilm Development……...……..11

EXPERIMENTAL HYPOTHESES…….. 13

MATERIALS AND METHODS…… 14

Diagnostic Verification of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*……... 14

Growth and Maintenance of *P. aeruginosa* and *S. maltophilia*……14

Media Preparation for Bacterial Growth and Biofilms....……... 15

Growth Assays.…… 15

Biofilm Assays……..… 16

Statistics……..……...… 17

RESULTS……... 18

Identification and Characterization of *P. aeruginosa* ATCC 27853 and *S. maltophilia* X26332 ……..……... 18

Effect of Iron and Temperature on the Growth of *S. maltophilia* X26332…… 20

Effect of Ferric Chloride on *P. aeruginosa* ATCC 27853 and *S. maltophilia* X26332 Culture Growth in PVC Microtiter Plates…….. 24

Effect of Ferric Chloride on *S. maltophilia* X26332 Biofilm Development in PVC Microtiter Plates……...….27

Effect of Sodium Chloride on *P. aeruginosa* ATCC 27853 and *S. maltophilia* X26332 Culture Growth in PVC Microtiter Plates ……... 31

Effect of Sodium Chloride on *S. maltophilia* X26332 Biofilm Development in PVC Microtiter Plates……...….32

DISCUSSION……..…… 33

Iron and Culture Growth……... 34

Iron and *S. maltophilia* Biofilms ….…….. 35

Sodium Chloride and *S. maltophilia* Biofilms ……. 38

***Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* Biofilms……. 39**

Future Directions……...…. 42

LITERATURE CITED……..… 43

List of Figures **Page**

Figure 1A: Biofilm Development of *P. aeruginosa*……10

Figure 1B: *S. maltophilia* Biofilm on a Glass Coverslip……... 10

Figure 2: API strip results of *S. maltophilia* and *P. aeruginosa*…..……18

Figure 3: Blood Agar and LBA plates of *S. maltophilia* and *P. aeruginosa* Cultures… 19

Figure 4A: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 4°C……..……... 21

Figure 4B: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 14°C……..……..……... 21

Figure 4C: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 30°C…..…….....… 22

Figure 4D: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 35°C…….…..… 22

Figure 5: Ferric Chloride and Temperature Growth Plates…….…….. 23

Figure 6: Growth of *S. maltophilia* in M9 Medium with Ferric Chloride…..... 25

Figure 7: Effect of Ferric Chloride on *S. maltophilia* and *P. aeruginosa* Culture Growth……26

Figure 8A: *S. maltophilia* Biofilm in PVC Plate……28

Figure 8B: Single Strip of *S. maltophilia* biofilm…….. 28

Figure 9: Effect of Ferric Chloride on *S. maltophilia* Biofilm Development……. 29

Figure 10: Effect of Sodium Chloride on *S. maltophilia* and *P. aeruginosa* Culture Growth…..... 31

Figure 11: Effect of Sodium Chloride on *S. maltophilia* Biofilm Development……. 32

ABSTRACT

Stenotrophomonas maltophilia is an emerging worldwide, opportunistic respiratory pathogen that is associated with a significant case-fatality ratio in immunocompromised patient populations and has the ability to cause serious infection in cystic fibrosis patients. Populations of *S. maltophilia* are able to produce biofilms, which complicates these infections and provides resistance to antimicrobials. Factors such as ferric chloride ($FeCl_3$), and sodium chloride (NaCl) have been shown to inhibit the biofilm development of another respiratory pathogen, *Pseudomonas aeruginosa*, without affecting culture growth. The effect of $FeCl_3$ and NaCl on *P. aeruginosa* biofilm formation has been well studied and characterized, but little is known about the effect of $FeCl_3$ and NaCl on the biofilm development of *S. maltophilia*. This study tested three different hypotheses. Based on the fact that *S. maltophilia* is in environmental organism which exists in various temperatures and requires iron, the first hypothesis was that temperature and ferric chloride would affect *S. maltophilia* growth. The second hypothesis was that ferric chloride has an effect on the biofilm development of *S. maltophilia* in the same way it affects *P. aeruginosa* biofilm development. The third hypothesis was that sodium chloride inhibits biofilm development of *S. maltophilia*. In the culture conditions used in this study, the growth and biofilm formation of *S. maltophilia* was not inhibited in the presence of $FeCl_3$ at final concentrations of 1 μM, 5 μM, and 100 μM or with NaCl, but was inhibited in the presence of concentrations ≥ 2% NaCl. In this current study, in contrast to *S. maltophilia*, a strain of *P. aeruginosa* demonstrated a decrease in growth when exposed to $FeCl_3$ at 100 μM. However, the effects of NaCl on *S. maltophilia* mirrored that of *P. aeruginosa*. Our data shows that $FeCl_3$ at the concentrations tested in the culture medium used on this study was not an effective inhibitor of the biofilm of *S. maltophilia*.

INTRODUCTION

Antibiotic-resistant bacterial infections are a primary cause of death in intensive-care units worldwide. Gram-negative bacterial strains such as *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* are antibiotic-resistant opportunistic pathogens which have been shown to be problematic in the clinical setting (Costerton et al., 1999). As more bacterial strains are becoming resistant to multiple antibiotics, such as vancomycin and co-trimoxazole (Cegelski et al., 2008; Looney et al., 2009), there is an even more urgent need for a new series of antibiotics that address different modes of action on their therapeutic targets (Cegelski, 2008). An additional problem that complicates treatment is that these bacteria all form dense cellular communities called biofilms, which can increase their antibiotic resistance, prevalence, and survival (Steward and Costerton, 2001). Researchers are thus trying to target biofilm development in an attempt to increase antibiotic sensitivity and as an alternative approach to the development of new antibiotics. This new approach in targeting biofilm development does not directly threaten bacterial growth or existence, and it may prevent the bacteria from acquiring drug resistance (Cegelski et al., 2008). One of the logical steps in this approach is to investigate the environmental or nutritional conditions that may have an effect and influence biofilm development. This thesis research investigates the influence of three factors that have been shown to have an effect on growth and biofilm formation in another respiratory bacterial pathogen, *P. aeruginosa.* The three factors studied were temperature, iron concentration, and sodium chloride concentration.

Background and Significance of Respiratory Pathogens: Pseudomonas aeruginosa and Stenotrophomonas maltophilia

There are two structural and physiological categories of bacteria in which most bacterial species belong: Gram-positive and Gram-negative. This distinction is important because these physiological differences help determine how these species exist, react to the environment, and how they may play a role in antibiotic resistance and biofilm development. These two categories of bacteria are distinguished by the characteristics of their cell wall. The Gram-positive cell wall has a relatively large amount of sugar and amino acid polymers known as peptidoglycan which reacts with the Gram stain causing the stained cells to appear purple in color (Tortura et al., 2004). Gram-negative bacterial cells have a relatively thin layer of peptidoglycan in their cell wall that is not as dense as that found in Gram-positive bacteria (Tortura et al., 2004). As a result, when the Gram stain is applied, these Gram-negative bacterial cells appear pink in color (Tortura, 2004). Gram-negative bacteria have an additional outer membrane made of lipopolysaccharide (Gould, 2008). Lipopolysaccharide (LPS) consists of lipid molecules and polysaccharide polymers which serve in both a structural and protective role in Gram-negative bacteria (Atlas and Bartha, 1998). This outer membrane layer plays a major role in the virulence of the Gram-negative bacteria and plays a critical role in their ability to develop biofilms (Stoodley et al., 2002).

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacterium that has been studied in the medical field for its relationship with chronic lung infections especially those affecting patients with cystic fibrosis (CF) (Millar et al., 2009; Spasenovski et al., 2010). *P. aeruginosa* has been shown to be the most prevalent and colonizeable bacterial isolate in CF patient sputum in the world (Denton and Kerr, 1998; Tré-Hardy et al., 2008). A large majority of approximately

80-90% of CF adults are chronically infected with *P. aeruginosa* (Tré-Hardy et al., 2009). CF is an autosomal recessive genetic disease which affects one in two thousand Caucasian individuals in North America (Coutinho et al., 2008). CF is a result of a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which plays an important role in electrolyte transport (Tré-Hardy et al., 2008). The presence of this mutation can have serious repercussions on organs throughout the body, especially the lungs (Tré-Hardy et al., 2008). A signature symptom of CF is thick mucus production in the lungs which is a result of abnormal viscous secretion of mucus and leads to chronic lung malfunction (Coutinho et al., 2008; Tré-Hardy et al., 2009). This dense mucosal layer makes the CF patient more susceptible to inhaled microbial pathogens and increases the risk of a bacterial infection in the lungs, with such infections responsible for 90% of the mortality rate (Spasenovski et al., 2010). CF patients can succumb to infections specifically caused by *P. aeruginosa* in the lungs. A high mortality rate with a decreased median life expectancy of approximately 30 years has been reported with CF patients (Coutinho et al., 2008). This highlights the importance of developing treatments for *P. aeruginosa* infection, and demonstrates why this organism has been highly researched.

Stenotrophomonas maltophilia is a worldwide, emerging, antibiotic-resistant, opportunistic pathogen that has only recently in the past two decades been the focus of research in the field of respiratory infections. *S. maltophilia* was first isolated in 1943 from pleural fluid of a patient and was initially named *Bacterium bookeri*, but it was eventually classified in the genus *Pseudomonas* in 1961 (Denton and Kerr, 1998; Looney et al., 2009). *S. maltophilia* was finally classified as its own genus in 1993 and, most likely due to this more recent classification, is still not as well characterized as *P. aeruginosa* (Denton and Kerr, 1998). The genus name *Stentrophomonas* comes from the Greek ‘unit feeding on few substances’ (Denton and Kerr,

1998). This reflects the characteristic that *S. maltophilia* can survive with a minimum of nutrients. *S. maltophilia* is a ubiquitous bacterium that can be found in a myriad of environments and geographical regions (Denton and Kerr, 1998). This is an organism that can commonly be found in a wide variety of aqueous-associated environments, such as rivers, wells, bottled water, sewage reservoirs, sink and fountain drains and soil sources (Denton and Kerr, 1998; Brooke et al. 2009).

S. maltophilia affects immunocompromised patient populations with a high case-fatality ratio. This bacterium has been attributed to a mortality rate of 26.7% in bacteremia (bacteria in the blood) cases (Nicodemo and Garcia Paez, 2007). Incidences of *S. maltophilia* isolates have increased worldwide in the past decade alone (Looney et al., 2009). Hospitals around the world have documented increases in blood isolated *S. maltophilia* including in England and Wales of 93% from 2000 to 2006 (Looney et al., 2009), hospitals in Taiwan have reported an 83% increase in isolates from 1999 to 2004 (Looney et al., 2009) and even the United States has reported a 13% increase from 1998 to 2004 (Safdar and Rolston, 2007). This specific pathogen has been associated with a variety of diseases including pneumonia, bacteremia, urinary tract infections, wound infections, bronchitis, endocarditis, meningitis, eye infections and catheter-associated infections (Schaumann et al., 2001; Brooke et al., 2007; Falagas et al., 2009). This bacterium is becoming of more importance in medicine because, in addition to causing complications in the hospital setting, it has also been recently identified as a possible community-acquired pathogen (Falagas et al., 2009). In the hospital setting, similar to *P. aeruginosa*, *S. maltophilia* also colonizes and complicates the prognosis of CF patients (Nicodemo and Garcia Paez, 2007).

However, since *S. maltophilia* is a recently identified microorganism with both biofilm-producing capabilities, antimicrobial-resistant properties and most importantly, an increasing infection rate, more research needs to be done on this bacterium in order to create new antimicrobial treatments or discover ways to block its biofilm-forming ability.

Biofilm Background

It is important to understand that there are both harmful and beneficial biofilms in nature. Harmful and virulent bacterial biofilms help cause the diseases and pathologies mentioned in the previous section of this thesis, but there are positive aspects of biofilm formation as well, especially pertaining to humans. Bacteria that produce beneficial biofilms have evolved to have a symbiotic relationship with their host. It has been shown that certain bacterial species such as *Escherichia coli* found in the human gastrointestinal tract naturally form biofilms in the mucosal epithelium (Macfarlane and Dillon, 2007). These biofilms can compete for resources and potentially prevent the proliferation of other harmful microorganisms.

Biofilms are microbial communities surrounded by an extracellular polysaccharide matrix material that causes them to readily attach to multiple surfaces (Brooke et al., 2007). This matrix is made up of many exopolysaccharides that in addition to allowing adherence to surfaces, also serves to facilitate communication between the bacteria (Stewart and Costeron, 2001). It was not until the 1970s that the bacterial biofilm mode of existence began to be explored (Costerton et al., 1999). Only in the past couple of decades have biofilms been more extensively studied and understood. As late as 1987, biofilms were thought of as simple slabs of matrix material in which bacterial cells randomly embedded themselves (Stoodley et al., 2002). Through the 1980s and 1990s, the intricacies of biofilm organization really began to be

understood and appreciated (Costerton et al., 1999). Now, it has been shown that this structured community lends itself to be a cause of persistent sources of infection (Cegelski et al., 2008; Stoodley et al., 2002). These biofilms preferentially develop on moist inert surfaces, medical devices, and living tissues. Another problem that the medical field faces with biofilms is that they can grow slowly in multiple locations and are slow to cause overt symptoms in the patients (Costerton et al., 1999).

