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Effect of Viscosity on Biofilm Phenotypic Expression

by

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ABSTRACT

Biofilms are adherent colonies of microorganisms growing within a polysaccharide cell surface glycocalyx. Antibiotics and biocides used at concentrations capable of killing planktonic organisms have little effect against biofilms. Bacterial and fungal growth has been observed in various viscous environments. It is hypothesized that a viscous environment could act as a mechanism responsible for the phenotypic expression of physiological factors characteristic of biofilms. The effect of viscosity on growth characteristics, morphology and antibiotic sensitivity of *Pseudomonas aeruginosa* and *Candida albicans* was evaluated. Growth curves demonstrated that bacteria and fungus grew better at higher viscosity. *P. aeruginosa* in viscous solutions were larger than the planktonic cells. *C. albicans* was reduced in size at higher viscosity. *P. aeruginosa* and*C. albicans* grown in viscous solutions demonstrated decreased susceptibility to antibiotics similar to the biofilm organisms indicating that viscosity of the environment is a possible mechanism for biofilm phenotypic expression.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AD = Air-displacement

BF = Biofilm

CBD = Calgary biofilm device

CFU = Colony forming units

cP = Centipoise

ddH_2O = Double distilled water

DMSO = Dimethyl sulfoxide

MBC = Minimum bactericidal concentration

MBEC = Minimum biofilm eradicating concentration

MFC = Minimum fungicidal concentration

MHB = Mueller Hinton Broth

MIC = Minimum inhibitory concentration

MIV = Minimum immobolizing viscosity

PD = Positive displacement

PVP = Polyvinylpyrrolidone

RP = Reverse pipetting

SGA = Sabouraud-Glucose agar

TEM = Transmission electron microscopy

TSA = Tryptic soy agar

TSB = Tryptic soy broth

w/v = weight per volume

CHAPTER 1: INTRODUCTION

1.1 Biofilms

Historically, the field of microbiology has focused on freely floating planktonic bacteria grown in batch culture. Studies of microorganisms *in situ* and *in vivo* have shown that they are more often surface-associated and existing as biofilms (Costerton et al, 1987). Biofilms are adherent colonies of microorganisms growing within a polysaccharide cell surface glycocalyx. Growing as a biofilm appears to result in modifications of physiological status as compared to the same organism growing in planktonic mode (Costerton et al, 1987). Adhesion triggers the expression of a large number of genes so that biofilm cells are clearly phenotypically distinct from their planktonic counterparts (Deretic et al, 1994; Marshall, 1992). Antibiotics and biocides used at concentrations capable of killing planktonic organisms have little effect against biofilms (Ceri et al, 1999; Nickel et al, 1985). Biofilm organisms also differ from planktonic organisms in growth rate (Brown et al, 1988), cell wall structure and composition, immunogenicity, and enzyme activity (Costerton and Lappin-Scott, 1989).

1.1.1 Structure

Bacterial and fungal cells appear to attach nonspecifically to substrata. No plastic or metal surface has been shown resistant to bacterial colonization (Costerton and Lappin-Scott, 1989). Cells initially adhere to surfaces by reversible association and then irreversible adhesion facilitated by exopolysaccharide glycocalyx polymers (Costerton et al, 1987). In reversible association, bacteria demonstrate Brownian motion and can be removed from surface by moderate shear force. When bacteria are irreversibly adhered,

bacteria lack Brownian motion, and cannot be disrupted by moderate shear force (Marshall, 1992). Bacteria divide to produce sister cells within glycocalyx matrix, initiating the development of microcolonies. A mature biofilm is achieved by division of cells within the microcolonies and recruitment of planktonic cells from the bulk fluid phase (Costerton et al, 1987; Hoyle et al, 1990). Biofilms consist of single cells and microcolonies of sister cells embedded within a polyanionic matrix (Costerton et al, 1987; Hoyle et al, 1990). The biofilm contains channels in which nutrients can circulate. Cells in different regions of the biofilm exhibit different patterns of gene expression (Costerton, 1999). According to Anwar et al (1992), biofilms consist of two major areas; surface cells and embedded cells. The surface biofilm cells are found in the upper regions. These cells have access to nutrients including oxygen and have little trouble discharging metabolic waste products. They are metabolically active and large and the cell envelopes are permeable to nutrients, much like their planktonic counterparts. The embedded biofilm cells in the glycocalyx are less metabolically active due to their poor access to nutrients. The cells are small and dormant (Anwar et al, 1992). The restriction to essential nutrients influences the physiology of the cell. However another study stated that direct examination of established biofilms demonstrated that more than 70% of cells within biofilms were metabolically active, and very active cells were found deep within the biofilm, adjacent to originally colonized surface (Nickel et al, 1985).

Biofilm bacteria are not static populations, they exhibit an adaptable and environmentally responsive phenotype. This has been documented in studies involving *P. aeruginosa* biofilms (Anwar et al, 1989; Brown et al, 1988). The molecular

composition of bacterial cell walls is affected by the growth environment. Changes in the bacterial cell wall also occur in response to changes in growth rate, exposure to subinhibitory concentrations of antibiotics and growth on solid surfaces. Transposon mutants of marine bacteria demonstrate that some genes not expressed in liquid or agar media are switched on at solid surfaces (Marshall, 1992).

1.1.2 Distribution

a) Natural environments

Biofilms have been found in almost all natural aquatic environments. Streams, rivers and sewage effluents have all been found to contain biofilms (Costerton et al, 1987). Adherent populations develop on virtually all surfaces immersed in natural aqueous environments such as aquatic plants and animals, stones and particles.

In marine environments, seawater bacteria attach to submerged surfaces such as oil rigs and ships. Fouling of ship hulls increases turbulence, which in turn increases fluid frictional resistance (Marshall, 1992). A biofilm a couple of micrometers thick over the hull of a ship can retard its speed by as much as 20% (Lewin, 1984). This results in reduced performance and the need for regular cleaning of ship surfaces which costs hundreds of millions of dollars per year (Marshall, 1992).

b) Industrial environments

Biofilms have been known to affect pipelines, ship hulls, computer chips and water conduits. Biofilms plug filters and injection faces, fouling products and generating of harmful metabolites such as H_2S. In oil recovery and delivery pipelines, biofilms generate ferrous sulfide particles and reduce S to H_2S which is corrosive to mild steel

pipes drilling tiny holes through pipe walls which result in the loss of a million dollars worth of oil a day in Alaska (Lewin, 1984). Biofilms also affect heat exchange in steam driven turbines. Bacteria colonize the water-cooled side of metal surfaces of heat exchangers resulting in insulation of the equipment. This can eventually reduce the exchange efficiency by more than 90% of its designed value. A 600 000 kilowatt fossil fuel power station loses half a million dollars worth of energy a year as a result of biofilm formation (Lewin, 1984). Biofilms form on computer chips and serve as conductors interfering with electronic signals (Potera, 1996). Bacterial biofilms of H_2S producing organisms block water injection wells causing the souring of oil and often leads to the closure of oil fields (Costerton and Lappin-Scott, 1989).

Biofilms are also industrially useful. They are capable of breaking down and transforming a wide range of chemical substrates. They are used in sewage and waste water management on trickling filters and fluidized bed reactors (Marshall, 1992) to consume organic material in plant effluents (Olson, 1997). They are also useful in bioremediation of toxic contaminants such as jet fuel and carbon tetrachloride (Potera, 1996). *Saccharomyces cerevisiae* biofilms have been used in alcoholic fermentation for the production of beer (Olson, 1997)

c) Medical biomaterials

Millions of surgical procedures each year require the permanent or temporary use of biomaterials such as implant devices and prostheses (Reid and Bailey, 1996). Surgical implantation of foreign devices results in increased susceptibility to bacterial infections (McDermid et al, 1993). The majority of nosocomial bacteremias are device related.

Insertion of medical devices into the human body often leads to colonization of surfaces resulting in biofilms (Reid and Bailey, 1996). Microorganisms appear to be able to adhere to practically all biomaterials. Bacteria involved in implant associated infections are most often endogenous skin microflora such as *Staphylococcus epidermidis* (Marshall, 1992; McDermid et al, 1993), bowel flora or ubiquitous environmental organisms such as *Pseudomonas aeruginosa* which are only pathogenic to immunocompromised patients (Costerton et al, 1987).

Implant associated bacterial infections demonstrate patterns of alternating quiescent and acute periods (Costerton et al, 1987). Patients initially respond to antibiotic therapy. The antibiotics treat the planktonic cells shed from the biofilm but the adherent cells are left intact resuslting in a constant nidus of infection. Recurring infections occur, often necessitating the removal of the device to control the infection. However, some biofilms do not give rise to symptomatic infections.

Many medical devices are affected by biofilms. Biofilms have been found on common everyday medical devices such as contact lenses which often times result in severe eye irritation and inflammation (Marshall, 1992). Toxic shock syndrome results from biofilm formation on tampons (Reid et al, 1995). Naturally occurring intravaginal organisms such as *Lactobacilli, Candida albicans,* and *Staphylococcus aureus* absorb to and grow on tampon fibers where they are protected from macrophage intermediated destruction (Olson, 1997). Biofilms can also be found on sutures, biliary stents, urinary catheters, cardiac catheters, peritoneal catheters, heart valves, artificial hearts, orthopedic devices, endo-ocular prosthetic devices and IUDs (Costerton et al, 1993). Biofilm

colonized catheters are a primary cause of nosocomial infections and primary septicemia (Raad et al, 1995).

d) Association with tissue surfaces

Human and animal gastrointestinal tracts are colonized by specific groups of bacteria that give rise to natural biofilms (Marshall, 1992). For example, bacteria exist within the rumen of cows to aid in digestion of material such as cellulose (Costerton et al, 1987). Biofilms have also been associated with other tissue surfaces such as on the epithelia of the human vagina and cervix (Costerton et al, 1987).

The field of dentistry is based on biofilms. Biofilms are the cause of plaque, dental caries and periodontal disease (Wilson, 1996; Slavkin, 1997).

1.1.3 Resistance

When organisms isolated from sites of infection, the routine procedure is to determine the MIC of an antimicrobial agent to counteract its growth to determine guidelines for treatment. This approach is flawed because bacteria in biofilm mode, are not eradicated by doses of antibiotics that inhibit planktonic cells (Ceri et al, 1999; Nickel et al, 1985).

Numerous studies have demonstrated that biofilms have an inherent resistance to antimicrobial agents, whereas planktonic cultures of the same organism are not (Ceri et al, 1999, Nickel et al, 1985, Schwank et al, 1998). When the biofilm is returned to conditions that allows growth of planktonic cells, the resistance is lost (Costerton et al, 1987). Biofilm infections are rarely resolved by host defense mechanisms (Nickel et al, 1985). In general, concentrations of antibiotics from 500 to 5000 times greater than those

required for killing planktonic strains are necessary. Often times, these concentrations are not feasible because they are toxic to the host. Antibiotic therapy of device-related biofilm infections typically reverses symptoms caused by planktonic cells but fails to kill the biofilm. Exposure of biofilm bacteria to antibiotic concentrations used to eradicate planktonic bacteria results in the opportunity for the biofilm to develop or import biofilm resistance mechanisms. Biocide resistance is also of increasing concern as biofouling caused by biofilms have been found in many industrial settings (Costerton et al, 1987). Bacteria in natural environments are resistant to bacteriophage and amoeba (Costerton et al, 1987).

The exact mechanism of resistance of biofilms has not yet been elucidated. Possible explanations for resistance include 1. Poor penetration of antimicrobial agent into the biofilm, 2. Altered biofilm physiology, and 3. Attachment-related changes in phenotype. The glycocalyx material allows the biofilm to resist the action of the drug (Hoyle et al, 1990; Nickel et al, 1985). The matrix of the biofilm is thought to retard the diffusion of antibiotics (Costerton et al, 1999). It has been proposed that the glycocalyx of the biofilm could act as a diffusion barrier. The exact mechanism of this is unclear but the presence of bound extracellular enzymes such as -lactamases within the glycocalyx reinforces its action as a diffusion barrier with respect to some antibiotics and molecular sieving properties enhanced through binding of divalent cations such as calcium from the environment (Wood et al, 1996). In addition, solutes have been found to diffuse at slower rates within a biofilm than in water (Costerton et al, 1999). It has been shown that some antibiotics penetrate biofilms more readily than others (Hoyle et al, 1992). Poor

penetration of antimicrobial agents has also been attributed to the charged exopolysaccharide matrix that binds antimicrobial agents through an ion exchange complex before they reach the target cells (Blenkinsopp et al, 1992; Reid and Bailey, 1996). Both the diffusion barrier and charged matrix theory assume that biofilm cells are not exposed to sufficient amounts of antibacterial agents to disrupt their metabolism and consequently kill them. A second mechanism for biofilm resistance deals with physiological differences between biofilm and planktonic cells. This proposed model suggests nutrient and oxygen constraints on biofilm cells result in the decrease in growth rate and a downregulation of metabolic rate thus reducing their susceptibility to agents (Anwar, 1992; Brown et al, 1988; Evans et al, 1991; Reid and Bailey, 1996). The third hypothesis proposes that resistance to antimicrobial agents is due gene expression unique from planktonic cells. According to Costerton et al (1987), bacteria respond to changes in environment by phenotypic variation in enzymatic activity, cell wall composition, and surface structure. These changes involve target molecules for biocides, antibiotics, antibodies, phagocytes and external structures that control access of these agents to targets. The biofilm adopts a distinct and protected biofilm phenotype in response to growth on a surface (Costerton, 1999; Suchett-Kaye et al, 1996).