Bacteria can adhere to a wide variety of surfaces and persist for an extended amount of time. For example, the Gram-positive bacterium *Staphylococcus aureus* has been shown to survive for months on dry surfaces (Kramer et al., 2006). Gram-negative organisms such as *P. aeruginosa* or *S. maltophilia* can also survive for months on surfaces such as glass, plastic surfaces or epithelial cells (Kramer et al., 2006; Brooke et al., 2007). It is also important to note that Gram-negative bacteria tend to have more persistent and virulent biofilms than Gram-positive bacterial biofilms because of the previously mentioned outer membrane structure signature to these kinds of bacteria (Stoodley et al., 2002).

Antimicrobial Resistance and Biofilms

Both *P. aeruginosa* and *S. maltophilia* have the capability of gaining antimicrobial resistance from other bacterial species through many mechanisms such as horizontal gene transfer, e.g. R (resistance) plasmids and transposons (Watson et al., 2008). *S. maltophilia* possesses several mechanisms that increase its resistance to antimicrobials including the ability to form biofilms and an intrinsic resistance to a wide variety of antibiotics including β-lactams, quinolones, and tetracycline (Nicodemo and Garcia Paez, 2007; Looney et al., 2009). The *S. maltophilia* genome has been sequenced, and this has provided insight on certain genes that have

been identified to help this bacterium gain antimicrobial resistance (Crossman et al., 2008). These identified genes encode for β-lactamases, efflux pumps and various other enzymes that promote drug resistance (Crossman et al., 2008; Looney et al., 2009). In addition to these intrinsic properties, biofilm formation provides resistance to antibiotics. For example, bacteria within biofilms formed by *P. aeruginosa* are 100-1000 times less susceptible to antibiotics than the same number of free-floating or planktonic bacterial populations (Tré-Hardy et al., 2009).

Factors such as nutrient concentration and temperature can influence biofilm development and may provide insights into how resistance to antimicrobials can occur in both *P. aeruginosa* and *S. maltophilia.*

Stages of Biofilm Development

Biofilm development can be divided into a five stage process (Figure 1). The initial step in the formation of biofilm is adherence of free (planktonic) organisms to a surface. A wide variety of surfaces can be utilized by different bacterial species which can lead to different morphologies of biofilm (Stoodley et al., 2002; Aslam, 2008). In the case of catheter-forming biofilms, a prominent problem in hospital-based infections, the surface would be the catheter (Raad et al., 2008). The exact nature of how the bacterial cells adhere to the surface depends on the specific bacterial species. One example of adherence is due to the twitching motility by *P. aeruginosa* which involves a cell surface component called type IV pili (Stoodley et al., 2002). Several other factors can influence the attachment of bacteria to a surface and biofilm production including hydrophobic and electrostatic forces, other cell surface structures, and shear stress from the fluid environment (Costerton et al., 1999).

The second stage is the production of extracellular polymeric substances (EPS) which provide the developing biofilm with structural support. The EPS is made up of various macromolecules such as polysaccharides, proteins, and nucleic acids and is the result of gene expression from the bacterial cells (Stoodley et al., 2002; Costerton et al., 1999). The production of the EPS secures the adhered biofilm more firmly to the surface (Stoodley et al., 2002; Cegelski et al., 2008).

The third stage consists of the early development of the biofilm architecture including the important extracellular polysaccharide matrix (Stoodley et al., 2002). This matrix is an essential part of the biofilm which contributes to its antibiotic resistance (Cegelski et al., 2008).

The fourth stage consists of the maturation of the biofilm (Stoodley et al., 2002). Once the biofilm has matured to this stage, it becomes very difficult to treat with antibiotics. Antimicrobial agents may fail to eradicate biofilms due to the failure of the agents to penetrate the full depth of this matured biofilm (Aslam, 2008; Cegelski et al., 2008).

The fifth stage occurs when the developed, mature biofilm colony disperses planktonic cells from itself; these cells then migrate to and adhere to another surface and can form another biofilm. Entire microcolonies can detach from the biofilm without provocation (Stoodley et al., 2002). This dispersal has been shown to occur concurrently with three interrelated processes: the production of nitric oxide or other stress inducing molecules inside biofilm structures, bacteriophage induction or cell lysis (Barraud et al., 2006).

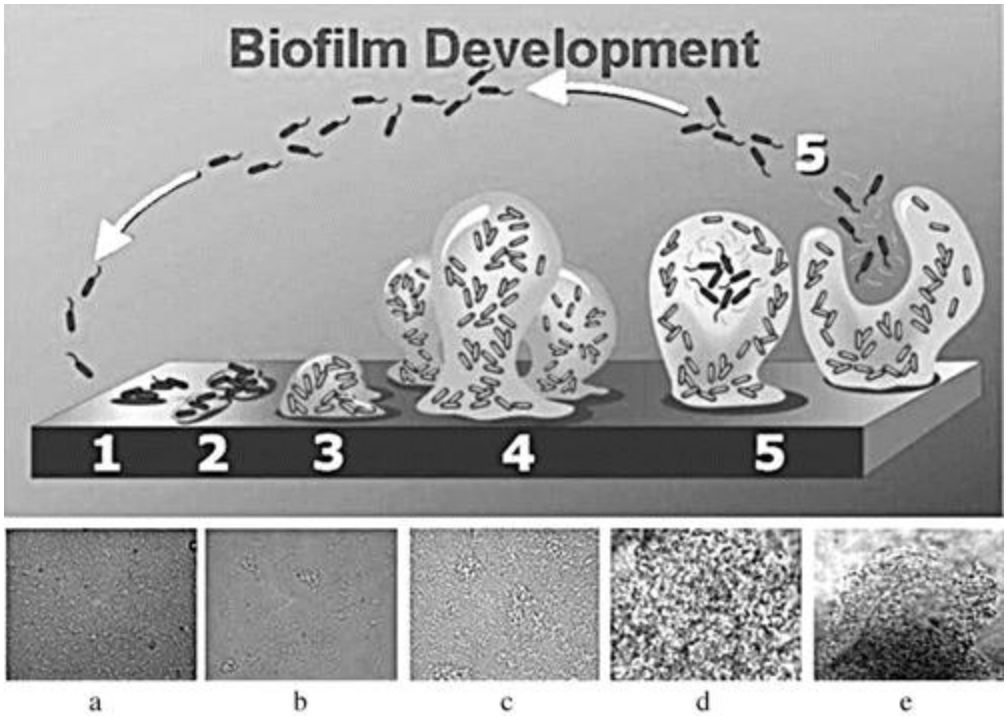


Figure 1A: The diagram above illustrates the five stages of biofilm development of *P. aeruginosa*. The panels below (a-e) the photomicrographs of *P. aeruginosa* growing on a glass surface in the respective 5 stages (Stoodley et al., 2002).

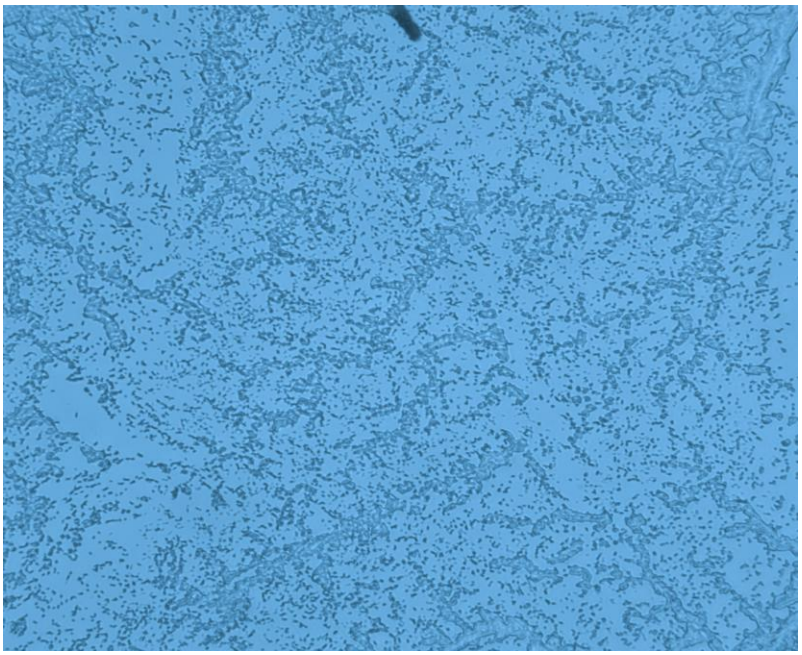


Figure 1B: *S. maltophilia* biofilm at 24 hours on a glass coverslip. Magnification 1000x. Cells were grown overnight in a Petri dish containing Luria-Bertani broth and a glass coverslip and incubated stationary for 24 hours at 35°C. Coverslip was stained with 1% (w/v) crystal violet and washed 3 times with phosphate buffered saline and air dried.

Factors Affecting Biofilm Development

One key aspect of biofilm production is the ability of the cells within the biofilms to communicate with each other so that the biofilm can develop appropriately (Horswill et al., 2006). This communication is known as quorum sensing. Quorum sensing refers to the chemical signaling that takes place among bacteria to process the environmental cues and keep track of their cellular density (Horswill et al., 2006; Cegelski et al., 2008). It is mediated by the production and subsequent recognition of small molecules called autoinducers. These autoinducers follow a signal transduction pathway which coordinates gene expression and regulates other cellular processes (Cegelski et al., 2008). Many factors that influence quorum sensing such as physical, chemical, or biological variables can impact signal production, stability and distribution (Horswill et al., 2006).

Another property affecting bacterial biofilm development is the nutritional conditions available to the cells. One common factor is temperature. Since *S. maltophilia* is an ubiquitous organism found in a variety of environments, it lends itself to survive in various temperatures (Denton and Kerr, 1998). Studies have shown that temperature can influence *S. maltophilia* cellular function (Rahmati-Bahram et al., 1997).

Most cells have an absolute requirement of iron because it is an essential metal for normal function and homeostasis of a wide range of cellular proteins (Reid et al., 2009). In bacterial cells, iron is used for several activities including aerobic respiration as an essential component important for some enzymes to function properly, and is important for cell growth. In the human host, bacteria have limited access to free iron as humans use several molecules (i.e. transferrin, lactoferrin, hemoglobin, etc.) that bind to iron (Berlutti et al., 2005).

Studies have shown that increases in iron, in the form of ferric chloride, all have a direct effect of decreasing *P. aeruginosa* biofilm development (Musk et al., 2005; Yang et al., 2007; Patriquin et al., 2008). Yang et al. (2007) showed that high concentrations of $FeCl_3$ (50 µM and 100 µM) decrease the amount of biofilm produced in cultures of *P. aeruginosa* grown in polystyrene microtiter plates.

Patriquin et al. (2008) tested biofilm development using a $FeCl_3$ µM range between 0.01 µM and 8 µM with *P. aeruginosa* on polyvinylchloride (PVC) pegs submerged in a standard 96-well microtiter plate. With increasing ferric chloride concentrations, biofilm mass significantly decreased (Patriquin et al. 2008).

Musk et al. (2005) tested the effects of various iron salts, including ferric chloride, on biofilm formation in *P. aeruginosa*. In addition to this experiment, the effect of iron salts on pre-formed biofilms was also tested. These biofilms were grown in 384-well plates. Consistent with the previously mentioned studies, increasing levels of iron salts, such as ferric chloride, have a direct effect of decreasing biofilm formation by *P. aeruginosa* (Musk et al., 2005).

Sodium chloride is another nutrient that has been tested to observe its influence on bacterial infection and biofilm development (Havasi et al., 2008). Havasi et al. (2008) tested the potential effectiveness of using hypertonic saline aerosols to treat CF patients with *P. aeruginosa* infections. They did this by testing the effect of NaCl concentrations of 0.27%, 0.9%, 2%, 4% and 7% on *P. aeruginosa* motility and growth. The test for growth and motility was performed on LB plates with 0.4% agar and determined by measuring the spread of the colony growth, and culture growth measured was simply done by spectrophotometry. Havasi et al. (2008) reported that NaCl concentrations greater than and equal to 2% showed decreased motility and growth. This study was promising because they claim that hypertonic saline (7% NaCl) is not harmful to

the host but has an effect on *P. aeruginosa*. This is also an important paper for this current study because Havasi et al. (2008) proposes that these same salt concentrations could have an effect on biofilm formation.

EXPERIMENTAL HYPOTHESES

A review of the literature shows that a preliminary understanding of *S. maltophilia* biofilm development has been achieved. Much is still not known about the factors that lead to its biofilm development. This thesis research tested the effect of ferric chloride and temperature, ferric chloride at various concentrations, and sodium chloride at various concentrations on *S. maltophilia* growth and biofilm development.

Since earlier studies showed that both sodium chloride and ferric chloride have a partial inhibitory effect on *P. aeruginosa*, we developed initial hypotheses to determine if the effect was similar with *S. maltophilia*. This thesis research was designed to test the hypothesis by first measuring how ferric chloride affects *S. maltophilia* growth at various temperatures. Then, we varied ferric chloride and sodium chloride concentrations to levels that affected *P. aeruginosa* biofilm development and observed the effect on *S. maltophilia*.