1.1.4 In vitro models

In order to determine what concentration of antibacterial agents should be used to eradicate a bacterial infection, clinicians determine the minimum inhibitory concentration (MIC). The MIC relates to the concentration of antibacterial agent required to inhibit the growth of a planktonic cell population. With knowledge that many infections result from

a biofilm population, the minimal biofilm eradicating concentration (MBEC) needs to be determined. The MBEC generally is significantly higher than the MIC. In most instances, the MBEC levels are difficult to achieve in patients due to antibiotic delivery mechanisms and may be toxic to the host. For example, the concentration of tobramycin needed to treat a *P. aeruginosa* biofilm is more than 1000 times greater than the concentration needed to treat the planktonic form (Nickel et al, 1985).

Several methods have been used to evaluate the effects of antibiotics and biocides on various bacterial biofilms. Prosser et al (1987) grew bacterial cells on Mueller-Hinton agar suspended in buffer and dispensed on catheter discs which are then transferred to petri dishes with broth and incubated for 20 to 22 hours at which time thick biofilms were established. The discs were then removed and placed in broth with the antibiotic in question and incubated for 4 hours. The discs were removed and viable counts were determined. The modified Robbins (mRD) device has been widely used but it is cumbersome and requires a significant amount of time and effort to perform an assay. The mRD is a perspex block with a central flow channel in which studs with a surface area of 0.5 cm^2 are exposed to flowing fluid from batch cultures. Once biofilms are formed on the stud, sterile medium containing antibiotic is passed though the colonized mRD. The number of living biofilm bacteria are determined by removing studs and detaching and dispersing adherent cells by scraping, vortex mixing, and sonication and plating to determine colony forming units (CFU) (Khoury et al, 1992). A drawback to this method is that one cannot control the large numbers of planktonic bacteria present that can inactivate the antibiotic. A simple system proposed by Ceri et al (1999) involves

growing a biofilm on pegs placed in a trough containing bacteria on a rocker. The shear force generated from the rocker allows even biofilm formation on the individual pegs. These biofilm pegs are placed in a standard 96-well plate containing antibiotics of difference concentrations and the growth is determined.

1.1.5 Control

Originally, biofilms were treated with antibacterial agents alone, however it was soon discovered that they were not sufficient. Bacterial biofilms have a profound resistance to antibiotic chemotherapy. When tobramycin at a concentration of 1000 μg/ml was used on catheter model in a mRD with a *Pseudomonas aeruginosa* biofilm, the biofilm was not killed. However, planktonic cells are killed with 0.4 μg/ml of tobramycin (Nickel et al, 1985). To combat this problem, new antibiotics need to be developed to specifically target biofilm bacteria. Fleroxacin, a fluroquinolone antibiotic is capable of killing common biofilms on urinary catheters in a rabbit by an unknown mechanism (Sheratz et al, 1993). Also, the antibiotics teicoplanin and daptomycin have been found to kill biofilms at much lower concentrations than traditional antibiotics requiring only 64 times the MIC (Potera, 1996). Few antibiotics on the market have been shown to penetrate effectively and eradicate biofilms

The inherent resistance of biofilms to conventional antibiotics and biocides has led to the application of new strategies to combat them. Besides the development of new antibacterial agents directed at biofilms, other strategies involve the prevention of adherence of organisms and increasing the permeability of the membrane.

Antibacterial molecules leaching from a biomaterial surface can influence bacterial adhesion and biofilm formation (Costerton et al, 1987). Studies have found that the coating of catheter segments with various antibiotics were effective in preventing bacterial and fungal colonization to catheter surfaces (Raad et al, 1995; Sheratz et al, 1993). The incorporation of silver into materials has also been successful at preventing bacterial adherence (Greenfeld et al, 1995; Leung et al, 1992; Lundeburg, 1986; Olson, 1997). An application of this technology would be the coating of plastic biliary stents. Late recurrent jaundice and cholangitis result from stent blockage by biofilms.

On the industrial side, it has been found that incorporation of toxic heavy metals such as copper and tin into antifouling paints helps to control biofilm formation on ship hulls (Marshall, 1992). Block copolymers and heparinized hydrophilic polymers prevent bacterial adhesion to surfaces of ship hulls by making them hydrophobic (Nagaoka and Kawakami, 1995). The prevention of adherence as a method of control to combat biofilms has also involved the use of metal catalysts (Wood et al, 1996), methyl and propyl parabens (Golomb and Sphigelman, 1991) and bile salts (Sung et al, 1994).

The permeability of the membrane can be influenced by electric fields and currents (Blenkinsopp et al, 1992; Costerton et al, 1993; Khoury et al, 1992; Jass et al, 1995; Jass and Lappin-Scott, 1996; Wellman et al, 1996), ultrasound (Pitt et al, 1994; Qian et al, 1996; Qian et al, 1997), magnetic fields (Benson et al, 1994), proteolytic enzymes (Selan et al, 1993) and protamines (Richards et al, 1990). These treatments combined with antibacterial agents have demonstrated increased eradication of biofilms. It is postulated that electrical fields overcome permeability problems by driving charged

antibiotic molecules through the biofilm matrix by electrophoresis (Blenkinsopp et al, 1992). It has been proposed that ultrasound enhances the transport of antibiotic into cells and results in higher antibiotic concentrations acting on biofilms (Qian et al, 1997). Magnetic fields cause the production of radical intermediates of membrane porin which may result in the increase of transport of antibiotics into the cell (Benson et al, 1994). Certain proteolytic enzymes work as anti-inflammatory drugs by enhancing the penetration of antibiotics to an infected site (Selan et al, 1993). The negatively charged biofilm matrix is disrupted by the membrane potential altering properties of protamines (Richards et al, 1990).

1.1.6 *Pseudomonas aeruginosa* biofilms

Numerous studies have focused on the formation of *Pseudomonas aeruginosa* biofilms. *P. aeruginosa* readily forms biofilms on many surfaces such as on the lung epithelium of cystic fibrosis patients (Costerton et al, 1999), and on catheter lines and contact lenses (Nickel et al, 1985). This ubiquitous organism is only pathogenic for compromised hosts such as immunocompromised patients, organ transplant patients and individuals with severe burns (Costerton et al, 1999). *P. aeruginosa* is an important pathogen in chronic respiratory tract infections such cystic fibrosis, chronic bronchitis and diffuse panbronchiolitis. The glycocalyx produced after colonization of respiratory tract is a potent factor leading to intractable infection (Hoyle et al, 1990).

A typical *P. aeruginosa* biofilm is found in the lungs of cystic fibrosis patients. CF patients are prone to lung infections because of a genetic defect which results in the loss of a CF transmembrane regulator (CFTR) chloride channel in the apical membranes

of epithelial cells. The absence of chloride channels results in the airway surface fluid having an elevated salt content (Feng et al, 1998). The salt inhibits the activity of antimicrobial peptides and proteins involved in innate immunity (Costerton et al, 1999). *P. aeruginosa* biofilms colonizing the lung epithelium release antigens while growing in microcolonies. In response, the host produces antibodies which react with the antigens on the surface of the biofilm matrix but the resistant biofilm is not cleared. The high concentration of antigen-antibody complexes result in pulmonary tissue damage from inflammation. *P. aeruginosa* lung infections in CF patients demonstrate resistance to multiple antibiotics (Saiman et al, 1999). Antibiotic therapies provide relief from symptoms but do not cure the infection as the antibiotics act only on the planktonic cells shed from the biofilm (Costerton et al, 1999). Pneumonia caused by *P. aeruginosa* in CF patients is a model of biofilm infections.

Research on the molecular and genetic basis of biofilm development has focused on *P. aeruginosa* biofilms. O'Toole and Kolter (1998) studied two classes of surface attachement deficient *P. aeruginosa* mutants. One class of mutants was deficient in flagella and the other lacked type IV pili. Both types of mutants were unable to form mature biofilms. The non-motile flagella mutants were unable to adhere to adhere to the test surface, suggesting that flagella and/or motility were essential in initial interactions with the surface. Organisms deficient in type IV pili formed a monolayer on the test surface but were unable to develop microcolonies, suggesting that type IV pili and/or twitching motility play a role in microcolony development. The attachment to surfaces is thought to result in the activation of certain genes. Davies et al (1998) report that *P.*

aeruginosa grown on a surface has increased expression of *algC*, a gene required for the synthesis of extracellular polysaccharides. Cell to cell signaling has been reported to be involved in the development of *P. aeruginosa* biofilms (Davies et al, 1998). In this study, two *P. aeruginosa* regulatory systems were characterized. The LasI-LasR system controls the expression of the RhlR-RhlI system and numerous virulence factors. The RhlR-RhlI system controls the expression of genes required for the production of secondary metabolites. The LasI and RhlI catalyze the synthesis of 3-oxododecanoylhomoserine lactone and butylhomoserine lactone respectively. It was reported that wild type, *lasI* mutants and *rhlI* mutants colonized surfaces and formed microcolonies. The wild type and *rhlI* mutants differentiated into mature biofilms whereas the *lasI* mutants resulted in thin and undifferentiated growth and was subject to dispersion by a weak detergent. When the *lasI* mutant was exposed to 3-oxododecanoylhomoserine lactone, biofilm development occurred. This study concluded that a specific quorum sensing signal is required for biofilm differentiation (Davies et al, 1998). In another study, *P. aeruginosa* biofilms produced on silicone catheters in a physical model of the bladder resulted in the production of acylated homoserine lactones (Stickler et al, 1998).

1.1.7 *Candida albicans* biofilms

The majority of implant infections are caused by gram-positive bacteria such as *Staphylococcus epidermidis*. Although not as common, infections due to Gram negative bacteria and fungi tend to be more serious (Dougherty, 1988). Fungal infections are most commonly caused by opportunistic *Candida* pathogens, particularly *C. albicans*, *C.*

tropicalis, and *C. parasilosis* (Cox and Perfect, 1993). One study found that *C. albicans* bloodstream infections represent 10% of all hospital-acquired septicemias (Wey et al, 1988). The majority of nosocomial bloodstream infections caused by *Candida* were due to the use of intravascular catheters (Goldmann and Pier, 1993). Also implicated in *Candida* infections are urinary catheters, prosthetic heart valves, cardiac pacemakers, silicone voice prosthesis, endotracheal tubes, and cerebrospinal fluid shunts (Odds, 1998). Despite the rising concern of device related fungal infections, few studies exist on the growth of *Candida* as biofilms.

In a study by Hawser and Douglas (1994), a model system for studying *Candida* biofilms on the surface of small discs of catheter material is described. Biofilms of numerous *Candida* sp. were formed on various catheter materials. Scanning electron microscopy demonstrated that a *C. albicans* biofilm exhibits yeast and hyphal morphologies such as germ tubes, pseudohyphae and hyphae. An extracellular polysaccharide matrix can be produced *in vitro* by incubating the catheter material with gentle shaking (Hawser et al, 1998). When *C. albicans* was grown on agar surfaces or in liquid broth, only the yeast morphology was seen. This suggests that adhesion to surfaces induces biofilm specific gene expression.