MATERIALS AND METHODS

Diagnostic Verification of Pseudomonas aeruginosa and Stenotrophomonas maltophilia

The *P. aeruginosa* strain used was ATCC 27853 and the *S. maltophilia* strain used was X26332. The *P. aeruginosa* strain was blood isolate obtained from the American Type Culture Collection. The *S. maltophilia* strain was a bloodstream isolate acquired from Children's Memorial Hospital, Chicago, IL. The identification of these organisms was verified using the Gram-negative non-enteric bacterial identification API system. Cultures of each organism were grown overnight at 37 ^{o}C in Luria-Bertani (LB) broth. LB is a standard medium used that has several essential nutrients that ensures healthy growth of both *P. aeruginosa* and *S. maltophilia*. Cultures were then centrifuged, and the pellet was resuspended in saline solution. This resuspended pellet was then standardized to a turbidity value of MacFarland standard 2 (bioMérieux) by addition to a fresh tube of sterile saline. An API strip was inoculated with each organism, covered and the incubated overnight at 37 ^{o}C. Observations were made after a 24 hour incubation, and identification of the organisms was made using the API system.

To test culture growth, production of hemolysins was tested by growing the cultures on blood agar plates. Each bacterium was also plated on Luria-Bertani agar (LBA) plates to observe the general colony appearance.

Growth and Maintenance of Pseudomonas aeruginosa and Stenotrophomonas maltophilia

P. aeruginosa and *S. maltophilia* and were grown on LBA plates at 37^{o}C overnight and passaged weekly onto fresh media plates.

Media preparation for Bacterial Growth and Biofilms

A Difco 5X M9 minimal salts medium solution (Becton, Dickinson Company; Sparks, MD) was prepared which contained essential ions/electrolytes such as sodium phosphate, potassium phosphate, ammonium chloride, and minimal amounts of sodium chloride. The medium was then supplemented with additional nutrients that promoted bacterial growth and biofilm development. Those additives included 20% glucose, 1 M magnesium sulfate, 1 M calcium chloride, and 10% casamino acids. The final concentrations of the medium were 0.36% glucose, 1.8 mM $MgSO_4$, 90 μM $CaCl_2$, and 0.9% casamino acids, respectively.

A stock of 100 mM ferric chloride was prepared through filter sterilization and then subsequently added at various concentrations to the M9 minimal salts medium to achieve the desired final iron concentrations of 0 μM, 1 μM, 5 μM and 100 μM (Yang et al, 2007; Patriquin et al, 2008).

For the sodium chloride experiments, Davis minimal broth (Fluka Analytical; Steinheim, Switzerland) was used instead of 5X M9 minimal salts medium because M9 already has sodium chloride and Davis minimal broth has none. Davis minimal broth contains ammonium sulfate, dextrose, dipotassium phosphate, magnesium sulfate, monopotassium phosphate, and sodium citrate. The Davis minimal broth was prepared with various salt concentrations to achieve the desired final sodium chloride percentages of 0.27%, 0.9%, 2%, 4% and 7% (Havasi et al., 2008)

Growth Assays

LBA plates with different concentrations of $FeCl_3$ (0 μM , 8 μM , and 100 μM) were prepared with 0.4% agar. Eighteen hour overnight cultures of *S. maltophilia* were centrifuged at the maximum speed of the microfuge (250 rpm) for 2 minutes and their pellets resuspended in

fresh LB. Each cell culture concentration was standardized by diluting it into another sterile tube of 5 ml of fresh LB using McFarland Standard #2 to reach desired density. Each agar plate was then inoculated with 100 μl of *S. maltophilia* in the center of the plate and then placed in one of four different temperature incubators: 4^{o} C, 14^{o} C, 30^{o} C, or 35^{o} C. Measurements of the diameter of colony growth were taken every 24 hrs for 6 days. Pictures were also taken to observe the behavior of the colony spreading. This experiment was conducted in a set of five replicates.

Biofilm Assays

In preparation of biofilm assays, overnight cultures were grown shaking at 250 r.p.m. overnight in the appropriate medium. For the ferric chloride experiment, 5X M9 minimal salts medium was used and supplemental nutrients were added to ensure growth in a 37^{o}C incubator. The nutrients added were the same concentrations stated earlier resulting in final concentrations of 0.36% glucose, 1.8 mM $MgSO_4$, 90 μM $CaCl_2$, and 0.9% casamino acids in the medium. For the sodium chloride experiment, Davis minimal broth was used and only casamino acids were added with a 0.9% final concentration.

Biofilm formation was measured using a Falcon plastic 96-well PVC microtiter plate (Watnik and Kolter, 1999). Fifteen to seventeen hour overnight cultures of *P. aeruginosa* and *S. maltophilia* were centrifuged for 2 minutes and their pellets resuspended in the various nutrient concentration media for inoculation. Each tube was standardized to an optical density reading of 0.1 at 600nm using a Beckman DU530 Life Science UV/Vis Spectrophotomer. This 0.1 absorbance reading was then diluted down with the appropriate media (M9 Minimal Salts medium with the iron experiment or Davis minimal broth with the sodium chloride experiment)

1:100 to get a final absorbance reading of 0.001. Once the desired absorbance was set, each well in the microtiter plate was inoculated with 135 µl of each medium containing cells. The plates were then supplemented with 15 µl of broth containing either ferric chloride or sodium chloride. Negative control wells were also inoculated with medium lacking cells, but each negative control contained the ferric or sodium chloride supplement.

Each plate had an initial OD_{590} reading and was then sealed with Parafilm and incubated at 35°C overnight for 24 hrs. Bacterial growth was then recorded at OD_{590} using an automated microplate reader (EL_x800 BioTek Instruments, Inc.). Media containing non-adherent cells were discarded and adhered cells were stained with 1% (w/v) crystal violet for 10 minutes at room temperature. The wells were washed with 200 µl phosphate buffered saline (PBS) 3 times for 1 minute each. The plate was then left to dry overnight. Once dry, the stained cells and biofilm material were then resuspended thoroughly by pipette with 160 µl of 80% ethanol and absorbance was again read at OD_{562} to measure the amount of biofilm.

100 microliters of each medium with cells at standardized turbidity readings were plated on LBA plates and incubated overnight at 35° C. Serial dilutions of the cultures used to inoculate the microtiter plate wells were plated in order to determine the number cells in each well. Colonies on the agar plates were counted and the number of colony forming units (CFU) was determined. This was performed to quantify the number of cells inoculated.

Statistics

In order to assess statistic significance, percent deviation and analysis of variation (ANOVA) were conducted on the results of each of the biofilm assays. ANOVA was performed using the Microsoft Data Analysis Toolpak.

RESULTS

Identification and Characterization of P. aeruginosa 27853 and S. maltophilia X26332

The non-enteric API 20 identification system verified the identity of *P. aeruginosa* and *S. maltophilia* (Figure 2). Growth of the two bacteria on Blood Agar at 37°C revealed that both *S. maltophilia* and *P. aeruginosa* are gamma-hemolytic isolates (Figure 3 A & C).

LBA plates illustrate the morphology of both organisms. *S. maltophilia* has tan, wet colonies while *P. aeruginosa* has colonies that are also tan and appear to diffuse outwards (Figure 3 B & D).

Figure 2: API strip of (A) *S. maltophilia* and (B) *P. aeruginosa*

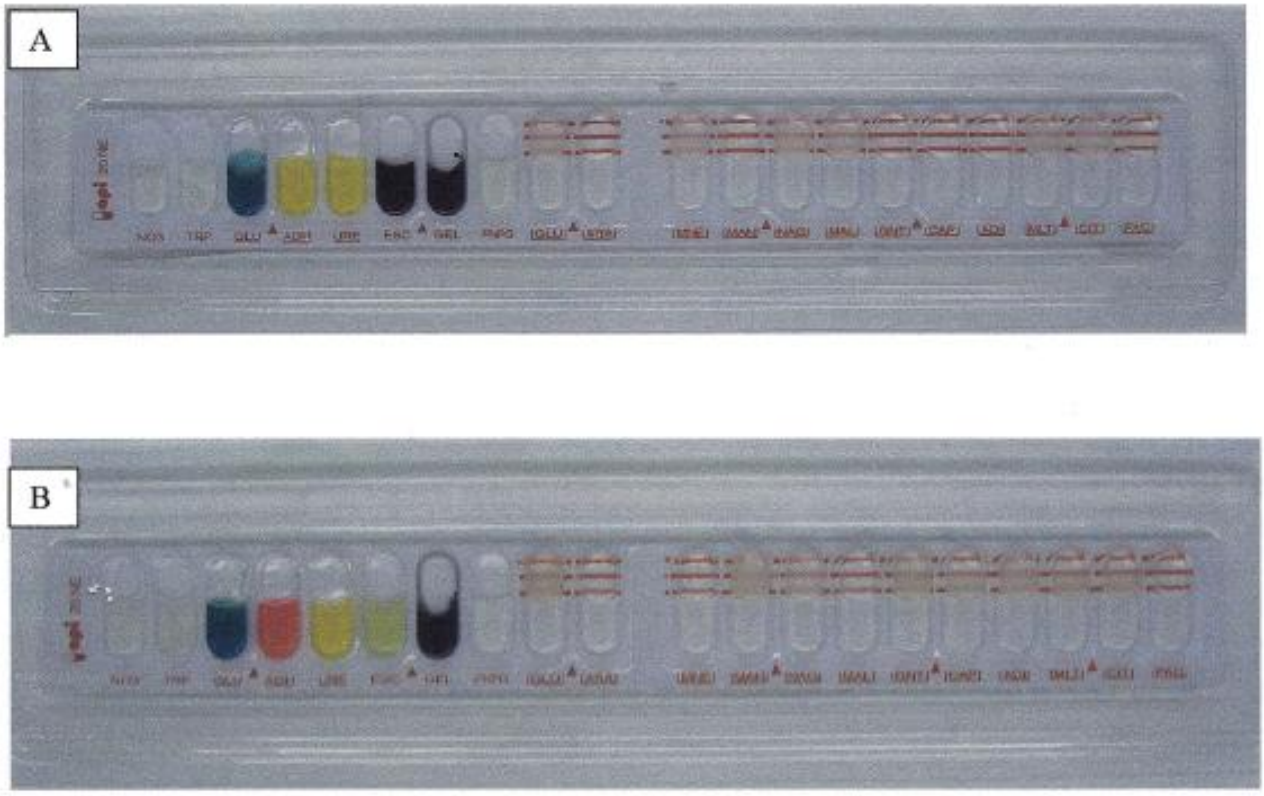


Figure 3: Blood Agar and Luria-Bertani Agar plates showing the colony growth of *S. maltophilia* and *P. aeruginosa*

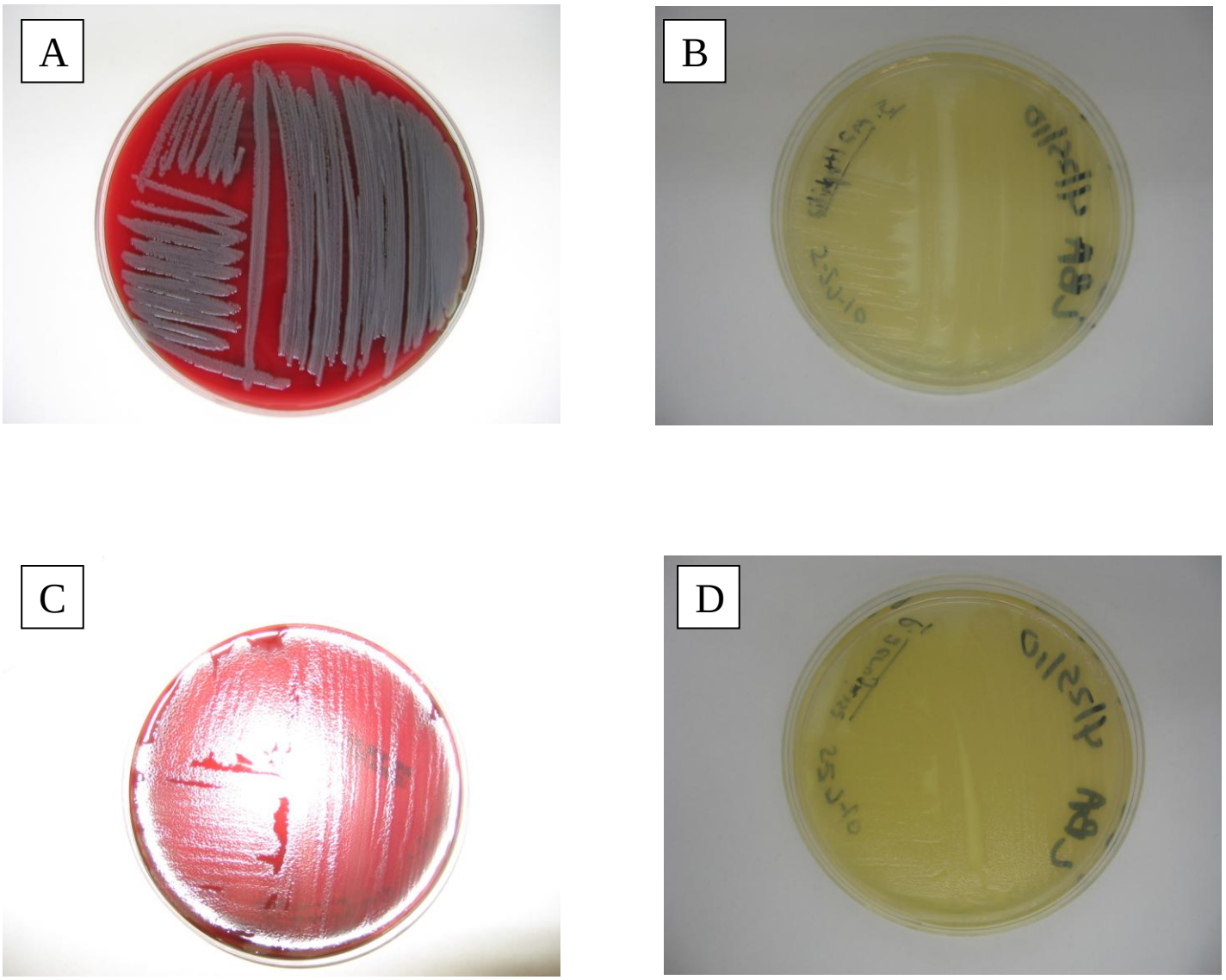


Figure 3 A shows *S. maltophilia* gamma-hemolytic colony growth on Blood Agar; Figure 3 B shows *S. maltophilia* colony growth on LBA; Figure 3 C shows *P. aeruginosa* gamma-hemolytic colony growth on Blood Agar; Figure 3 D shows *P. aeruginosa* growth on LBA. Note the diffuse colony growth of *P. aeruginosa* when compared to *S. maltophilia*.