Similar to bacterial biofilm, fungal biofilms are also resistant to antimicrobial agents. Hawser and Douglas (1995) reported *C. albicans* biofilms formed on catheter material were resistant to the action of five clinically important antifungal agents. They found biofilm values to be 30 to 2000 times higher than the MICs of the same organism in planktonic mode. Fluconazole exhibited the greatest activity, whereas amphotericin B

showed the least activity against biofilm cells. The mechanisms by which *Candida* biofilms resist the action of antifungal agents are unknown. One mechanism proposed related to the slow growth rate of biofilms as a result of nutrient limitation. Growth of *C. albicans* in a perfused biofilm fermentor allowed the growth rate of adherent populations to be controlled. Amphotericin B susceptibility was tested against *C. albicans* biofilms growing at different rates. The biofilms were resistant to the drug over a range of low growth rates demonstrating that the resistance is not due to a low growth rate alone (Baillie and Douglas, 1998). *C. albicans* biofilms found *in vivo* are likely to be limited by iron. Iron deprivation has been reportedto alter the surface composition of *C. albicans* (Sweet and Douglas, 1991). This change in surface composition could affect antimicrobial agent susceptibility (Costerton et al, 1987). However, one study found that glucose-limited and iron-limited *C. albicans* biofilms were equally resistant to amphotericin B (Baillie and Douglas, 1998).

1.2 Viscosity

1.2.1 Organisms in viscous environments

In addition to existing as freely floating and surface adherent organisms, some bacteria and fungi are found in a variety of viscous environments. A number of organisms are able to live in the mucus layer of mammals. *Campylobacter jejuni* move and adhere in the viscous environment of the thick mucus layer lining the epithelial surface of intestinal tract of humans and other mammals (Shigematsu et al, 1998; Szymanski et al, 1995). *Treponema* are also found in the mucus of vertebrate gut (Kimsey and Spielman, 1990). *Helicobacter pylori* is a gram negative microaerophilic

bacteria which colonizes and persists in the mucous layer of the human stomach (Nakamura et al, 1998). *Borrelia burgdoferi* is thought to reside in viscous sites within hosts such as joint fluids (Kimsey and Spielman, 1990). Most pathogenic spirochaetes such as *Leptospira* and *Treponema* possess the ability to negotiate viscous fluids (Pietrantino et al, 1988). *Cristispira* is another example of an organism existing in a viscous environment. *Cristispira* is found the highly viscoelastic matrix of the crystalline style of mollusks (Kimsey and Spielman, 1990)

Bacteria and fungi can also be found viscous environments such as aqueous and vitreous humour of the eye (Kimsey and Spielman, 1990), heavy oil (Huu et al, 1999), sputum (Feng et al, 1998), ointments and cosmetics (Lenczewski et al, 1996). The effects of existing in viscous environments on the organisms is not well-studied.

1.2.2 Viscosity experiments

Many experiments involving viscosity and bacteria dealt with bacterial motility, swimming patterns, or flagella expression. Bacteria were reported to increase in velocity in solutions of higher viscosity (Greenberg and Canale-Parola, 1977; Schneider and Doetsch, 1974; Shigematsu et al, 1998; Shoesmith, 1960). After a maximal velocity is reached at a specific viscosity, the velocity decreases with further increases in viscosity until motility ceases at the minimum immobilizing viscosity (MIV) (Greenberg and Canale-Parola, 1977; Lawrence et al, 1992; Mitchell et al, 1991; Shoesmith, 1960). The MIV value of various bacteria depends on the type of flagellation and cell shape (Greenberg and Canale-Parola, 1977). Spirochaetes have MIV values much higher than flagellated bacteria due to the helical shape of the cell. Polarly flagellated bacteria have

maximum velocities at lower viscosities than peritrichously flagellated bacteria (Greenberg and Canale-Parola, 1977; Schneider and Doetsch, 1974). Loss of translational motility also correlates with viscosity (Kimsey and Spielman, 1990). Swimming patterns of organisms change in response to changes in viscosity. For example, *Campylobacter jejuni* possesses a unique mode of swimming in solutions of high viscosity (Szymanski et al, 1995). The ability to swim in viscous environments is important for the colonization of areas of high viscosity such as the mucous layer of the intestinal tract. Flagella expression is affected by viscosity. Organisms such as *Vibrio* swim in liquid environments propelled by a constitutively expressed polar flagellum. When found on surfaces, *Vibrio* swarm using numerous lateral flagella (Kawagashi et al, 1996). Lateral flagella are also synthesized at elevated viscosities. The expression of lateral flagella occurs when the polar flagellum senses a decrease in the rotation rate of the motor. The relationship between swimming speed and viscosity of the medium for each polar system has been explored. *Vibrio* expressing only the polar flagellum were not able to swim at high viscosity whereas lateral flagella-expressing organism could (Atsumi et al, 1996; Lawrence et al, 1992). *Vibrio* cells were elongated in the presence of a viscous environment similar to the phenotype expressed when found on surfaces (Lawrence et al, 1992).

1.3 Research hypothesis and objectives

The growth of microorganisms on solid surfaces as biofilms is well-studied. Biofilm formation occurs when microorganisms adhere to solid surfaces. Upon adhesion to surfaces, genes unique to the biofilm are expressed. It has not yet been determined if

other factors can serve as signals for biofilm expression. The objective of this study is to determine whether the expression of a biofilm phenotype can be modulated by a viscous environment as opposed to adherence to a surface. It is hypothesized that microorganisms become biofilms when adherent to surfaces due to its sessile nature. By restricting movement of the organisms in a viscous environment, a biofilm phenotype should be expressed.

The specific objectives of this study are:

1. To assess the antibiotic susceptibility of *Pseudomonas aeruginosa* grown in solutions of different viscosity and in planktonic and biofilm mode to gentamicin and piperacillin.
2. To assess the antifungal agent susceptibility of *Candida albicans* grown in solutions of different viscosity and in planktonic and biofilm mode to amphotericin B.
3. To determine if there are any morphological differences between *Pseudomonas aeruginosa* and *Candida albicans* grown in solutions of different viscosity and in planktonic and biofilm mode.
4. To determine if the growth rate of *Pseudomonas aeruginosa* and *Candida albicans* is affected by growth in viscous solutions.

CHAPTER 2: METHODS AND MATERIALS

2.1 Organisms

Pseudomonas aeruginosa PAO1 and *Candida albicans* ATCC 14053 were used for all studies. *P. aeruginosa* PAO1 is a piliated non-mucoid laboratory strain, whose adherence characteristics have been studied (Bradley, 1979; Saiman et al, 1992) and readily forms biofilms (Mathee et al, 1999). *C. albicans* has been used in numerous fungal biofilm studies (Baillie and Douglas, 1998; Hawser and Douglas, 1995). The organisms were maintained on polystyrene spheres (Microbank™, Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at –70°C until plating. *P. aeruginosa* was subcultured onto tryptic soy agar (TSA) (Becton Dickinson, Cockeysville, MD, USA) and incubated for 18- 24 hours at 37°C. *C. albicans* was grown for 24 – 36 hours on 2% Sabouraud glucose agar (SGA) (Mikrobiologie, Germany) at 37°C. The organisms were passaged at least twice to ensure purity and viability.

2.2 Viscous media

2.2.1 Polyvinylpyrrolidone (PVP)

Viscous media was produced by the addition of polyvinylpyrrolidone (PVP) (K-360 (Sigma Diagnostics, St. Louis, MO, USA) to Mueller-Hinton broth (MHB) (Becton Dickinson). PVP (K-360) has a molecular weight of 360 000 Da and an intrinsic viscosity of 80-100. Five different solutions of PVP/MHB were produced: MHB containing 0% (w/v) PVP, 7.5 % PVP, 10 % PVP, 12.5 % PVP and 15 % PVP. PVP was used to increase viscosity because it is readily soluble in water (Guner, 1996) and produces Newtonian solutions (Rubinstein, 1975). PVP is used commercially in

disinfectants such as iodine-povidone, cosmetics, pharmaceuticals and catheter surface coatings. Numerous studies involving bacteria in viscous environments utilized PVP to increase the viscosity of the system (Greenberg and Canale-Parola, 1977; Schneider and Doetsch, 1975; Stecchini et al, 1998).

2.2.2 Viscosity measurements

The absolute viscosity of the MHB/PVP solutions was determined using a dial viscometer (Brookfield Syncro-Lectric viscometer, Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA). The absolute viscosity is defined as the viscosity that is measured by any system geometry which is not under the influence of gravity for obtaining the measurement (Howard, 1991). 600 ml of each MHB/PVP solution was placed in a 37^0C water bath for 1 hour before a dial reading was taken. The dial reading was multiplied by the factor appropriate to the viscometer model/spindle/speed combination being used to calculate the viscosity (Brookfield Dial viscometer Operating Instructions). The accuracy of the viscometer was verified using viscosity standard fluids (Brookfield) of 10 cP and 100 cP. 500 ml of the viscosity standard and the viscometer spindle were immersed in the water bath (25^0C) for 1 hour before the reading was taken. The acceptable range of viscosity for each standard was calculated.

2.2.3 Osmolality measurements

The osmolality of the viscous solutions containing different quantities of PVP-360 was determined by freezing point compression using The Advanced™ micro-osmometer model 3MO (Advanced Instruments, Inc., Needham Heights, Mass., USA). The osmometer was calibrated using 50 mOsm/kg H_2O and 850 mOsm/kg H_2O standards

(Advanced Instruments) prior to measuring the viscous solutions. Twenty microliters of each test solution was dispensed into the osmometer using a 20 µl positive-displacement sampler (Advanced) with disposable plastic sampler tips. The osmolality of the viscous solutions are reported in mOsm/kg H_2O.

2.2.4 Accurate delivery of viscous solutions

Three different pipettes were tested for accurate delivery of test solutions ranging in viscosity from <1 cP to 1600 cP (H_2O, 12.5% PVP (w/v), and 15% PVP). A traditional air-displacement pipette (Eppendorf Reference 100 Pipette, Brinkmann Instruments, Inc., Mississauga, ON, Canada) with disposable tips was tested. An electronic air-displacement pipette (Eppendorf® Model 4850 Electronic Pipette), with a reverse pipetting function designed for more accurate delivery of viscous solutions was also tested. The reverse pipetting function allows the pipette to take up the desired volume of liquid plus an excess volume and then dispenses only the desired volume. Lastly, a positive displacement pipette (Microman Bio M100, Gilson Medical Electronics S.A., Villiers-le-Bel, France) consisting of disposable plastic capilleries and pistons was also tested. Replicate one hundred microliter aliquots of each test solution were dispensed from the different pipettes was weighed on an analytical scale (Mettler AJ 100, Mettler-Toledo AG, Greifensee, Switzerland).

2.3 Growth Curves

Growth curves were performed for both *P. aeruginosa* and *C.albicans* grown at each viscosity. For *P. aeruginosa*, 1 colony selected from an 18-24 hour TSA plate was inoculated into 10 ml MHB and incubated for 3.5 hours at 37°C. A 1/ 100 dilution of

this suspension was made into each media by adding 100 µl of suspension into 9.9 ml of viscous media. These suspensions were incubated at 37^{0}C and tested at 1, 2, 3, 4, 5, 6, 8, 10, and 12 hour time points. At each time point, 20 µl was removed from the suspension and placed in 180 µl of 0.9% saline in a 96 well microtiter plate (Nunc™ Brand Products, Denmark). Serial dilutions were performed in 0.9% saline from 10^{-1} to 10^{-6}. Twenty microlitres of each serial dilution (10^{-1} to 10^{-6}) was spot plated onto TSA and incubated overnight. The bacterial concentration in CFU/ml was determined by counting the number of colonies after incubation and multiplying by 10 and the dilution factor.

The *C. albicans* growth curve was performed in a similar fashion. One colony selected from a 24-36 hour 2% SGA plate was inoculated into 10 ml MHB and incubated for 4 hours at 37^{0}C. A 1/100 dilution was made into each viscous solution. The suspensions were incubated at 37^{0}C and tested at 1, 2, 4, 6, 8, 10, 12, 14, 20, 26, 32, 38, and 48 hours. At each time point, 20 µl was removed from the suspension and placed in 180 µl of 0.9% saline in a 96 well microtiter plate. Serial dilutions from 10^{-1} to 10^{-6} were performed in 0.9% saline. Twenty microliters of each dilution was spot plated onto 2% SGA and incubated overnight. The number of CFU/ml were determined by counting the number of colonies after incubation and multiplying by 10 and the dilution factor.

2.4 Microscopy

P. aeruginosa and *C. albicans* grown in the solutions of different viscosities were followed for changes in morphology.

2.4.1 Negative staining

Negative staining was done to visualize flagella in *P. aeruginosa* grown in MHB at the different viscosities. *P. aeruginosa* was grown in MHB containing 0% (w/v) PVP, 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP for 18- 24 hours at 37^{0}C. A 1/100 dilution was performed in sterile double distilled water (ddH_2O). Biofilm bacteria was grown following the procedure described by Ceri et al (1999) using the Calgary Biofilm Device (CBD) (MBEC Biofilm Technologies Ltd., Calgary, AB, Canada). The biofilm bacteria were removed by manually scraping the pegs on the device. The bacteria were stained with a 2% phosphotungstic acid solution (Electron Microscopy Sciences, Fort Washington, PA, USA), placed on Formvar (polyvinyl formaldehyde) (Electron Microscopy Sciences) coated copper grids and visualized by transmission electron microscopy (Hitachi 7000 TEM, Japan). Morphometric measurements of *P. aeruginosa* grown in the different solutions and under biofilm conditions were taken from the photographs of the negative stains and converted to micrometers.

2.4.2. TEM

P. aeruginosa was fixed and embedded for transmission electron microscopy. One colony from an 18-24 hour TSA plate was inoculated into 10 ml of MHB (0% PVP) and viscous MHB (7.5% PVP, 10% PVP, 12.5% PVP, 15% PVP) for 12 hours at 37^{0}C. The suspensions were centrifuged for 1.5 hours at 10 000 r.p.m. (Beckman J-6B centrifuge, Beckman Instruments, Palo Alto, CA, USA) to pellet out the bacteria. The pellets were resuspended and fixed in a 5% gluteraldehyde (Electron Microscopy Sciences) in 0.1 M cacodylic buffer (pH 7.2) (Sigma) with 0.15 % ruthenium red (Aldrich Chem. Company, Milwaukee, WI, USA). Biofilm bacteria were produced using

the CBD as described in section 2.4.1. Biofilm bacteria were manually scraped from the device and suspended in 5% gluteraldehyde in 0.1 M cacodylic buffer with 0.15% ruthenium red. The suspensions were enrobed in 4% agar (Difco, Detroit, MI, USA). The samples were washed 5 times in 0.1 M cacodylic buffer with 0.05% ruthenium red and post-fixed in 4% osmium tetroxide (Polysciences Inc., Warrington, PA, USA). The samples were dehydrated through a series of acetone washes (Fisher Scientific Company, Fair Lawn, NJ, USA) at concentrations of 30%, 50%, 70%, 90% and 100%. Further dehydration was done with propylene oxide (Fisher). The samples were embedded SPURR low-viscosity embedding resin (Electron Microscopy Sciences) (Spurr, 1969). The specimens were thin sectioned using an LKB 8800 Ultrotome III^{R} (LKB-Produkter, Bromma, Sweden) and the sections were placed on copper grids. The grids were stained with a uranyl acetate solution (Fisher) followed by a lead citrate solution (Fisher). The bacteria were viewed using a transmission electron microscope (Hitachi 7000 TEM).

2.4.3 Light Microscopy

C. albicans grown in the different viscous solutions for 24 hours at 37^{0}C were examined under light microscope. Wet mounts of the organism were prepared and stained with a 1% (w/v) crystal violet solution (Difco). The slides were examined with light microscopy (Zeiss, Germany) under oil immersion at 1000X magnification. Morphometric measurements of *C. albicans* grown in the different solutions were taken using the ocular micrometer.

2.5 Antibiotic susceptibility tests

2.5.1. Drugs

Gentamicin (Sigma) and piperacillin (Sigma) were prepared as stock solutions in sterile ddH_2O at concentrations of 1280 μg/ml and 5120 μg/ml respectively. The stock solutions were stored at –70^{0}C. Working solutions were prepared in MHB (0% PVP) or viscous MHB containing 7.5 % (w/v) PVP, 10% PVP, 12.5% PVP or 15 % PVP at a concentration of 256 μg/ml (gentamicin) and 1024 μg/ml (piperacillin). From these working solutions serial two-fold dilutions were made in MHB and viscous MHB in the wells of a 96 well microtiter plate (Nunc).

Amphotericin B (Sigma) was prepared as a stock solutions in full strength dimethyl sulfoxide (DMSO) (Fisher) at a concentration maximum of 51.2 mg/ml. From this solution serial two-fold dilutions were made in DMSO to a concentration of 0.0015625 mg/ml. The amphotericin B stock solutions were stored at –70^{0}C. Working solutions were prepared by adding 1 μl of the amphotericin B stock solutions to 99 μl of media in a 96 well plate to give a 1% DMSO solution in concentrations ranging from 512 μg/ml to 0.015625 μg/ml.

2.5.2 MIC (NCCLS method)

The minimum inhibitory concentrations (MIC), which is defined as the concentration of antibiotic required to prevent the growth of a planktonic population, were determined following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for broth microdilution (NCCLS, 1997). The MIC values obtained were used as a means of comparison with the MIC and minimum bactericidal

concentration (MBC) or the minimum fungicidal concentration (MFC) of the same organism grown in a viscous solution.

2.5.2.1 Bacteria

Colonies of *P. aeruginosa* were picked from 18-24 TSA plates and placed in MHB (Becton Dickinson) such that the turbidity matched a McFarland standard of 0.5 (Dalynn Laboratory Products, Calgary, AB, Canada). This suspension was diluted 1/10 in MHB in order to obtain a concentration of approximately 1×10^6 to 5×10^6 cells ml. The concentration of the inoculum was confirmed by determination of the number of CFU/ml on TSA plates. Five microliters of the 1/10 suspension were added to a 96 well microtiter plate with wells containing 100 µl of antibiotic serially diluted in MHB. Gentamicin concentrations ranged from 256 µg/ml to 0.5 µg/ml. The range of piperacillin concentrations was from 1024 µg/ml to 2 µg/ml. The microtiter plates were incubated for 20 hours at 37^0C. Following incubation, the optical density was measured on a Thermomax microplate reader (Molecular Devices, Menlo Park, CA, USA) at 650 nm. Turbidity was used as an indication of bacterial growth to determine the minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of an antimicrobial agent that prevents growth. Optical densities of 0.100 or higher were considered as growth. Twenty microlitres of each well were spot plated onto TSA and incubated overnight to determine the minimum bactericidal concentration (MBC). The MBC is defined as the lowest concentration of an antimicrobial agent that kills a microbial population, as indicated by the absence of growth following subculturing in the dilution method. Zero colony forming units was considered no growth.

2.5.2.2. Fungi

Colonies of *C. albicans* were picked from 24-36 hour 2% SDA plates and placed in MHB such that the turbidity matched a McFarland standard of 0.5. This suspension was diluted 1:100 followed by a 1:20 dilution with RPMI 1640 media (Sigma) in order to obtain a concentration of approximately 0.5×10^3 to 2.5×10^3 cells per ml. Five microliters of this suspension was added to a 96 well microtiter plate with wells containing 100 µl of amphotericin B serially diluted in RPMI 1640 media (Sigma) ranging in concentration from 512 µg/ml to 0.015625 µg/ml. The microtiter plates were incubated for 24 hours at 37^0C. Following incubation, the MIC was determined by measuring the optical density on a Thermomax microplate reader at 650 nm. The MFC was determined by spot plating 20 µl from each well onto 2% Sabouraud-Dextrose agar and incubating overnight.

2.5.2.3 Reference strain

The MIC of gentamicin and piperacillin were also determined for the reference strain *P. aeruginosa* ATCC 27853 as quality controls. The MICs obtained were compared to an accepted range determined by the NCCLS.

2.5.3 MBEC

The minimum biofilm eradicating concentration (MBEC) of gentamicin and piperacillin for *P. aeruginosa* and amphotericin B for *C. albicans* were determined for comparison to the MIC and MBC/MFC of the same organisms grown in viscous solutions. The MBEC was determined following the procedure described by Ceri et al (1999).

2.5.3.1 Bacteria

The inoculum was established by direct colony suspension of *P. aeruginosa* from 18-24 hour cultures on TSA and placed in TSB (Becton Dickinson) such that the suspension equaled the turbidity of a McFarland standard of 1.0 (Dalynn). The suspension was diluted 1:30 in TSB. The concentration of the inoculum was confirmed by determination of the number of CFU/ml on TSA plates. Twenty-five milliliters of the 1:30 suspension was pipetted into the channelled trough chamber of the Calgary Biofilm Device (CBD). The biofilm peg lid was placed in the trough and biofilm formation was carried out at 37^0C and 95% relative humidity on a rocking platform (Red Rocker variable speed rocking platform, Hoefer Scientific Instruments, San Francisco, CA, USA) such that fluid flowed along the channels of the CBD, generating the required shear force across all pegs. The peg lid and trough were incubated for 5 hours in order to achieve a biofilm of 1 X 10^5 to 1 x 10^6 CFU per peg. The biofilm formation was determined by obtaining the number of CFU/ml on TSA plates following disruption of the biofilm by sonication using and Aquasonic Sonicator model 250 HT (VWR Scientific, Westchester, PA, USA). The biofilms formed on the lid were transferred to a 96 well plate containing the dilutions of gentamicin and piperacillin prepared in MHB. The antibiotic plates were incubated for 20 hours, after which the lid was removed, rinsed in 0.9% saline to remove planktonic cells and placed in a second 96 well plate containing MHB (recovery plate). The biofilm was removed from the CBD lid by sonication as described above, a new plate cover was added. The viability of the biofilm was determined after 20 hours of incubation at 37^0C by obtaining plate counts (spot plate 20 µl of each well of recovery

plate) and reading the optical density on a plate reader. The MBEC is defined as the minimal concentration of antibiotic required to eradicate the biofilm.

2.5.3.2 Fungi

C. albicans inoculum was obtained in the same manner as above. Colonies from 24-36 hour 2% SGA plates were placed in MHB. A 1:30 dilution of the inoculum was made and placed in the CBD trough. The CBD trough and lid were placed on a rocking platform for 24 hours. Following incubation, 96 physiologically identical biofilms were formed on the pegged lid of the CBD. The lid was rinsed once with 0.9 % saline and transferred to a 96 well plate containing amphotericin B dilutions in 1%DMSO RPMI 1640 media. The pegs were exposed to the antifungal for 24 hours in a 37^0C incubator The peg lid was then removed and rinsed twice in 0.9% saline to remove planktonic cells, and then transferred to a 96 well plate containing RPMI 1640 recovery media. The lid and the recovery plate were sonicated for 5 minutes in an ultrasonicator to dislodge adherent cells into the recovery medium. Twenty microliters from each well of the recovery plate was spot plated onto SGA and incubated overnight to obtain the MBEC. The MBEC was also determined by measuring turbidity on a plate reader.

2.5.4 MIC and MBC/MFC in viscous solutions

The MIC and MBC/MFC of organisms grown in MHB containing PVP was performed in order to determine if a viscous environment had an effect on antibiotic susceptibility.

2.5.4.1 Bacteria

Two different *P. aeruginosa* inocula were prepared for the determination of the MIC and MBC of gentamicin and piperacillin in viscous media. One colony was inoculated into 10 ml of MHB and viscous MHB (containing 7.5 % (w/v) PVP, 10% PVP, 12.5 % PVP and 15%) and incubated for a) 6.5 hours b) 20 hours followed by 1:100 dilution in MHB and viscous MHB and further incubation of 3 hours at 37^0C. The number of CFU/ml of the inocula were determined by performing serial dilutions from 10^{-1} to 10^{-6} of each inoculum using 0.9 ml 0.9% saline blanks. Twenty microliters of the serial dilutions were spot plated onto tryptic soy agar and incubated overnight.

Five microliters of the different inocula were added to a 96 well plate containing the same antibiotic concentrations as the MIC and MBEC above diluted in MHB and viscous MHB. The dilutions of the antibiotics in viscous MHB were performed using a positive displacement pipette (Gilson). The plates were incubated for 20 hours at 37^0C. Following the 20 hour incubation, the microtiter plate were read at 650 nm (Softmax ^{TM} microtiter plate reader).

2.5.4.2 Fungi

The MIC and minimum fungicidal concentration (MFC) of amphotericin B (Sigma) for *C. albicans* was determined through broth microdilution. The inoculum was prepared by inoculating 10 ml Mueller-Hinton broth and viscous Mueller-Hinton broth (containing 7.5% (wt./vol.) PVP, 10 % PVP, 12.5% PVP and 15% PVP) with one colony selected from a 24-36 hour 2% SGA plate. The suspensions were incubated for 28 hours at 37^0C. The antifungal DMSO stock solutions were thawed and diluted 1/100 in 99 µl of

the different MHB/PVP solutions in a microtiter plate resulting 1% DMSO solution. The antifungal dilutions took place in columns 3-12 of plate A and columns 1-6 of plate B. Column 1 (plate A) served as the sterile control. A growth control well containing 1% DMSO was done in column 2 (plate A) to ensure that DMSO did not affect fungal growth. Five microliters of the corresponding inocula was added to columns 2-12 (plate A) and columns 1-6 (plate B). The plates were incubated at 37^0C for 24 hours. Following incubation, the MIC was determined by measuring the optical density on a Softmax™ plate reader at 650 nm. The MFC was determined by spot plating 20 µl from each well onto 2% Sabouraud-Dextrose agar and incubating overnight.