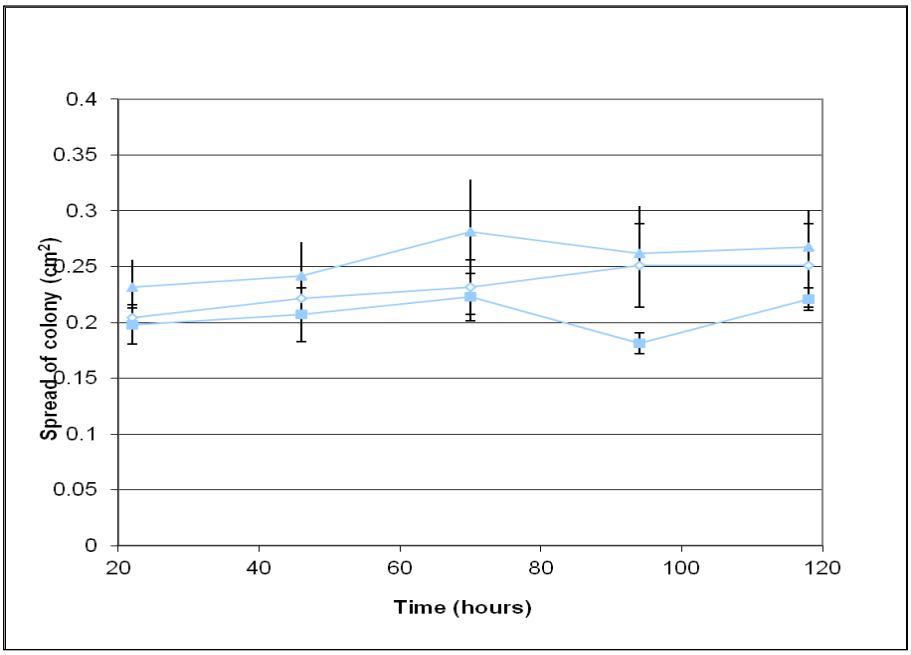
Effect of Iron and Temperature on the Growth of S. maltophilia X26332

Observations of three independent cultures of *S. maltophilia* showed that the concentration of iron and temperature influenced the colony growth (Figure 4). All cultures grown at either 30°C or 35°C displayed growth. The cultures grown on LB and 0.4% agar with a concentration of 100 μM $FeCl_3$ demonstrated the same diameter of growth spreading when compared to the cultures grown in LB with 0.4% agar and with no supplemented $FeCl_3$. The 8 μM iron plates showed a slight decrease in growth when compared to the control plates of no iron supplementation. The cultures incubated at 4°C and 14°C had little or no growth. The growth of each colony at 30°C and 35°C was similar.

The appearance of the bacterial colony growth can be seen on the following page in Figure 5. In the 30°C and 35°C plates, which had the most growth, the colonies radiated in a circular shape outwards.

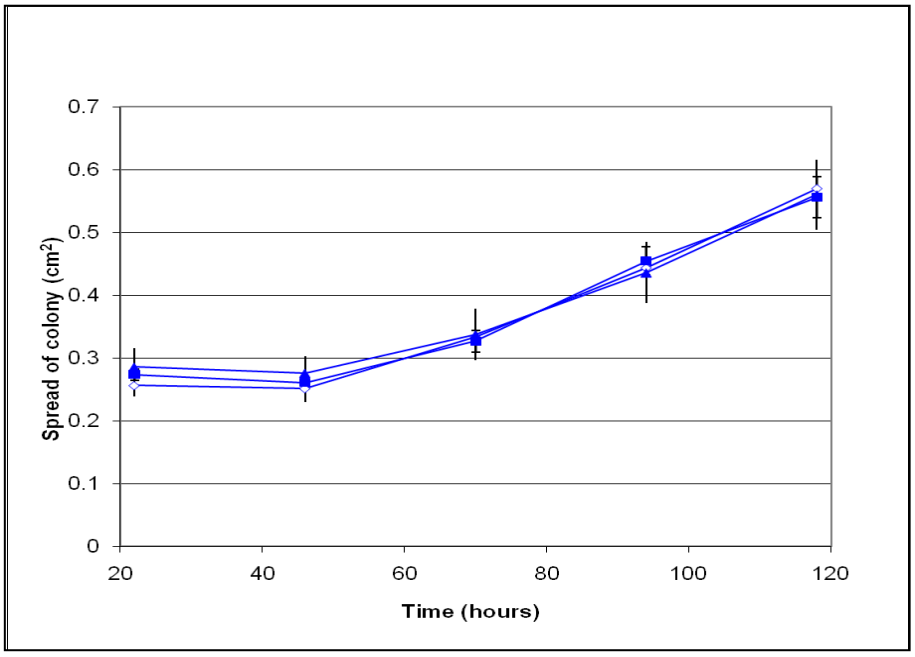
Each graph in Figure 4 represents cultures of *S. maltophilia* (1.03×10^5 cells) that were point inoculated into the center of LB containing 0.4% agar plate supplemented with ferric chloride and incubated at select temperatures. The colony growth was determined by measuring the diameter using a ruler at 22, 46, 70, 94 and 118 hours. The data shown are a representative average of experiments done 5 times. Vertical bars represent standard error.

Figure 4A: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 4°C



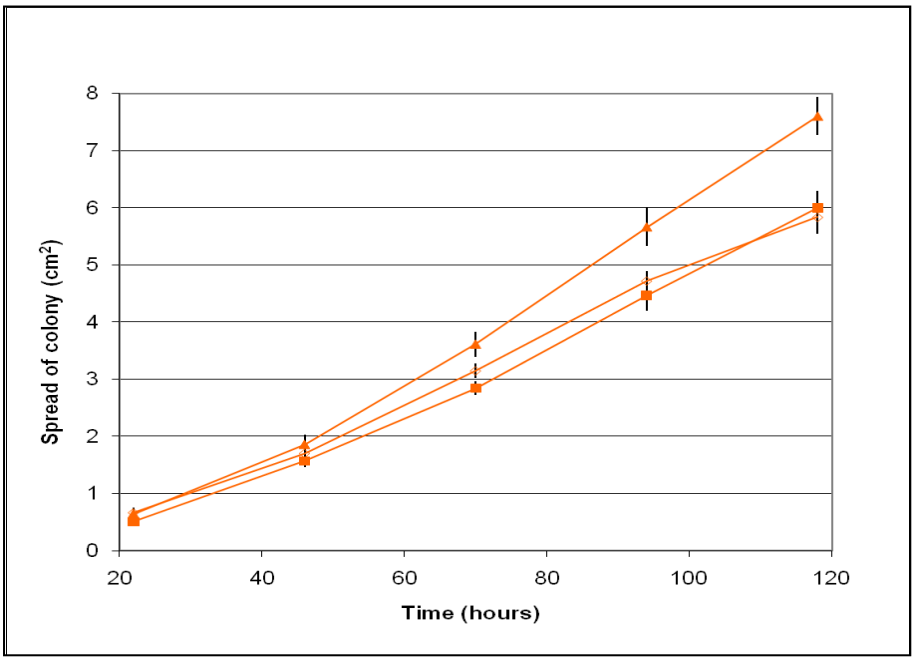
In the figure above, lines represent *S. maltophilia* colony growth at 4°C over 120 hours at various iron concentrations: 0 µM $FeCl_3$ (○),8 µM $FeCl_3$(■), 100 µM $FeCl_3$(▲)

Figure 4B: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 14°C



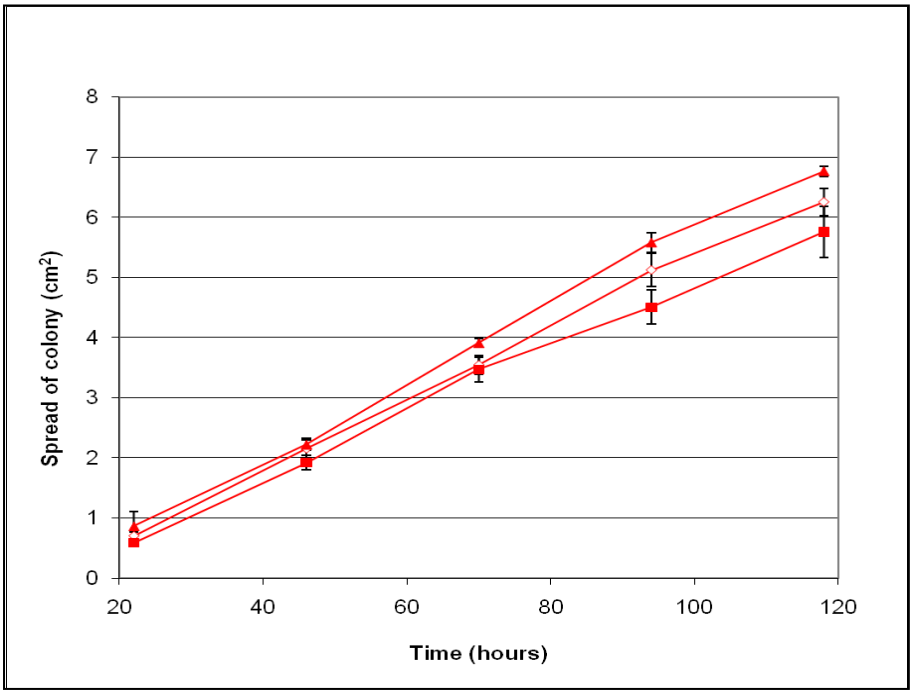
In the figure above, lines represent *S. maltophilia* colony growth at 14°C over 120 hours at various iron concentrations: 0 µM $FeCl_3$ (○),8 µM $FeCl_3$(■), 100 µM $FeCl_3$(▲)

Figure 4C: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 30°C



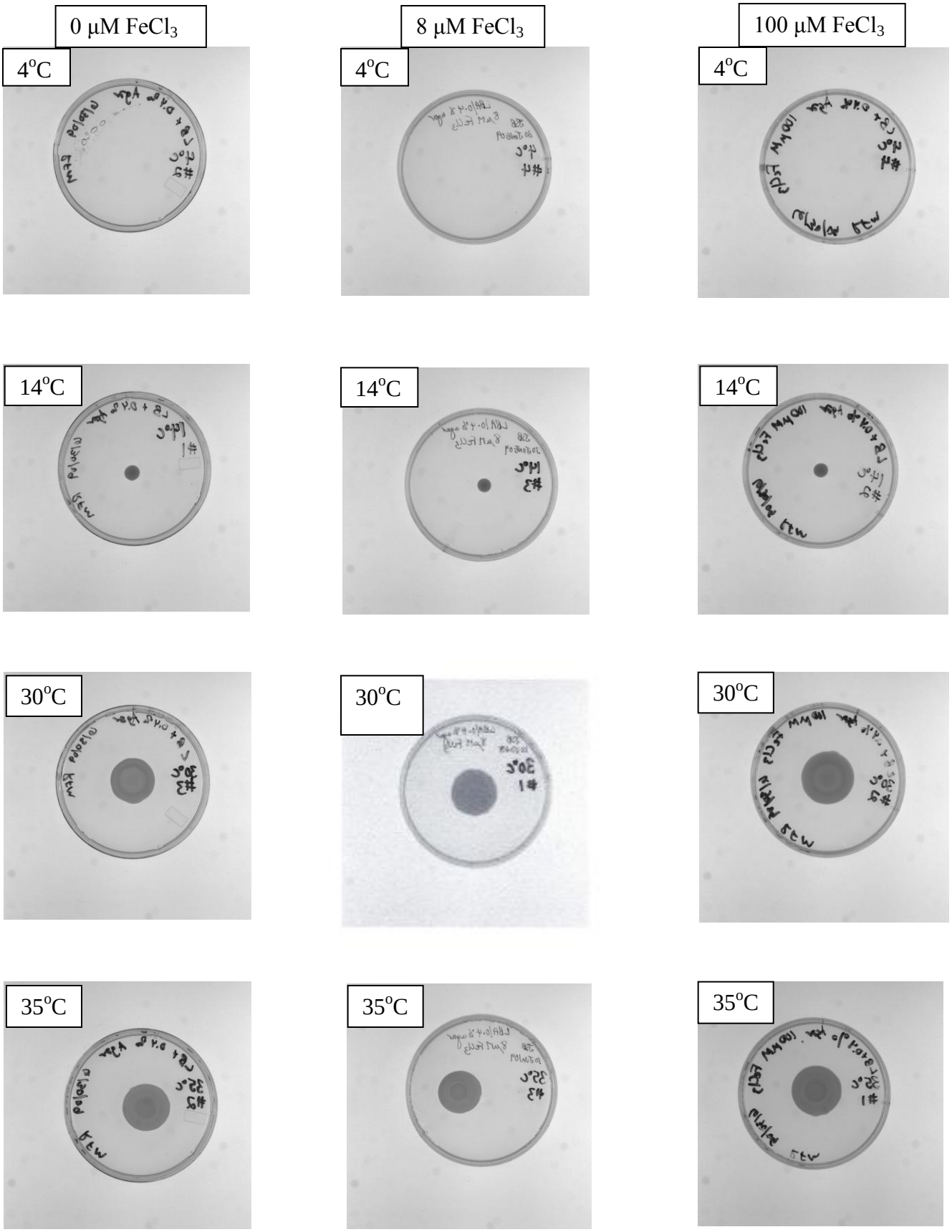
In the figure above, lines represent *S. maltophilia* colony growth at 30°C over 120 hours at various iron concentrations: 0 µM $FeCl_3$ (○),8 µM $FeCl_3$(■), 100 µM $FeCl_3$(▲)

Figure 4D: Effect of Ferric Chloride on *S. maltophilia* X26332 Colony Growth at 35°C



In the figure above, lines represent *S. maltophilia* colony growth at 35°C over 120 hours at various iron concentrations: 0 µM $FeCl_3$ (○),8 µM $FeCl_3$(■), 100 µM $FeCl_3$(▲)

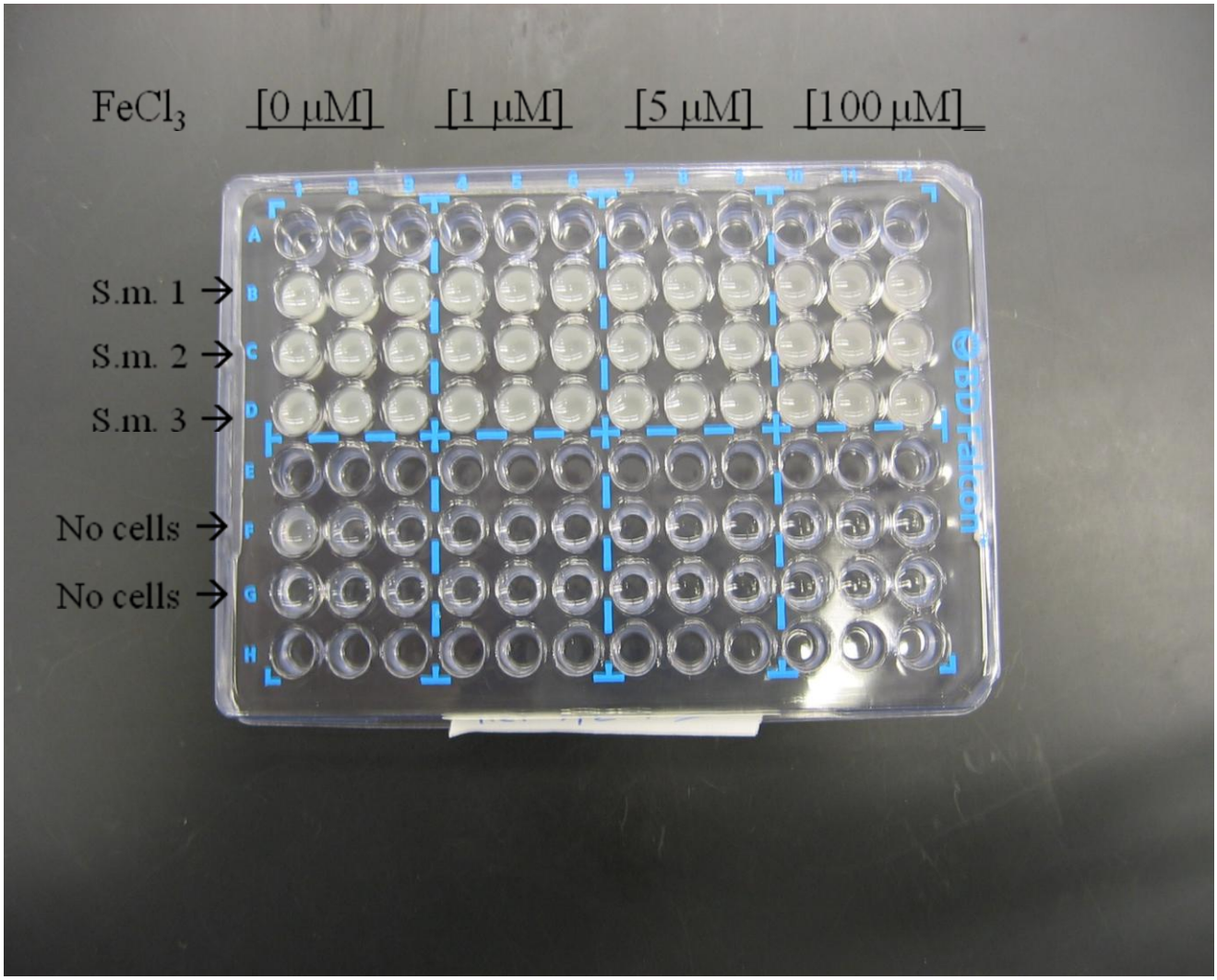
Figure 5: Plates showing diameter of growth for each temperature and iron concentration



Effect of Ferric Chloride on P. aeruginosa ATCC 27853 and S. maltophilia X26332 Culture Growth in PVC Microtiter Plates

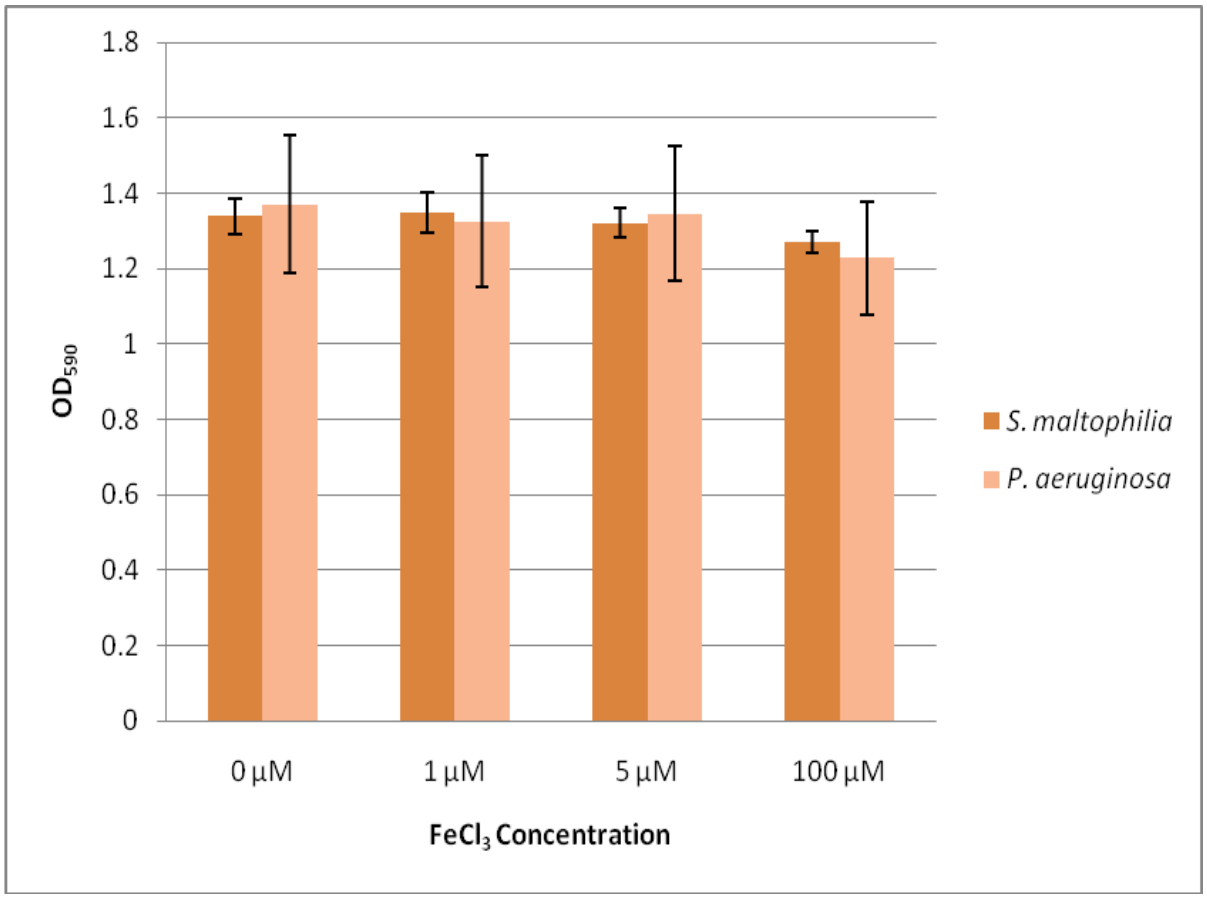
For the biofilm assays, cultures were grown at 35°C in PVC microtiter plate wells containing M9 minimal salts medium supplemented with ferric chloride at the final concentrations of 0, 1, 5 and 100 μM. At 24 hour incubation, growth of the *S. maltophilia* cultures maintained consistent across the wells as the concentration of iron chloride increased (Figure 6). ANOVA analysis verified that the culture growth for *S. maltophilia* was statistically insignificant. A slight decrease in *S. maltophilia* culture growth in M9 containing 100 μM $FeCl_3$ was observed, but it was not statistically significant. The *P. aeruginosa* culture plate at 24 hours had an orange/red hue to it when compared to the *S. maltophilia* culture. The *S. maltophilia* culture had a light tan color. This difference between the *P. aeruginosa* and *S. maltophilia* cultures became more noticeable with increasing levels of iron (Figure 7).

Figure 6: PVC Microtiter plate showing 24 hour culture growth of *S. maltophilia* in M9 medium supplemented with various concentrations of ferric chloride



Lanes B, C and D contain independent cultures of *S. maltophilia*. Lanes F and G contain medium and no cells. The following columns contain $FeCl_3$ at final concentrations: 1-3 = 0 µM, 4-6 = 1 µM, 7-9 = 5 µM, and 10-12 = 100 µM

Figure 7: Effect of ferric chloride on *S. maltophilia* X26332 and *P. aeruginosa* ATCC 27853 culture growth in iron supplemented M9 medium.

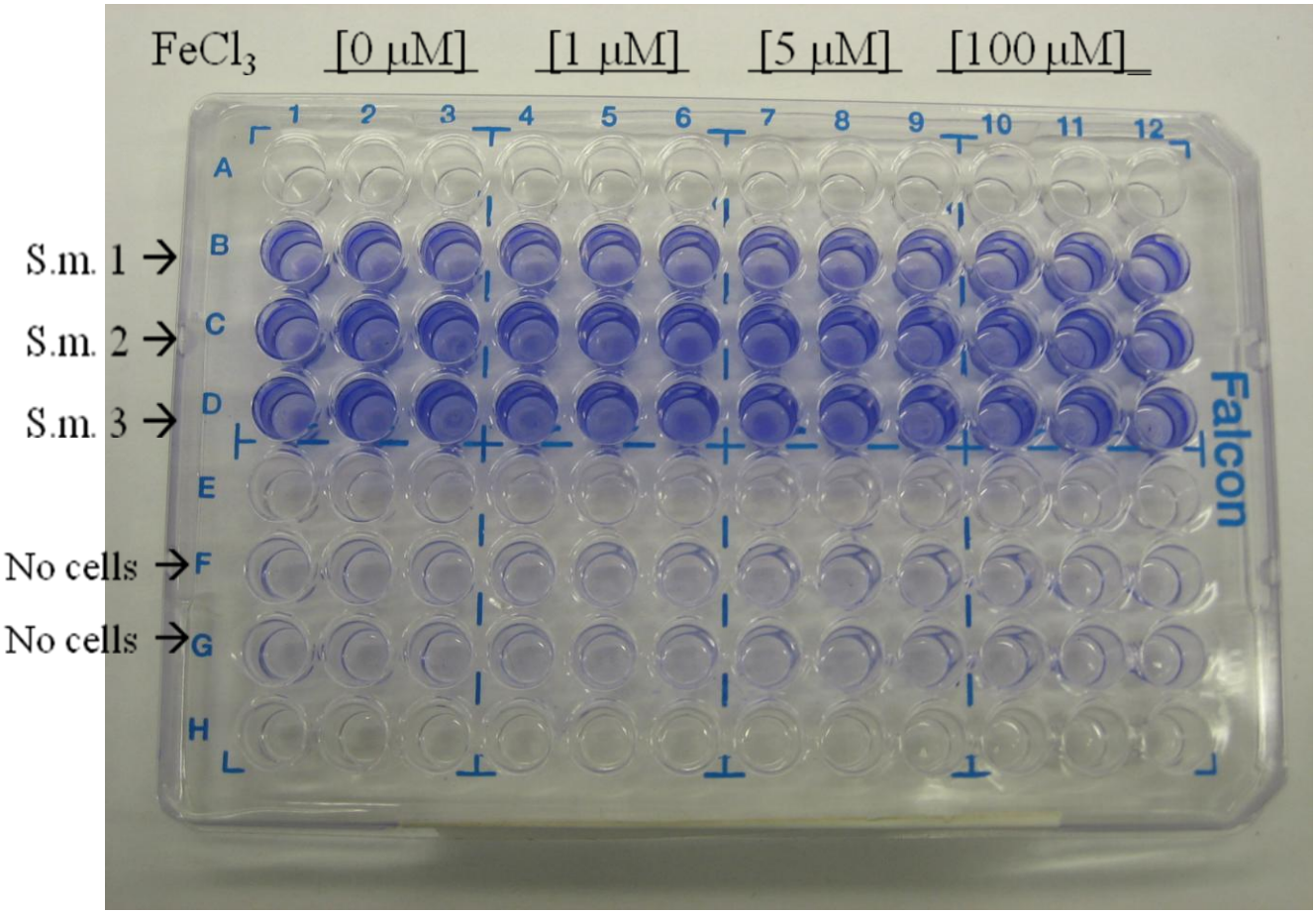


Cultures of *S. maltophilia* (5.87×10^4 cells) and *P. aeruginosa* (2.35×10^4 cells) were inoculated into PVC microtiter plate wells with ferric chloride concentrations as noted above, sealed with Parafilm and incubated at 35°C for 24 hours. The optical density for each well was recorded at 590 nm. The experiement for *S. maltophilia* was performed with 4 independent trials, each time in triplicate. The *P. aeruginosa* experiement was performed twice, each time in triplicate. The vertical bars represent ± standard error. ANOVA analysis verified that there is no significant difference in growth of *S. maltophilia* cultures incubated with different concentrations of $FeCl_3$. *P* value = 0.52 for *S. maltophilia* and $P<0.05$ for *P. aeruginosa*. This indicates that there is a significant difference in growth for *P. aeruginosa*.