2.6 Statistical analysis

The viscosity, osmolality and yeast cell measurement results were expressed as mean +/- SEM and compared by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. The growth curves were expressed as $\log_{10}$ mean and compared by ANOVA and Tukey Kramer test. The antibiotic susceptibility results in 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP, and 15 % PVP were expressed as geometric mean ($\log_2$). The antibiotic susceptibilities in each of the viscous solutions (7.5% PVP – 15% PVP) were compared to the antibiotic susceptibility of the planktonic population in MHB (0 % PVP) using an unpaired t-test. $P < 0.05$ was considered significant in all statistical tests.

CHAPTER 3: RESULTS

3.1 Viscosity

The absolute viscosity as expressed in centipoise (cP) was determined for the solutions of MHB containing 0% PVP (w/v), 2.5% PVP, 5% PVP, 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP. Similar viscosity values were obtained in previous studies (Nakamura et al, 1988; Petrino and Doetsch, 1979; Schneider and Doetsch, 1974). An exponential increase in viscosity in relation to the increasing polyvinylpyrrolidone content was observed (Figure 1A). The linear relationship between log (absolute viscosity) and PVP concentration (Figure 1B) is supported by published literature (Rubinstein, 1977). The viscosity of the solutions ranged from <1 cP for 0% PVP to 1600 cP for 15% PVP.

The calibration procedure found the viscometer to be accurate. A 10 cP and 100 cP standard were tested. It was determined that any viscosity reading between 8.905 cP and 10.095 cP of the 10 cP standard indicated the viscosmeter was accurate. A reading of 9 cP was taken. The 100 cP standard was accurate between 89.03 cP and 110.97 cP. The 100 cP standard gave a reading of 100 cP.

3.2 Osmolality

Osmolality as expressed in mOsm/kg H_2O was determined for the solutions of MHB containing 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP. A linear increase in osmolality in relation to the increasing polyvinylpyrrolidone content was observed (Figure 2A). The correlation coefficient was 0.93. There was also an

increase in osmolality in relation to viscosity (Figure 2B). PVP is reported to have negligible effects on osmolality (Petrino and Doetsch, 1978). The osmolality of the solutions ranged from 298.67 mOsm/kg H_2O to 425.3 mOsm/kg H_2O.

3.3 Accuracy of pipettes

The air-displacement, electronic, and positive displacement pipettes all delivered H_2O with precision and reproducibility. There was no significant difference between the weights of H_2O delivered by the three different pipettes (Figure 3). Accurate delivery of viscous solutions has been an inherent problem. Pipetting of viscous solutions using a traditional air-displacement pipette and an electronic pipette was ineffective. Both pipettes were not able to void the entire contents of the pipette tip. When dealing with the 12.5% PVP and 15% PVP solutions, the positive displacement pipette delivered sample weights around 0.1 g (Figure 3). The air-displacement and electronic pipette delivered approximately 30% less (around 0.7 g) of the viscous solutions than the positive displacement pipette (Figure 3). There was no significant difference in the viscous solutions weights when using the air-displacement and electronic pipette. The unique tip design of the positive displacement pipette accurately dispensed viscous test materials up to 1600 cP. The positive displacement pipette was used for dispensing small volumes (20 µl-100µl) of viscous solutions for the remaining experiments. A 10-ml syringe was used to measure and dispense larger volumes of PVP solutions. Lenczewski et al (1996), found that a positive displacement autodilutor involving disposable tips and a tip wipe feature delivered accurate sample weights of viscous test materials whereas an air-displacement pipette delivered approximately 30% of the expected amount. When

dispensing viscous solutions, a type of plunger or piston was needed to physically push the solution from the delivery vessel.

3.4 Growth Curves

The growth kinetics of *P. aeruginosa* and *C. albicans* grown in solutions of different viscosity are shown in Figures 4 and 5 respectively. *P. aeruginosa* concentrations in the various MHB/PVP solutions started at 1.2×10^3 CFU/ml at hour 0 and reached concentrations ranging from 1.15×10^7 CFU/ml to 3.34×10^7 CFU/ml at hour 12. A minimal lag phase was observed in all populations. Exponential growth occurred between hours 1 and 8 for all solutions. The bacterial population in MHB appeared to reach stationary phase at hour 10 while the organisms in the viscous solutions had not yet reached stationary phase at hour 12. The lowest number of colony forming units per ml occurred when *P. aeruginosa* was incubated in MHB alone ($1.15 \times 10^7 \pm 1.2 \times 10^6$ CFU/ml). The most growth occurred in the 10% PVP solution with a concentration $3.34 \times 10^7 \pm 1.01 \times 10^7$ CFU/ml. The cell yield at 12 hours was significantly higher in 7.5% PVP, 10% PVP, and 12.5% than in 0% PVP.

The growth curves for *C. albicans* all began with a concentration of 6×10^2 CFU/ml at hour 0. No lag phase was observed for the organisms in the viscous solutions, however the planktonic population had a lag phase between hour 0 and hour 3. *C. albicans* grew exponentially until hour 10 and remained stationary afterwards. From hour 32 to hour 48, the cell yield was significantly higher in the viscous solutions with concentrations ranging from 8.4×10^5 to 3.1×10^6 CFU/ml as compared with 0% PVP (2×10^5 to 3×10^5 CFU/ml). The 10% PVP solution had the highest number of CFU/ml.

3.5 Microscopy

3.5.1 Negative staining

Negative staining was done on *P. aeruginosa* to determine if flagella were expressed under viscous and biofilm conditions. The planktonic cells grown in MHB were stained as a positive control. A polar flagellum was observed on planktonic bacteria (Figure 6). Flagella was also seen on the organisms grown at various viscosities (7.5% PVP, 10% PVP, 12.5% PVP, and 15% PVP) (Figure 6). The negative stain of the biofilm bacteria exhibited a glycocalyx layer surrounding the cell as well as a monotrichous polar flagellum (Figure 6). Morphometric measurements of the negatively stained cells were made. The planktonic cells were significantly smaller than the bacteria grown in 7.5% PVP, in 12.5% PVP, in 15% PVP and as a biofilm population (Figure 7). *P. aeruginosa* cells were largest in the 7.5% PVP solution (2.23 μm ± 0.19) followed by the biofilm cells (2.21 μm ± 0.29). The planktonic cells grown in MHB measured 1.34 μm ± 0.067.

3.5.2 Transmission electron microscopy

Transmission electron microscopy was performed on *P. aeruginosa* to determine if there were any significant morphological differences between planktonic and biofilm cells as well as the organisms grown in the different viscous solutions. No differences were observed. Figure 8 shows transmission electron micrographs of planktonic cells, cells grown in 10% PVP and biofilm cells. The presence of glycocalyx was not observed because the samples were not antibody stabilized for glycocalyx visualization

3.5.3 Light microscopy

C. albicans cells grown in the different viscous solutions were measured using light microscopy (Figure 9). A linear decrease in the cell size of *C. albicans* occurred as the concentration of PVP increased (Figure 10). The organisms grown in the viscous solutions were significantly smaller at 4.82 μm ± 0.16 (7.5% PVP), 4.76 μm ± 0.14 (10% PVP), 4.48 μm ± 0.16 (12.5% PVP), 4.24 μm ± 0.20 (15% PVP) than the planktonic cells in MHB (5.36 μm ± 0.15). The measurements are the mean values of the diameters obtained from those of at least 25 cells from each of the viscous solutions using the ocular micrometer on the light microscope.

3.6 Antibiotic susceptibility

Antibiotic susceptibility tests following published procedures were performed to determine the efficacy of a number of antibiotics on *P. aeruginosa* and *C. albicans* grown under planktonic and biofilm conditions (NCCLS and Ceri et al, 1999). The values obtained were used for comparison to antibiotic susceptibility of organisms grown in viscous solutions.

3.6.1 *P. aeruginosa*

After colonization was complete at 5 hours, the mature *P. aeruginosa* biofilms on the Calgary Biofilm Device (CBD) were exposed to an aminoglycoside (gentamicin) and a β-lactam (piperacillin) for 20 hours to determine the minimal biofilm eradicating concentration (MBEC). Two pegs from the CBD lids were sonicated and plate counts were determined to ensure that a sufficient biofilm of 1.5×10^6 CFU/peg had developed.

P. aeruginosa planktonic cells were diluted in MHB to a concentration of 1 X 10^7 CFU/ml for the determination of the minimum inhibiting concentration (MIC) and minimum bactericidal concentration (MBC) of gentamicin and piperacillin. This was determined by serially diluting the inoculum and determining the number of CFU/ml though plate counts.

The geometric mean (log_2) of the MIC, MBC and MBEC of gentamicin and piperacillin for *P. aeruginosa* PAO1 are presented in Table 1. The biofilm formed by *P. aeruginosa* was less susceptible to the antibiotics than a planktonic culture of the same organism. The MBEC of gentamicin (16 μg/ml) was more than five-fold greater than the MIC (3.08 μg/ml). The minimum bactericidal concentration was 5.2 μg/ml. *P. aeruginosa* biofilms were less susceptible to piperacillin. The MBEC of piperacillin was 2048 μg/ml which was more than 300 times greater than the MIC (5.7 μg/ml). The MBC (45.3 μg/ml) was also significantly greater than the MIC.

Values greater than 1024 μg/ml were designated as 2048 μg/ml which was one dilution greater than the maximum value. Values less than 2 μg/ml were designated as 2 μg/ml which was the minimum value used. These values were for the determination of the mean and for statistical analysis.

The MIC of gentamicin and piperacillin of reference strain *P. aeruginosa* ATCC 27853 was 2 μg/ml and 4 μg/ml respectively. These values were within the NCCLS quality control (QC) range of 0.5 –2 μg/ml for gentamicin and 1 – 4 μg/ml for piperacillin.

3.6.2 *C. albicans*

C. albicans biofilms of 1 x 10^5 to 1 x 10^6 CFU/peg on the CBD were formed after 24 hours of incubation for the determination of the MBEC of a polyene, amphotericin B.

A *C. albicans* ATCC 14053 suspension containing 1 x 10^5 to 5 x 10^5 CFU/ml of MHB was made for MIC and minimum fungicidal concentration (MFC) determination of amphotericin B. The concentration was verified by serial dilution and plate counts.

The MIC, MFC and MBEC of amphotericin B is shown in Table 2. The planktonic yeast cells were very susceptible to amphotericin B with a mean MIC of 0.094 μg/ml and an MFC of 0.024 μg/ml. The biofilm cells were less to amphotericin B. The MBEC was more than 100 times greater than the MIC and MFC with a concentration of 16 μg/ml.

3.7 MIC and MBC/MFC in viscous media

The antibiotic susceptibility of organisms grown in viscous solutions were performed in order to determine if viscosity caused increased resistance similar to biofilm populations.

3.7.1 *P. aeruginosa*

The MIC and MBC assays were performed on *P. aeruginosa* inocula incubated for different periods of time to determine if these assays are inoculum-size dependent.

3.7.1.1 6.5 hour inoculum

P. aeruginosa PAO1 was inoculated in MHB containing 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP. Concentrations of these suspensions ranging

from 5.0 x 10^7 to 5.0 x 10^8 CFU/ml were achieved with a 6.5 hour incubation at 37^0C. These suspensions were used for susceptibility testing.

The procedure for antibiotic testing in viscous solutions was the same for all viscosities except that the antibiotics were diluted in the corresponding viscous media. The MICs obtained from the planktonic *P. aeruginosa* in 0% PVP for gentamicin and piperacillin were not significantly different from the MIC following the NCCLS method. The gentamicin MIC in 0% PVP was 2.6 μg/ml compared to 3.1 μg/ml obtained in the NCCLS method (Table 1) (P value=0.4710). The MIC of piperacillin (0% PVP) was 3.5 μg/ml which was comparable to the MIC by the NCCLS method (5.7 μg/ml) (Table 1) (P value = 0.0900). This indicates that the assay developed for viscous solutions is suitable for antibiotic susceptibility testing.