Effect of Ferric Chloride on S. maltophilia X26332 Biofilm Development in PVC Microtiter Plates

Visual observations of the microtiter wells at 24 hour incubation revealed that both *P. aeruginosa* ATCC 27583 and *S. maltophilia* X26332 formed biofilms at all of the culture conditions tested – M9 minimal salts medium supplemented with various concentrations of ferric chloride (Figure 8A). One strip was isolated from the microtiter plate showing the effect of ferric chloride on *S. maltophilia* (Figure 8B). Recorded measurements of the *S. maltophilia* X26332 biofilms showed that the absorbance values at OD_{562} were insignificantly different for the different concentrations of $FeCl_3$ (Figure 9). However, the biofilms formed by *P. aeruginosa* were too viscous and optically dense to quantify using the procedure described in the materials and methods.

Figure 8A: PVC Microtiter plate showing *S. maltophilia* biofilms stained with 1% crystal violet.



Lanes B, C and D contain independent cultures of *S. maltophilia*. Lanes F and G contain medium and no cells. Columns 1-12 contain $FeCl_3$ at the following final concentrations: 1-3 = 0 µM, 4-6 = 1 µM, 7-9 = 5 µM, and 10-12 = 100 µM

Figure 8B: A side view displaying one strip of a 96-well microtiter plate showing *S. maltophilia* biofilms formed in the presence of $FeCl_3$

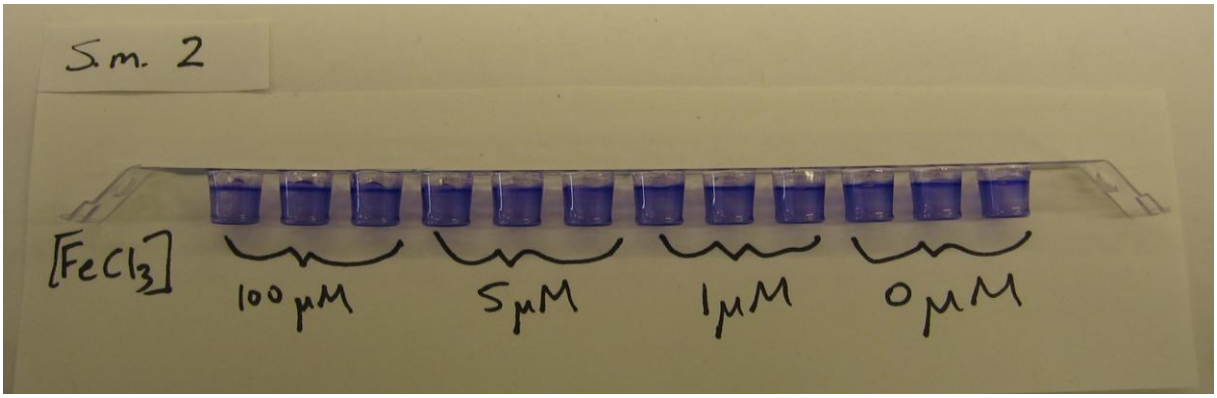
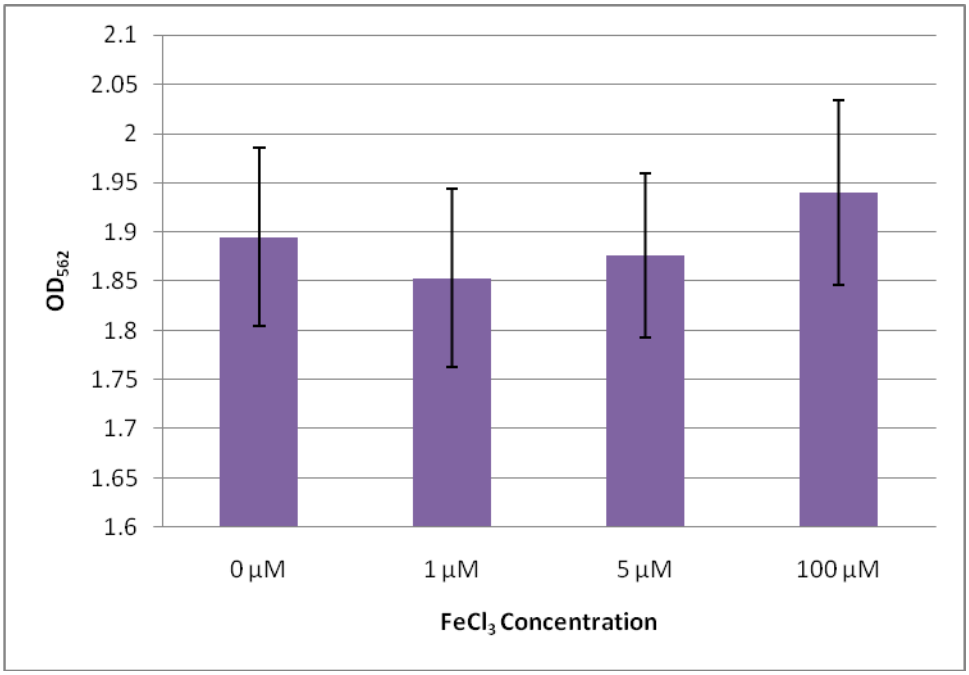


Figure 9: Effect of ferric chloride on *S.* maltophilia biofilm development in M9 medium.



Cultures of *S. maltophilia* ($5x10^4$ cells) were inoculated into PVC microtiter plate wells with ferric chloride concentrations as noted, sealed with Parafilm and incubated at 35°C for 24 hours. Non-adhered cells were removed and the remaining adhered cells were stained with 1% crystal violet (w/v) and washed three times with PBS. Wells were allowed to air dry overnight. Wells were resuspended with 80% ethanol as described in Methods and Materials and OD_{562} readings were recorded. Graphs represent data from 4 trials, each performed in triplicate. Vertical bars represent ± standard error. ANOVA analysis verified no significant difference in biofilms between the different concentrations of ferric chloride. *P* value = 0.917

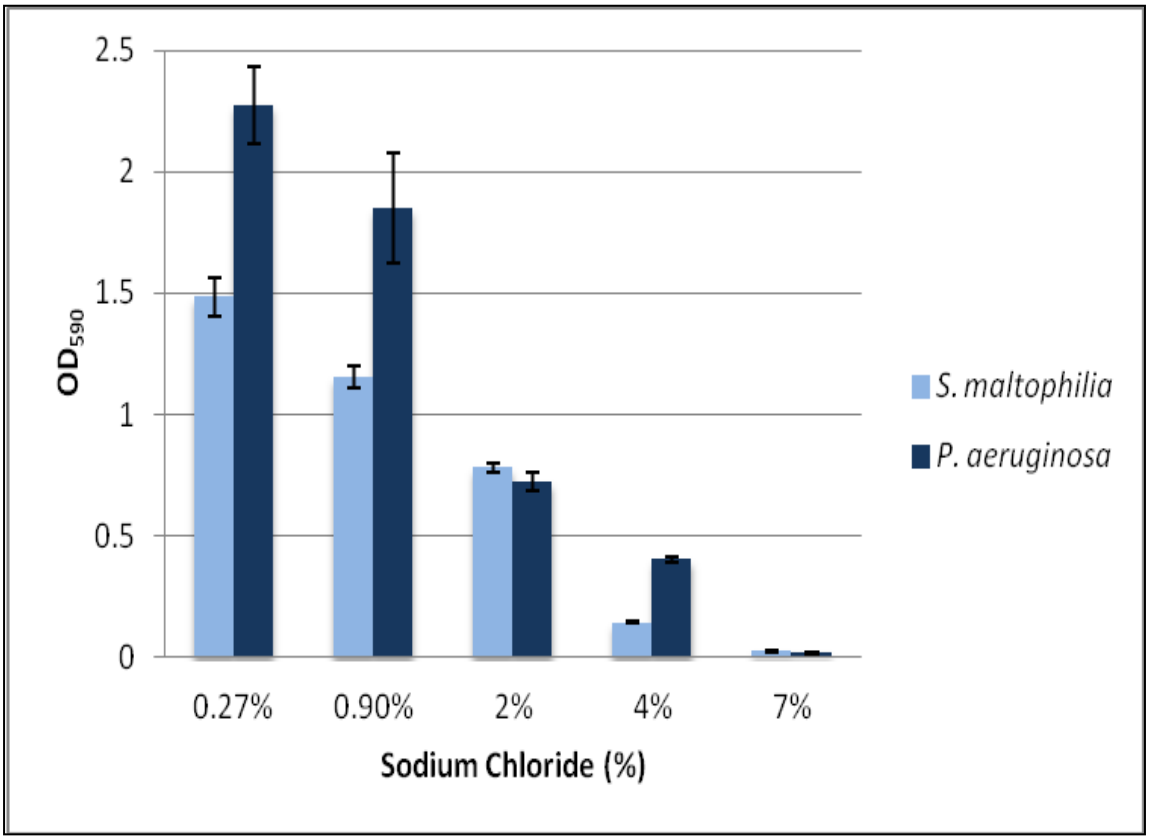
Effect of Sodium Chloride on P. aeruginosa ATCC 27853 and S. maltophilia X26332 Culture Growth in PVC Microtiter Plates

For the biofilm assays, *S. maltophilia* and *P. aeruginosa* cultures were grown stationary at 35°C in M9 minimal salts medium containing NaCl at final concentrations of 0.27%, 0.9%, 2%, 4% or 7%. As the media increased in salinity, a marked decline of culture growth after 24 hours was observed for both *S. maltophilia* and *P. aeruginosa* (Figure 10). The cultures grown in the presence of 4% and 7% NaCl had the lowest turbidity readings at $OD_{590\ nm}$ in comparison to the turbidity of the cultures grown in M9 containing 0.27% and 0.9% NaCl. The culture at 4% NaCl had growth at approximately 10% of 0.27% NaCl and the cultures at 7% NaCl had almost no observable growth.

Effect of Sodium Chloride on S. maltophilia X26332 Biofilm Development in PVC Microtiter Plates

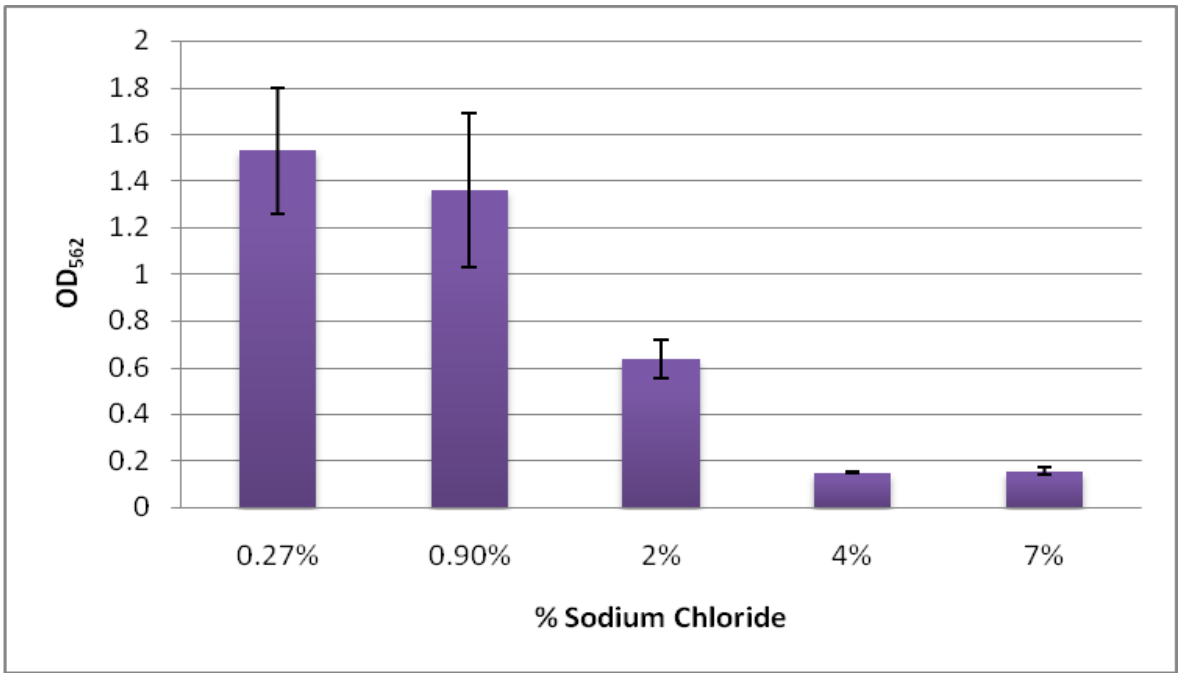
The trend in biofilm formation paralleled the trend in culture growth for both *P. aeruginosa* and *S. maltophilia*. The data of this experiment mirrored that of what was reported in that there is a decrease in growth of *P. aeruginosa* as NaCl concentration increased. As the NaCl concentration increased, the culture growth and biofilm formation of *S. maltophilia* was inhibited (Figure 11). The 24 hour readings at OD_{562} of *S. maltophilia* showed a marked decline for biofilms formed by cultures grown in Davis minimal salts medium with sodium chloride at final concentrations of 4% and 7%. OD_{562} readings of biofilms produced by *S. maltophilia* X26332 in the presence of medium containing 0.27% and 0.9% saline were similar. Biofilm formation of *S. maltophilia* X26332 was reduced in culture medium containing greater than 2% sodium chloride.