The geometric means of the MIC and MBC of gentamicin at each viscosity are presented in Table 3. The MIC of gentamicin increased as the viscosity of the medium increased. The bacteria grown in the15% PVP had an mean MIC of 10.6 μg/ml as compared to the planktonic MIC of 2.64 μg/ml. Statistical analysis indicates that the MIC in 12.5% PVP (9.2 μg/ml) and 15% PVP (10.6 μg/ml) was significantly higher than the MIC in 0% PVP (2.6 μg/ml). Viscosity also had an effect on the bactericidal activity of gentamicin. The lowest MBC (6.1 μg/ml) occurred in the 0% MHB. An increase in resistance to the bactericidal activities with increasing viscosity occurred. However, the MBC value was higher in the 10% PVP solution (21.1 μg/ml) than in the 12.5 % PVP solution (16 μg/ml). The highest MBC was seen in the 15% PVP solution with a value of

36.8 μg/ml. These values were greater than the biofilm MBEC (16 μg/ml) of the same organism (Table 1).

P. aeruginosa exhibited decreased susceptibility to piperacillin when grown in solutions with viscosity higher than that of MHB (Table 4). The MICs in 7.5% PVP and 10% PVP solutions were the same (8 μg/ml), these values were higher than the planktonic (0% PVP) MIC (3.5 μg/ml). The greatest MIC value was in the solution of 15% PVP with a value of 12.1 μg/ml. The MBCs in the viscous solutions were 6 to 11-fold greater than the MBC in 0% PVP (128 μg/ml) (Table 4). These MBCs (776.0 μg/ml to 1448.2 μg/ml) are difficult to achieve in a clinical situation. The MBCs were not quite as high as the concentration required to eradicate a biofilm of the same organism (2048 μg/ml) (Table 1). *P. aeruginosa* grown in viscous solutions ranging from 7.5% PVP to 15% PVP are resistant to the bactericidal effect of piperacillin.

3.7.1.2 20 hour inoculum followed by 1/100 dilution and 3 hour incubation

P. aeruginosa was inoculated in MHB containing 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP. Concentrations of these suspensions ranging from 5 x 10^6 to 5 x 10^7CFU/ml were achieved with a 20 hour incubation at 37^0C followed by a 1/100 dilution and further 3 hour incubation at 37^0C. These suspensions were lower in concentration than those obtained with 6.5 hours of incubation (5.0 x 10^7 to 5.0 x 10^8 CFU/ml) (Section 3.7.1.1).

The antibiotic susceptibility testing of gentamicin saw no significant differences between the MIC values of bacteria grown in 0% PVP, 7.5% PVP, 10% PVP, 12.5% PVP and 15% PVP (Table 5). The highest MIC value was seen in the non-viscous solution

(2.2 μg/ml). This value was comparable to the planktonic MIC value obtained with the 6.5 hour inoculum. The MBCs ranged from 1.6 μg/ml (7.5% PVP) to 5.7 μg/ml (10% PVP). The planktonic control (0% PVP) had an MBC of 3.2 μg/ml. The bactericidal activity of gentamicin was not significantly different in the viscous solutions compared to the 0% PVP.

The piperacillin MIC values ranged from 2.2 μg/ml (7.5% PVP) to 4 μg/ml (15% PVP) when *P. aeruginosa* was grow in the different solutions (Table 6). The MIC was 2.5 μg/ml in 0% PVP. There was not a significant difference in the inhibitory activities of piperacillin in the viscous solutions as compared to 0% PVP. A similar trend was observed in the MIC values of piperacillin (Table 6). The MBC of piperacillin increased with an increase in viscosity (Table 6) with the exception of 7.5% PVP (3.2 μg/ml). The MBC at the lowest viscosity (0% PVP) was 3.6 μg/ml. The 15% PVP only demonstrated an MBC of 7.1 μg/ml. However, the MBC values in the viscous solutions were not found to be significantly different from the 0% PVP control. These values obtained were dramatically lower than those obtained with the 6.5 hour inoculum (Section 3.7.1.1).

The antibiotic susceptibility of gentamicin and piperacillin, as determined by the MIC and MBC, of *P. aeruginosa* grown in the viscous solutions overnight followed by a 1:100 dilution and further incubation for 3 hours was dramatically different than the 6.5 hour inoculum. The MIC and MBC of gentamicin for the planktonic populations (0% PVP) were not significantly different from those obtained with a 6.5 hour inoculum. The MIC of piperacillin was not significantly different either. However, the MBC of

piperacillin using the 6.5 hour inoculum was more than 32-fold greater than the MBC obtained in this experiment (Table 4).

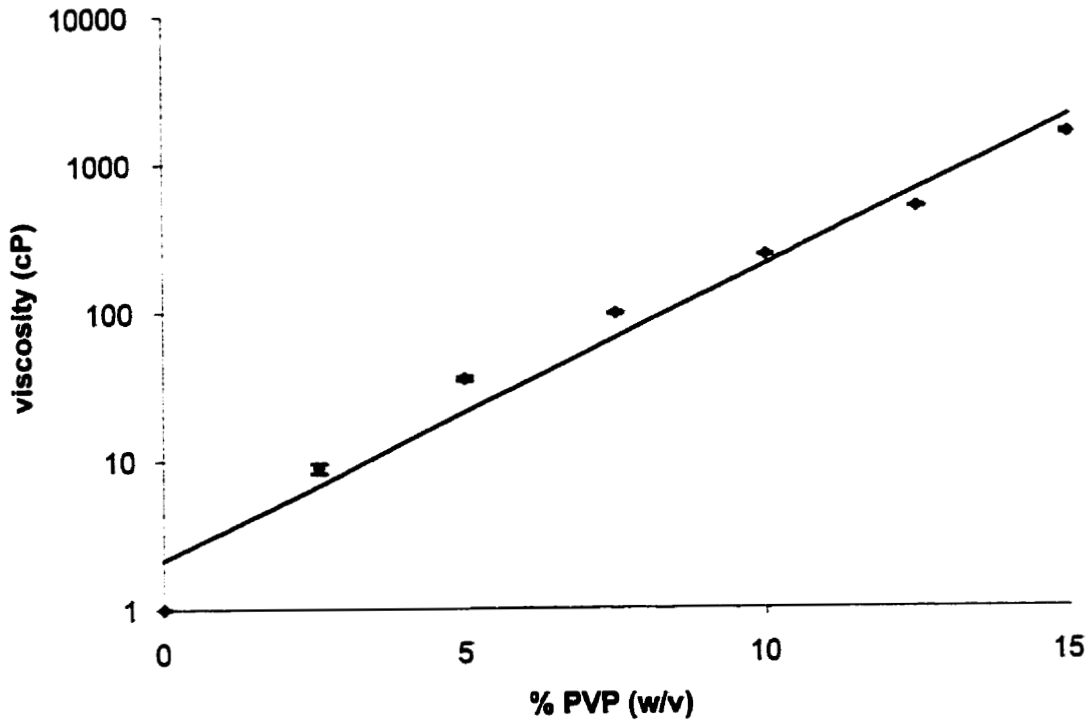
3.7.2 *C. albicans*

The MIC and MFC values of amphotericin B for *C. albicans* increased as the viscosity increased (Table 7). The planktonic MIC value (0.063 μg/ml) was the same as the MIC at low viscosity (7.5% PVP). The maximum MIC (0.125 μg/ml) for amphotericin B was reached at 15% PVP. This value was significantly greater than the planktonic MIC (0% PVP). The MFC value of planktonic organisms (0% PVP) was 0.035 μg/ml. The yeast grown in 15% PVP (4 μg/ml) demonstrated the most resistance to amphotericin B fungicidal activity. The 15% PVP MFC value was not as high as the MBEC value obtained (16 μg/ml) (Section 3.6).

The antifungal agent susceptibility of yeast grown in 0% PVP were different from those obtained following the NCCLS method. The MIC of amphotericin B was significantly less at 0.063 μg/ml in 0% PVP compared to 0.094 μg/ml obtained in the NCCLS assay. Interestingly, the MFC was greater in 0% PVP (0.35 μg/ml) than the NCCLS assay (0.024 μg/ml). This discrepancy in numbers could be attributed to the different procedures used.

Figure 1. Viscosity of Mueller-Hinton broth with increasing polyvinylpyrrolidone content. A) linear y –axis. B) log y-axis. (n=3).

A



B

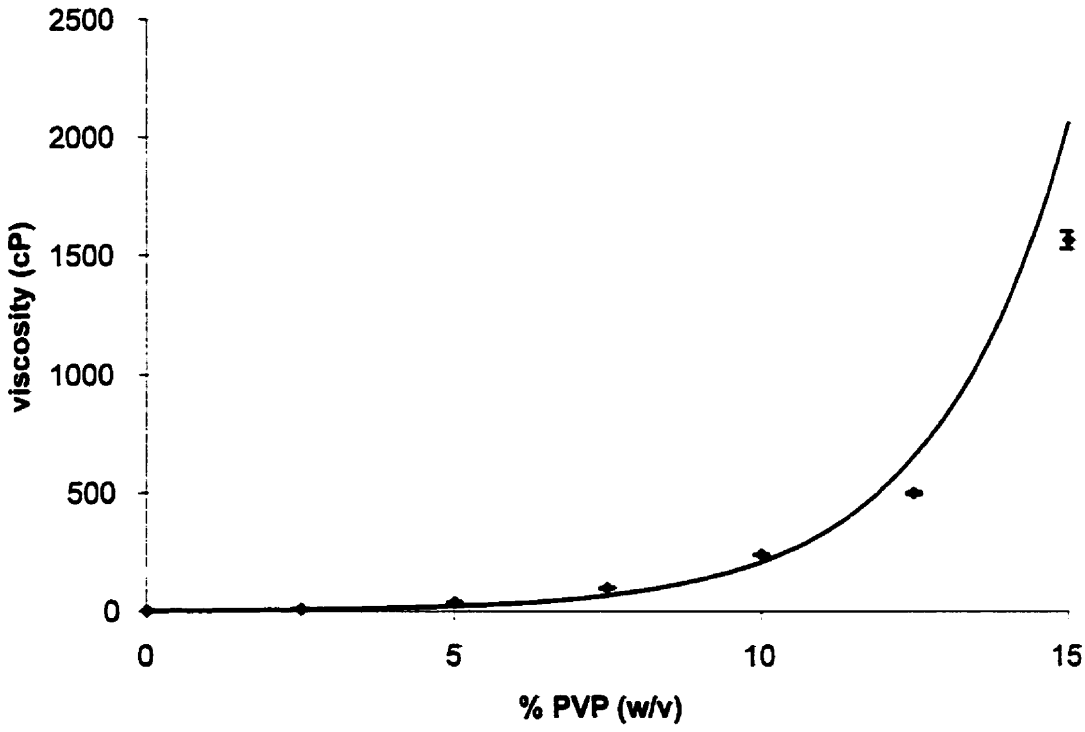
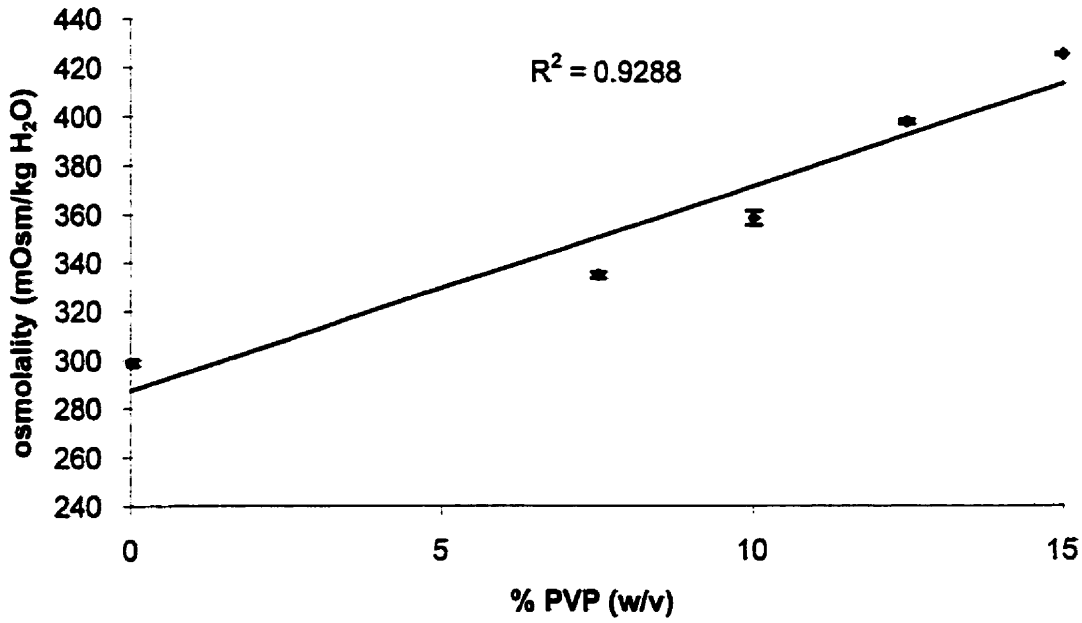


Figure 2. Osmolality of Mueller-Hinton broth with increasing polyvinylpyrrolidone content. A) in relation to PVP content (% w/v), B) in relation to viscosity. (n=3)

A


440
420
400
380
360
340
320
300
280
260
240
osmolality (mOsm/kg H_2O)
R^2 = 0.9288
0
5
10
15
% PVP (w/v)

B

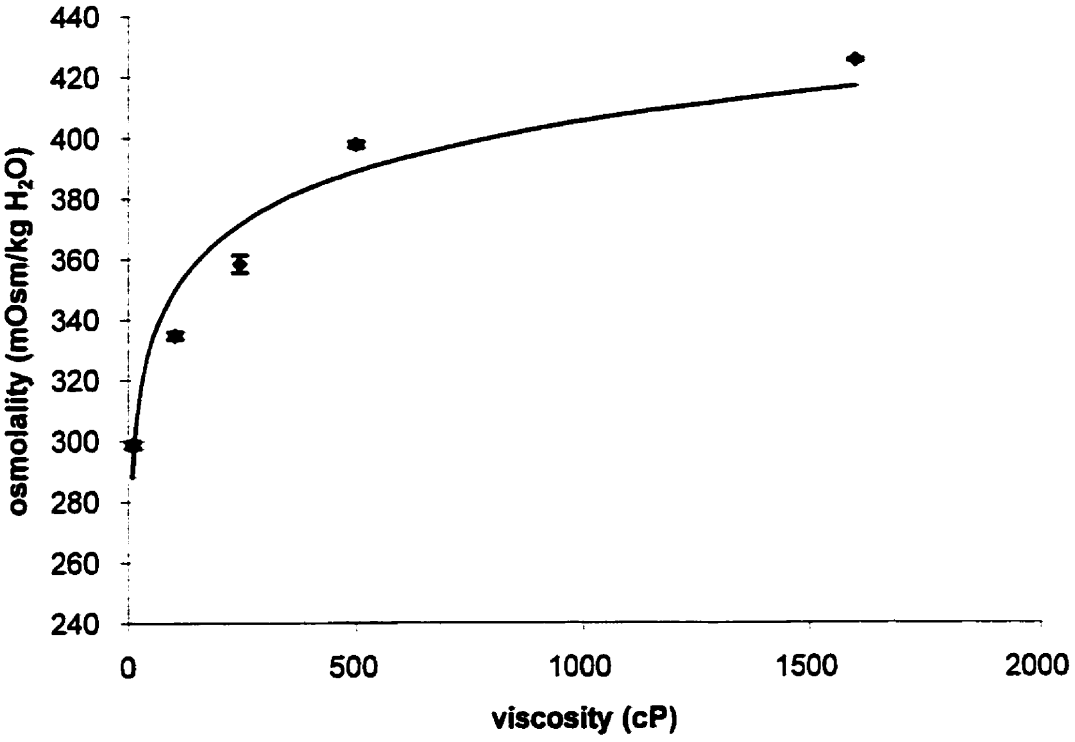

440
420
400
380
360
340
320
300
280
260
240
osmolality (mOsm/kg H_2O)
0
500
1000
1500
2000
viscosity (cP)

Figure 3. Comparison of accuracy of pipetting viscous solutions by using an air-displacement pipette () an electronic pipette with reverse pipetting function () and a positive displacement pipette (). (n=5) * P<0.05 compared with all groups.

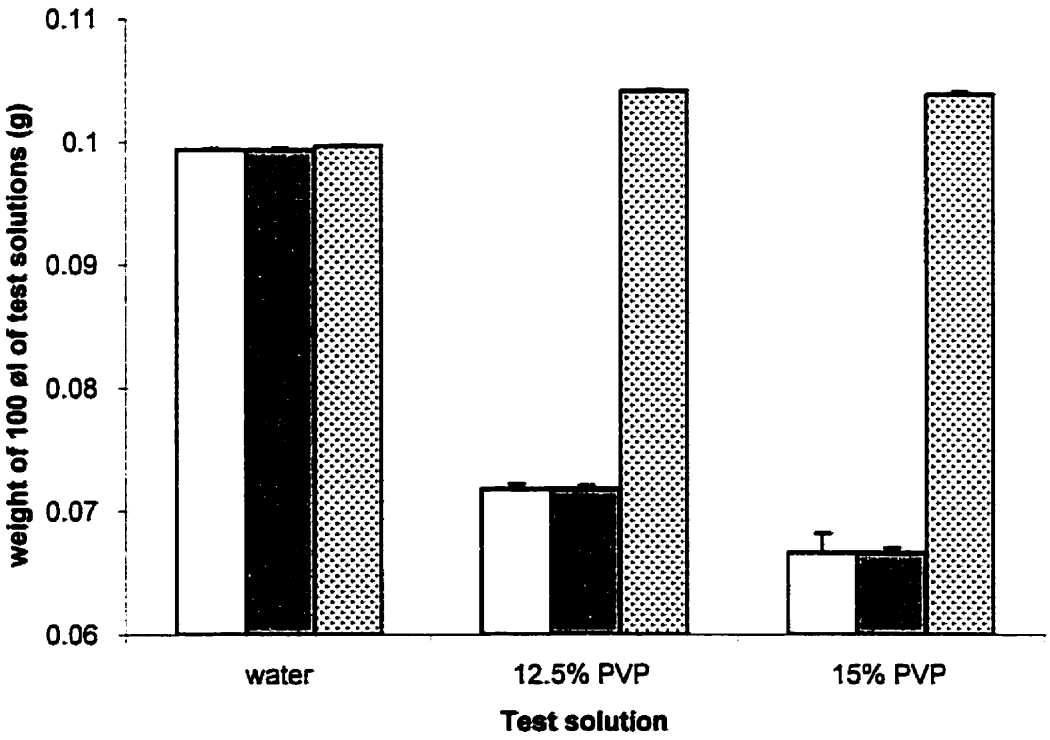

0.11
0.1
0.09
0.08
0.07
0.06
weight of 100 ø1 of test solutions (g)
water
12.5% PVP
15% PVP
Test solution

Figure 4. Growth curves (log_{10}CFU/ml) of *P. aeruginosa* PAO1 incubated in MHB with 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5 % PVP and 15% PVP. 0% PVP (—■—) ,7.5% PVP (——◆——), 10% PVP (---▲---), 12.5% PVP (—×—), and 15% PVP (——●——). (n= 3) * $P<0.05$ compared with 0% PVP.

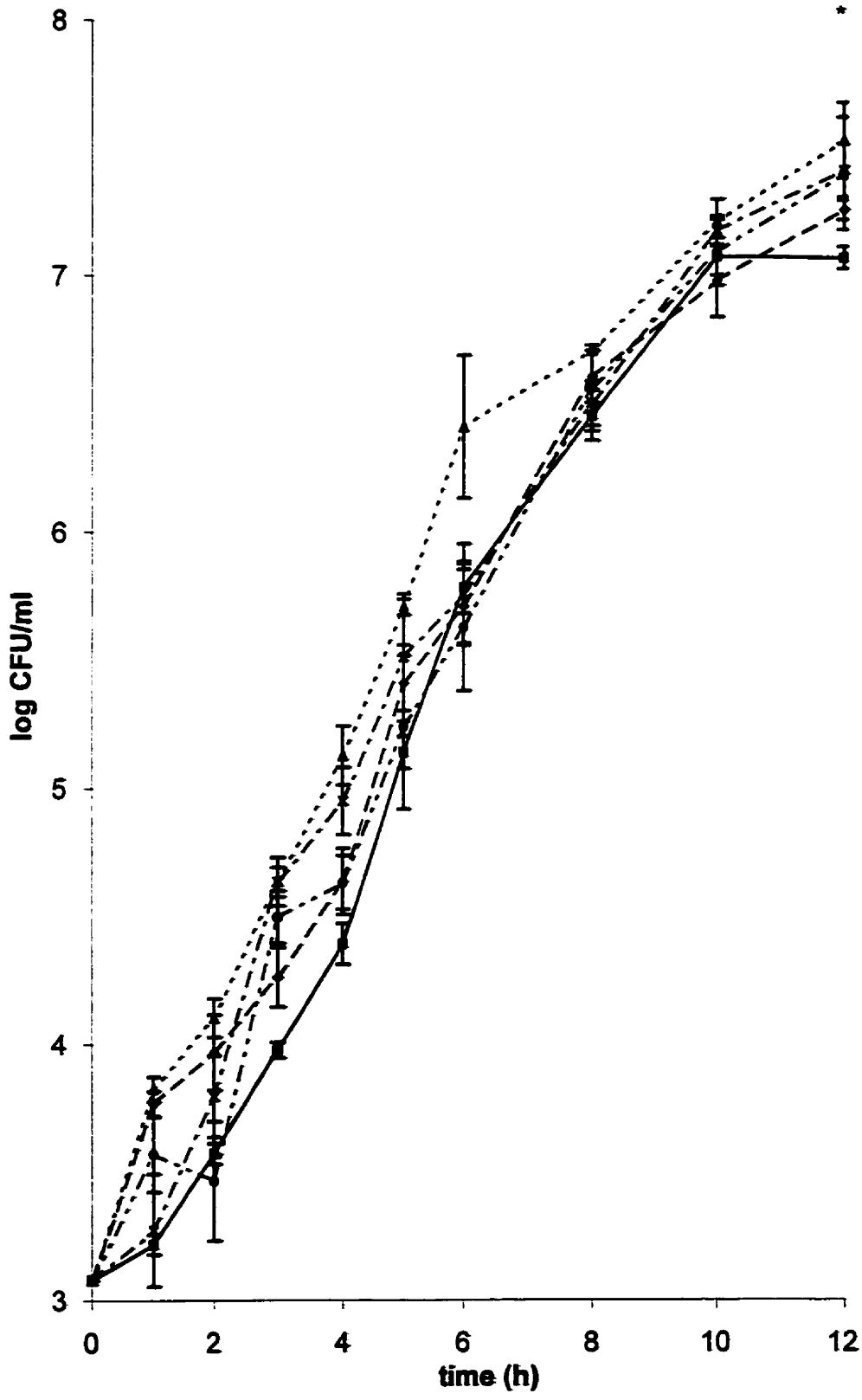

8
7
6
5
4
3
log CFU/ml
0
2
4
6
8
10
12
time (h)

Figure 5. Growth curves (log_{10}CFU/ml) of *C. albicans* ATCC 14053 incubated in MHB with 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5 % PVP and 15% PVP. 0% PVP (—■—), 7 .5% PVP (——◆——), 10% PVP (---▲---), 12.5% PVP (—✕—), and 15% PVP (——●——). (n=3) * $P<0.05$ compared with 0% PVP.