Figure 10. Effect of sodium chloride on culture growth of *S. maltophilia* X26332 and *P. aeruginosa* ATTC 27853 in PVC microtiter plates.



Cultures of *S. maltophilia* ($2.42x10^6$ cells) and *P. aeruginosa* ($3.12x10^6$ cells) were inoculated into PVC microtiter plate wells containing Davis medium supplemented with indicated sodium chloride concentrations, sealed with Parafilm and incubated at 35°C for 24 hours. The optical density $OD_{590\ nm}$ was recorded at 24 hours. The experiment was performed 2 independent times in triplicates. $P<0.05$ for both *S. maltophilia* and *P. aeruginosa*. The vertical bars represent ± standard error.

Figure 11. Effect of sodium chloride on *S. maltophilia* X26332 biofilm formation in PVC microtiter plate wells.



Cultures of *S. maltophilia* ($2.42x10^6$ cells) were inoculated into PVC microtiter plate wells with indicated sodium chloride concentrations, sealed with Parafilm and incubated at 35°C for 24 hours. Non-adhered cells were removed and the remaining adhered cells were stained with 1% crystal violet (w/v) and washed three times with PBS. Wells were allowed to air dry overnight. Wells were resuspended with 80% ethanol and optical density at 562 nm was recorded. The experiment was performed in 2 trials, each time in triplicate. Error bars represent ± standard error. $P<0.05$

DISCUSSION

The importance for new avenues in antimicrobial treatments is evident by the alarming increase of both nosocomial (hospital-based) and community-acquired infections. *P. aeruginosa* and *S. maltophilia* are two bacterial pathogens that have been reported to cause these nosocomial and community-acquired infections and in the process form biofilms which decrease treatment effectiveness and increase antimicrobial resistance (Costerton et al., 1999). The goal of biofilm eradiation or prevention in *P. aeruginosa* infections has become increasingly important because of the bacterium's antimicrobial-resistant properties and its high prevalence in the medical setting. As mentioned in the introduction, *P. aeruginosa* is especially problematic towards patients with cystic fibrosis (Tré-Hardy et al., 2009).

The production of biofilms proves to make antimicrobial treatment even more problematic, so new approaches in treatment are being investigated. This new approach of either preventing biofilm formation or decreasing the antimicrobial properties of biolfims is a novel one that will hopefully show promise in the medical setting. This is why our lab investigated the growing number of factors that influence *S. maltophilia* biofilm development. Our investigations will hopefully contribute to the growing understanding of biofilm formation and susceptibility. This is potentially insightful because new antibiotics are constantly being researched and produced to address the growing number of infections, especially those by antimicrobial resistant microorganisms. The idea is that if bacteria, like *S. maltophilia* or *P. aeruginosa*, somehow lose their ability to easily produce biofilms, then they could potentially not be as harmful to the patient. If the bacteria are able to remain alive, but simply be less virulent, then the organism would presumably not receive the selective pressure to gain resistance to an antibiotic.

This research thesis focused on both *P. aeruginosa* and *S. maltophilia* in order to directly compare these organisms and contribute to the growing body of research of applicable data. While it would be easier to control and isolate each individual species, a common problem in a real-world medical setting is that bacterial infections may include multiple bacterial species acting concurrently, and infected tissues may be colonized with several species of microorganisms. *P. aeruginosa* and *S. maltophilia* are shown to co-colonize within a tissue, such as the respiratory tract in cystic fibrosis (CF) patients (Denton and Kerr, 2002; DiBonaventura et al., 2007; Nazik et al., 2007). Much of the previous literature has focused on how iron and salt affect *P. aeruginosa* growth and biofilm development, and this thesis research has attempted to determine how these factors affect *S. maltophilia* growth and biofilm development. Several papers have suggest iron as a prospective treatment for *P. aeruginosa* biofilms, and by observing the effect of ferric chloride on *S. maltophilia* biofilm development one would be able to better determine its potential efficacy as a treatment for the biofilms of *S. maltophilia*.

Iron and Culture Growth

The first hypothesis tested in this study was that supplementation of the culture medium with ferric chloride would increase the growth of *S. maltophilia* when incubated at certain temperatures. This hypothesis was supported. The highest culture densities were observed at the highest concentration 100μM of ferric chloride and the higher temperatures of 30°C and 35°C. This experiment served as a basis as to whether we could control and see a difference in *S. maltophilia* X26332 growth with increasing ferric chloride concentrations. Several previous studies (Musk et al., 2005; Yang et al., 2007) showed that increasing iron concentrations,

especially in the form of ferric chloride, had an inversely proportional effect on *P. aeruginosa* ATCC 27853 growth.

However, interpretation of the data from this growth experiment must be treated with care. The LB medium used in the agar plates had an unquantifiable concentration of iron. One of the essential components of LB is soy extract and the exact concentration of iron could therefore not be controlled. After much search for a medium that that lacks iron, M9 minimal salts medium was discovered. Unfortunately, the M9 minimal salts medium came with its own complications as it did not initially appear to be conducive to culture growth of either *P. aeruginosa* or *S. maltophilia*. Eighteen hour overnight cultures proved to be difficult, and when attempted on a biofilm assay, did not even form measureable biofilms. The M9 minimal salts medium had to be supplemented with magnesium sulfate and calcium chloride as essential nutrients and glucose as a carbon source along with some essential amino acids, in the form of casein enzymatic hydrolysate or casamino acids to provide a suitable growth medium for both *S. maltophilia* and *P. aeruginosa*. The overnight growth of both *S. maltophilia* X26332 and *P. aeruginosa* ATCC 27853 in this new modified M9 medium was comparable to the growth of both of these organisms when cultured in LB broth.

Iron and S. maltophilia Biofilms

The second hypothesis in this study tested if the supplementation of the culture medium with ferric chloride would have an effect on the bacterial growth and biofilm formation of *S. maltophilia*. This hypothesis was not supported. Based on the research performed with *P. aeruginosa* PAO1 (Musk et al., 2005; Yang et al., 2007; Patriquin et al., 2008), it was expected

that increasing ferric chloride concentrations would have a significant effect on biofilm development of *S. maltophilia*.

Data obtained in this current study shows that iron chloride does not inhibit or increase biofilm formation by *S. maltophilia* X26332. At all ferric chloride concentrations tested in this thesis study, OD_{562} readings of biofilm were not significantly different and the OD_{590} readings for growth were not significantly different. The *P* value readings were 0.52 and 0.917, respectively. These results are in contrast to reports that ferric chloride inhibits *P. aeruginosa* biofilm development.

It has been reported that the physiological free iron concentration in the lower respiratory tract of a healthy human is 10^{-18} M, which is relatively low when compared to the free iron concentration of $6.3x10^{-5}$ M (or 63 µM) from the sputum of a CF patient (Berlutti et al., 2005). Berlutti et al. (2005) tested the effect of supplementation of the culture medium with various Fe^{3+} concentrations (1 µM and 10 µM) on *P. aeruginosa* adhesion and biofilm formation on polystyrene microtiter plates. They reported that 1 µM Fe^{3+} induced motility in *P. aeruginosa* and 10 µM Fe^{3+} stimulated biofilm formation. This information is particularly useful to this current study because it provides a quantifiable comparison of the iron concentrations used in this experiment when compared to the physiological iron concentration. The 100 µM $FeCl_3$ concentration used in the experiment in this thesis is higher than that found in the CF lung, so this further affirms the results of the past studies indicating that this concentration of iron could be effective in *P. aeruginosa* biofilm prevention. However, data from the experiments in this thesis indicate that treatments may not be effective in the prevention of *S. maltophilia* biofilm formation.

The information obtained from this specific experiment of the effect of ferric chloride on *S. maltophilia* biofilm development could be applicable in the clinical setting. Current research is targeting iron treatment in the prevention of *P. aeruginosa* biofilms in the CF lung. As this thesis data indicates, treatments with iron do not inhibit *S. maltophilia* growth and biofilm production. Therefore, treatments of *S. maltophilia* infections with iron will likely be ineffective. Treatment of polymicrobial infections with iron could be potentially problematic. In polymicrobial infections, if two naturally occurring organisms cohabitate an environment and are usually kept in check by competition and availability of resources, the inhibition of growth of one organism could impact the growth and development of the other.

Pompilio et al. (2010) showed that *P. aeruginosa* can directly influence the ability of *S. maltophilia* to adhere to a surface and grow. Their study isolated bronchial epithelial cells from a CF patient and tested the ability of colonization and biofilm development of *S. maltophilia* on these cells. It was reported that *S. maltophilia* cells do form biofilms on this epithelial cell surface. The study further showed that epithelial cells pre-colonized by *P. aeruginosa* and then subsequently removed actually caused the *S. maltophilia* cells to adhere more readily to the bronichial epithelial surface. This supports the idea that these two bacteria can have an influence on each other.

Since both *P. aeruginosa* and *S. maltophilia* are found in the lung, it is reasonable to conclude that a single treatment, such as ferric chloride, that affects one but not the other could cause other unforeseen health problems. *S. maltophilia* is an opportunistic pathogen, so the removal of *P. aeruginosa* biofilms could provide more nutrients and space for *S. maltophilia* biofilms to form. *S. maltophilia* infection and disease may then occur in the host. Drug treatments usually have more than one component, so there could be treatments that do affect

both *P. aeruginosa* and *S. maltophilia*. This thesis study just shows that, at least for iron, other avenues have to be addressed for treatments aiming to prevent *S. maltophilia* biofilm development.

Lastly, it was noted that the increasing levels of iron concentrations had an additional effect of producing a pigment in *P. aeruginosa* cultures in the microtiter wells. It has been reported that iron can cause *P. aeruginosa* to produce pigments (Palumbo, 1972). While production of this pigment is not further studied in this paper, it does confirm that 100 µM $FeCl_3$ wells in the microtiter plates did have an inherent difference when compared to the lower ferric chloride concentrations. The difference measured in this thesis was culture growth and subsequent biofilm development, but this qualitative difference in color suggests that other differences may be present.

Sodium Chloride and S. maltophilia Biofilms

The third hypothesis tested in this study proposed that increases in sodium chloride concentration would decrease the bacterial growth and biofilm formation of *S. maltophilia*. This hypothesis was supported by data obtained in this study. As the percentage of sodium chloride in the culture medium increased, a marked decrease in both growth and biofilm production was observed for cultures of *S. maltophilia* X26332. This finding is consistent with the results shown in the Havasi study on the inhibitory effect of hypertonic saline on *P. aeruginosa* motility and growth (Havasi, 2008).

In this thesis study, the same salt concentrations were tested for their effect on *P. aeruginosa* and *S. maltophilia* culture growth and biofilm development. The results of culture growth agree with the reported findings at the high salt concentrations (2%, 4% and 7%) with *P.*

aeruginosa (Havasi et al., 2008). In the biofilm assay, microtiter plate cultures were used to monitor growth and biofilm development of both organisms. These same higher salt concentrations hindered biofilm development in *S. maltophilia* with virtually no biofilms being observed at 4% and 7% salt concentrations. Biofilms were not observed in the negative control microtiter plate wells. The negative controls consisted of wells filled with medium and salt concentrations, but no cells. The data are promising because they suggest that the hypertonic saline aerosol treatment to prevent *P. aeruginosa* growth would have a similar effect on the growth and biofilm development of *S. maltophilia.*

This observed effect of sodium chloride on *S. maltophilia* biofilm formation is also promising because of the inherent antimicrobial effect of select concentrations of saline. Saline concentrations in tears for example have been shown to be an effective antimicrobial agent against organisms that could potentially infect the eyes (Cowell et al., 1999; Kwong et al., 2007). Kwong et al. (2007) tested the hypothesis of human tears as an effective antimicrobial agent against *P. aeruginosa*. In a translocation assay, tears from healthy human volunteers were able to protect corneal epithelial cells against colonization by *P. aeruginosa* (Kwong et al., 2007).

This saline treatment approach is also applicable to the idea of using saline aerosols as an antimicrobial treatment. While potentially not harmful to the host, increased salt concentrations could have a direct effect on diminishing infections caused by microbial growth and biofilm production of *P. aeruginosa* or *S. maltophilia*.

Pseudomonas aeruginosa and Stenotrophomonas maltophilia Biofilms

The role of iron in the development of *P. aeruginosa* biofilm is a current topic that is being studied intensely. As stated previously, the need for alternative antibiotic treatments is of

utmost importance because of the increasing incidences of emerging antimicrobial-resistant microorganisms that include *P. aeruginosa* and *S. maltophilia*. This rise in incidence is one of the motivations at looking at the role of iron and the biofilm-producing abilities of these microorganisms. Iron is shown to be required in signaling *P. aeruginosa* biofilm development (Banin et al., 2005). Studies have shown that iron is a significant factor in the cystic fibrosis lung (Lamont and Konnings, 2009; Reid et al., 2009). These reports demonstrate that the role of iron acquisition, while vital to the cellular function of *P. aeruginosa*, is still not well characterized. Lamont and Konnings suggest that *P. aeruginosa* may secrete siderophores which are iron-scavenging molecules with high affinities for Fe^{3+} ions. They are shown to be made by pathogenic bacteria and are required for normal infection (Lamont and Konnings, 2009). *P. aeruginosa* is known to produce two siderophores, pyoverdines and pyochelins (Lamont and Konnings, 2009). Pyoverdine-mediated iron transport appears to be essential for the production of biofilms by *P. aeruginosa* (Lamont and Konnings, 2009).

Musk and Hergenrother (2008) demonstrated that chelated iron sources, like ferric chloride, inhibited *P. aeruginosa* biofilm development *in vitro*. They were able to show that high levels of iron salts, including 50μM ferric chloride, sufficiently decreased biofilm production in a microtiter plate (Musk and Hergenrother, 2008). Use of an iron chelator implies that all of the free, available iron will be taken up and not readily available for cells to use. This experiment provides additional evidence of the crucial role of iron in *P. aeruginosa* growth and biofilm development. This study determined that iron had no effect on *S. maltophilia* biofilm development, and a search of the Pubmed database has not revealed any reports of siderophores produced by *S. maltophilia*.

One of the problems experienced in this current thesis study was that a reliable, quantifiable reading for *P. aeruginosa* biofilm was not obtained. This was due to the type of growth and biofilm development that the organism demonstrated in the PVC microtiter plate. It would have been advantageous to mirror the previously reported experiments as our positive control, but unfortunately, other than taking pictures and taking visual observations, the exact degree of biofilm development could not be ascertained.

Biofilm formation has been shown to be dependent on the type of bacterial strain used in studies. This current thesis study used *P. aeruginosa* ATCC 27533. Patriquin et al. (2007) and Yang et al. (2007) both used *P. aeruginosa* strain PAO1. Musk et al. (2005) and Musk and Hergenrother (2008) used *P. aeruginosa* strain PA14. Musk et al. (2005) partially addressed this issue of bacterial strain specific biofilms by testing the biofilm formation of other *P. aeruginosa* strains. Twenty different *P. aeruginosa* strains taken directly from the sputum of CF patients were used to test biofilm development in the presence of iron. The clinical isolates used in the Musk et al. (2005) study showed a wide range of results of the effect of iron on adhered bacterial cells. Some strains appeared to be unaffected by the presence of increased iron, while many were affected and displayed little or no biofilm. It is important to note that this study used ferric ammonium citrate, which could have a different effect than ferric chloride.

The issue of strain specificity and biofilm formation could also be applied to *S. maltophilia*. The strain used in this current study was X26322, a blood isolate from a patient. This strain could behave differently compared to a *S. maltophilia* strain isolated from somewhere else in the body or an environmental strain of *S. maltophilia*. Community-based infections of *S. maltophilia* are increasing in incidence (Falagas et al., 2009), so it would be important to test whether environmental strains would behave the same or differently than clinical strains.

Future Directions

One of the most logical next steps to the experiments presented here would be to see the effect of ferric chloride and sodium chloride on *S. maltophilia* biofilm development *in vivo*. Pompilio et al. (2010) proposed that biofilms produced on abiotic surfaces might not compare with how these bacterial cells act *in vivo*. Reid et al. (2009) also suggests that mechanisms of iron acquisition could be much different *in vivo* compared to *in vitro* models.

This thesis study helps show that the factors, $FeCl_3$ and NaCl, affect biofilm development in *S. maltophilia*. The fact that $FeCl_3$ does not inhibit or increase the biofilm formation of *S. maltophilia* is important for researchers attempting to create new antimicrobial treatments for patients at risk of *S. maltophilia* infections. As previously mentioned, a new series of treatments is currently being used to treat the biofilm-forming ability of bacteria like *S. maltophilia*. This study shows that alternative methods need to be approached, like NaCl, treatment, instead of using $FeCl_3$, to specifically target *S. maltophilia*.

A myriad of factors remain to be tested that influence biofilm development by *S. maltophilia*. Studies have suggested that various other metals and salts can affect *P. aeruginosa* growth and biofilm development. It is important to study other co-localized microorganisms that reside with *P. aeruginosa* and *S. maltophilia* that are also opportunistic and could prove to be problematic for infections, especially in the immunocompromised patient population that is afflicted most by *S. maltophilia*.

LITERATURE CITED

Aslam S. 2008. Effect of antibacterials on biofilms. American Journal of Infection Control 36: 9-11.

Atlas RM, Bartha R. 1998. Microbial Ecology Fundamentals and Applications: Fourth Edition. Menlo Park, California: Addison Wesley Longman.

Ballmann M, von der Hardt H. 2002. Hypertonic saline and recombinant human DNase: a randomized cross-over pilot study in patients with cystic fibrosis. Journal of Cystic Fibrosis 1:35-37.

Banin E, Vasil ML, Greenberg EP. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proceedings of the National Academy of Sciences 102(31): 11076-11081.

Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. Journal of Bacteriology 188(21): 7344-7353.

Berlutti F, Morea C, Battiston A, Sarli S, Cipriani P, Superti F, Ammendolia MG, Valenti P. 2005. Iron availability influences aggregation, biofilm, adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. International Journal of Immunopathology and Pharmacology 18(4): 661-670.

Brooke JS, Vo A, Watts P, Davis N. 2007. Mutation of a lipopolysaccharide synthesis gene results in increased biofilm of *Stenotrophomonas maltophilia* on plastic and glass surfaces. Annals of Microbiology 58: 35-40.

Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ. 2008. The biology and future prospects of antivirulence therapies. Nature Reviews Microbiology 6: 17-27.

Costerton JW, Stewart P, Greenberg EP. 1999. Bacterial biofilms: A common cause of persistent infections. Science 284: 1318-1323.

Coutinho HD, Falcao-Silva V, Goncalves G. 2008. Pulmonary bacterial pathogens in cystic fibrosis patients and antibiotic therapy: a tool for the health workers. International Archives of Medicine 24:1-7.

Cowell BA, Willcox MDP, Herbert B, Schneider RP. 1999. Effect on nutrient limitation on adhesion characteristics of *Pseudomonas aeruginosa*. Journal of Applied Microbiology 86: 944-954.

Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB. 2008. The complete genome, comparative and functional analysis of *Stentrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biology 9: R74.1-R74.13.

Denton M, Kerr KG. 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clinical Microbiology Reviews 11(1):57-80.

DiBonaventura G, Stepanović S, Picciani C, Pompilio A, Piccolomini R. 2007. Effect of environmental factors on biofilm formation by clinical *Stenotrophomonas maltophilia* isolates. Folia Microbiologica 52(1): 86-90.

Falagas ME, Kastoris AC, Vouloumanou EK, Dimopoulos G. 2009. Community-acquired *Stenotrophomonas maltophilia* infections: a systematic review. European Journal Clinical Microbiology Infectious Diseases 28:719-730.

Gould I. 2008. The epidemiology of antibiotic resistance. International Journal of Antimicrobial Agents 32: S2-S9.

Havasi V, Hurst CO, Briles TC, Yang F, Bains DG, Hassett DJ, Sorscher E. 2008. Inhibitory effects of hypertonic saline on *P. aeruginosa* motility. Journal of Cystic Fibrosis 7: 267-269.

Horswill A, Stoodley P, Stewart PS, Parsek MR. 2006. The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. Analytical and Bioanalytical Chemistry 387: 371-380.

Huang TP, Somers EB, Lee Wong AC. 2006. Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in *Stenotrophomonas maltophilia*. Journal of Bacteriology 188(6): 3116-3120.

Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infectious Diseases 6: 130.

Kwong MSF, Evans DJ, Ni M, Cowell BA, Fleiszig SMJ. 2007. Human tear fluid protects against *Pseudomonas aeruginosa* keratitis in a murine experimental model. Infection and Immunity 75(5): 2325-2332.

Lamont IL, Konings AF. 2009. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. Biometals 22:53-60.

Looney WJ, Narita M, Mühlemann K. 2009. *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. The Lancet Infectious Diseases 9:312-23.

Macfarlane S, Dillon J. 2007. Microbial biofilms in the human gastrointestinal tract. Journal of Applied Microbiology 102: 1187-1196.

Millar FA, Simmons NJ, Hodson ME. 2009. Trends in pathogens colonizing the respiratory tract of adult patients with cystic fibrosis, 1985-2005. Journal of Cystic Fibrosis 8: 386-391.

Musk DJ, Banko DA, Hergenrother PJ. 2005. Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. Chemistry and Biology 12: 789-796.

Musk DJ, Hergenrother PJ. 2008. Chelated iron sources are inhibitors of *Pseudomonas aeruginosa* biofilms and distribute efficiently in an in vitro model of drug delivery to the human lung. Journal of Applied Microbiology 105: 380-388.

Nazik H, Öngen B, Erturan Z, Şalcıoğlu M. 2007. Genotype and antibiotic susceptibility patterns of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* isolated from cystic fibrosis patients. Japanese Journal of Infectious Diseases 60: 82-86.

Nicodemo AC, Garcia Paez JI. 2007. Antimicrobial therapy for *Stentrophomonas maltophilia* infections. European Journal of Clinical Microbiology and Infectious Diseases 26: 229-237.

Patriquin GM, Banin E, Gilmour C, Tuchman R, Greenberg EP, Poole K. 2007. Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. Journal of Bacteriology 190(2): 662-671.

Pompilio A, Crocetta V, Confalone P, Nicoletti M, Petrucca A, Guarnieri S, Fiscarelli E, Savini V, Piccolomini R, DiBonaventura GD. 2010. Adhesion to and biofilm formation on IB3-1 bronchial cells by *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. BMC Microbiology 10:102.

Raad I, Reitzel R, Jiang Y, Chemaly RF, Dvorak T, Hachem R. 2008. Anti-adherence activity and antimicrobial durability of anti-infective-coated catheters against multidrug-resistant bacteria. Journal of Antimicrobial Chemotherapy 62: 746-50.

Rahmati-Bahram A, Magee JT, Jackson SK. 1997. Effect of temperature on aminoglycoside binding sites in *Stentrophomonas maltophilia*. Journal of Antimicrobial Chemotherapy 39: 19-24.

Reid DW, Anderson GJ, Lamont IL. 2008. Cystic fibrosis: ironing out the problem of infection? American Journal of Physiology – Lung Cellular and Molecular Physiology 295: 23-24.

Reid DW, Anderson GJ, Lamont IL. 2009. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. American Journal of Physiology – Lung Cellular and Molecular Physiology 297:795-802.

Reid DW, O'May C, Roddam LF, Lamont IL. 2009. Chelated iron as an anti-*Pseudomonas aeruginosa* biofilm therapeutic strategy. Journal of Applied Microbiology 106: 1058.

Safdar A, Rolston KV. 2007. *Stenotrophomonas maltophilia*: changing spectrum of a serious bacterial pathogen in patients with cancer. Immunocompromised Hosts 45: 1602-1609.

Schaumann R, Stein K, Eckhardt C, Ackermann G, Rodloff AC. 2001. Infections Caused by *Stenotrophomonas maltophilia* – A Prospective Study. Infection 29: 205-208.

Spasenovski T, Carroll MP, Lilley AK, Payne MS, Bruce KD. 2010. Modelling the bacterial communities associated with cystic fibrosis lung infections. European Journal of Clinical Microbiology and Infectious Diseases 29: 319-328.

Stewart P, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilm. The Lancet 358: 135-138.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. Annual Review of Microbiology 56: 187-209.

Tortura GJ, Funke BR, Case CL. 2004. Microbiology: An Introduction – Eighth Edition. San Francisco, CA: Pearson Education, Inc., publishing as Benjamin Cummings.

Tré-Hardy M, Vanderbist F, Traore H, Devleeschouwer MJ. 2008. In vitro activity of antibiotic combinations against *Pseudomonas aeruginosa* biofilm and planktonic cultures. International Journal of Antimicrobial Agents 31: 329-336.

Tré-Hardy M, Macé C, El Manssouri M, Vanderbist F, Traore H, Devleeschouwer MJ. 2009. Effect of antibiotic co-administration on young and mature biofilms of cystic fibrosis clinical isolates: the importance of the biofilm model. International Journal of Antimicrobial Agents 33: 40-45.

Watnick PI, Kolter R. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Molecular Microbiology 34(3): 586-595.

Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick R. 2008. Molecular Biology of the Gene – Sixth Edition. San Francisco, CA: Pearson Education, Inc., publishing as Benjamin Cummings.

Yang L, Barken KB, Skindersoe ME, Christensen AB, Givskov M, Tolker-Nielsen T. 2007. Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. Microbiology 153: 1381-1328.