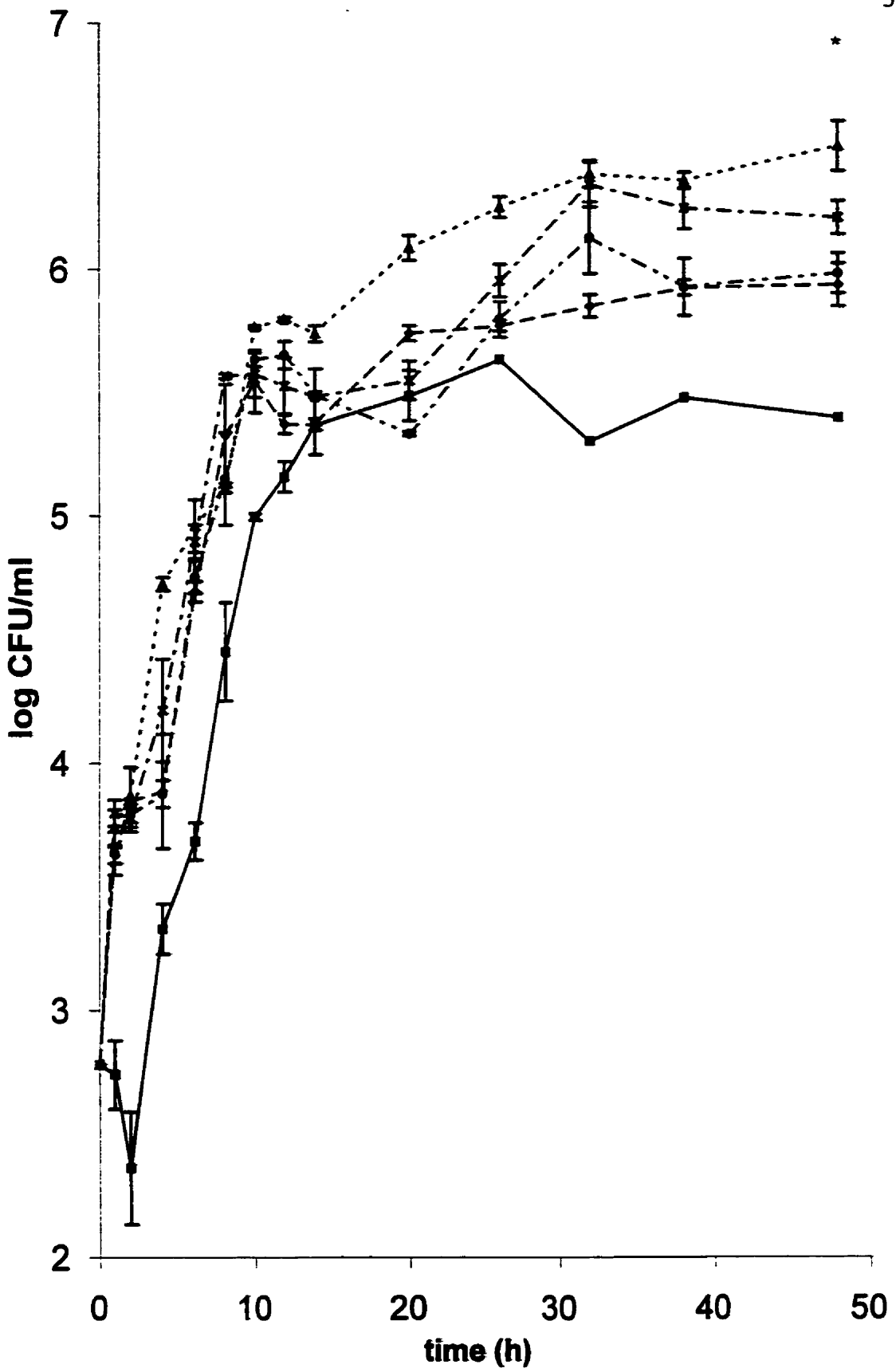
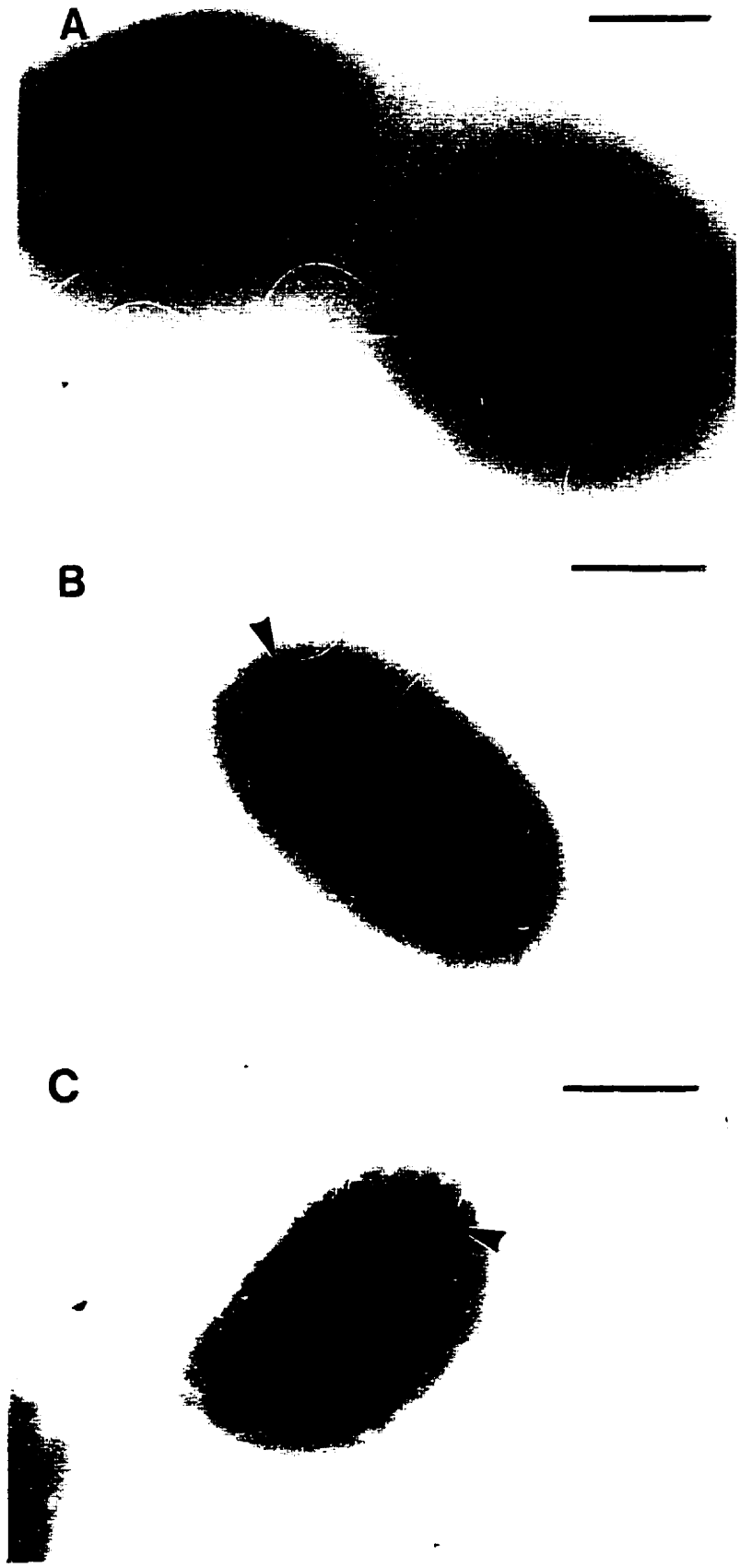

7
6
5
4
3
2
log CFU/ml
0
10
20
30
40
50
time (h)

Figure 6. Transmission electron micrographs (18 000 X) of negatively stained *P. aeruginosa* PAO1 to visualize flagella grown A) as planktonic bacteria, B) in 7.5% PVP, C) in 10% PVP, D) in 12.5% PVP, E) in 15% PVP, F) as biofilm bacteria. Arrows indicate flagella. Bar = 1.0 μm.


A
B
C

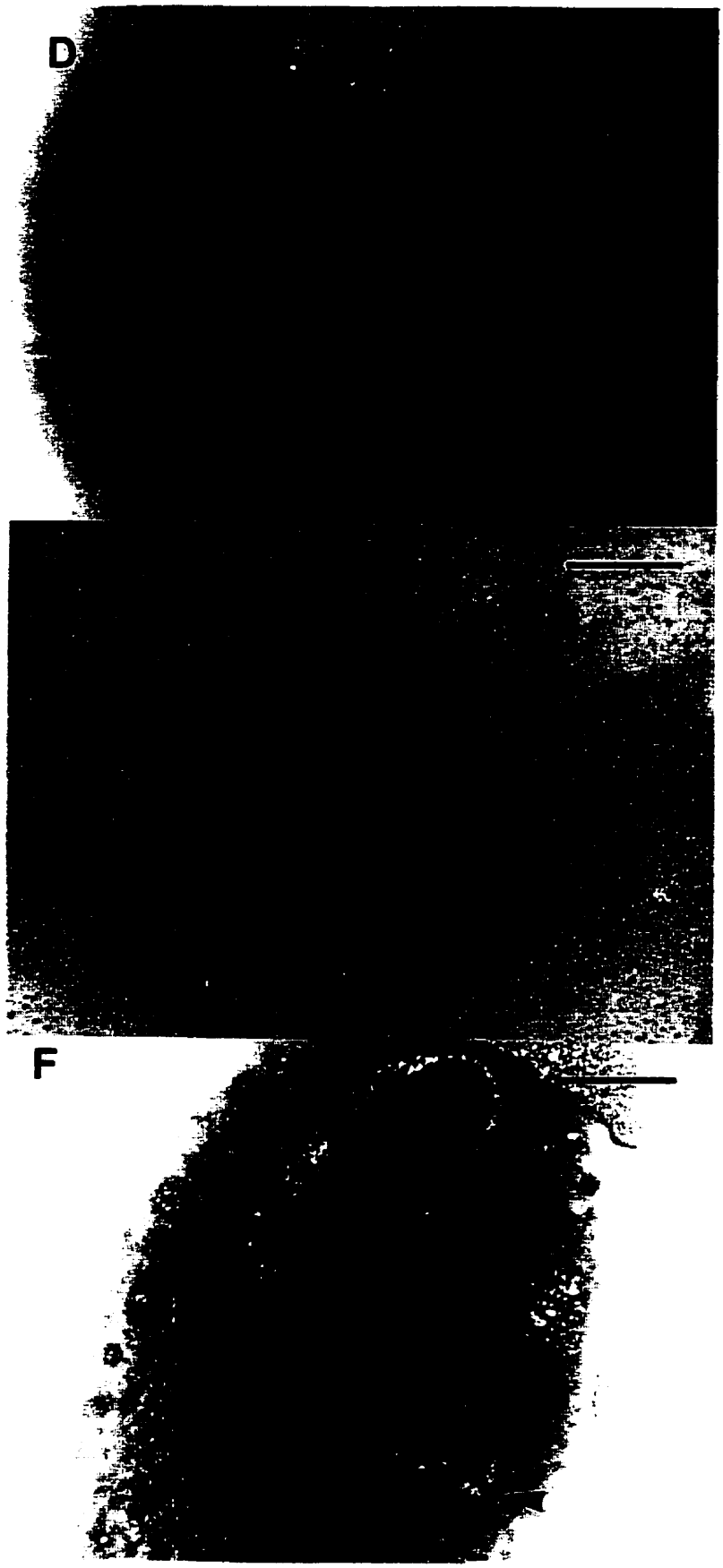

D
F

Figure 7. Morphometrical measurements of *P. aeruginosa* PAO1 grown in MHB with 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP, 15% PVP solutions and as a biofilm. *P<0.05 compared with 0% PVP.

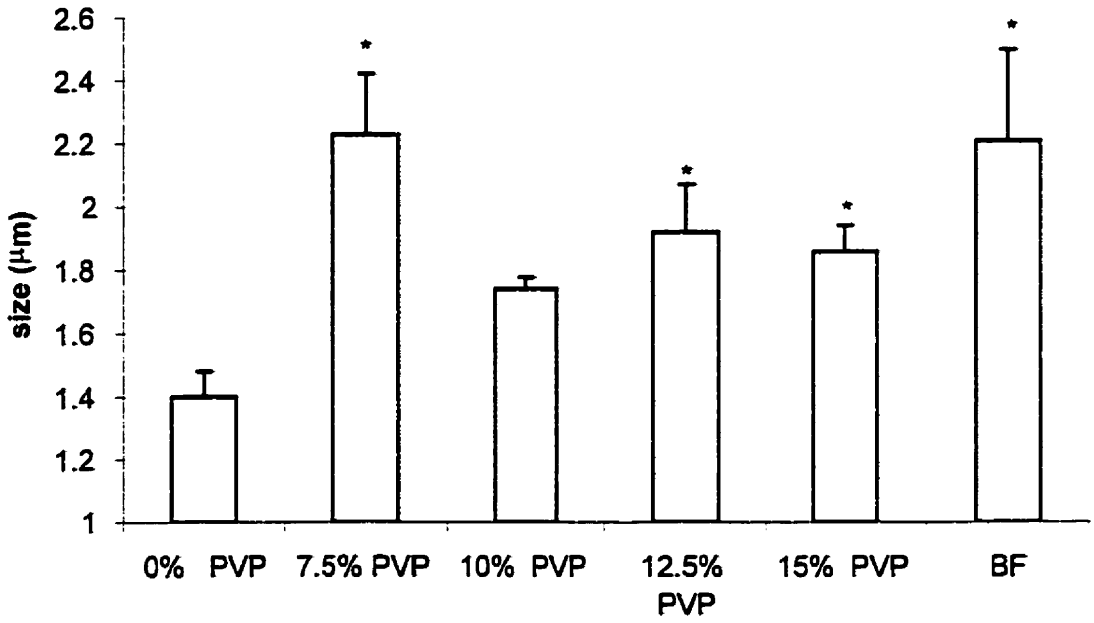

2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
size (μm)
*
*
*
*
*
0% PVP
7.5% PVP
10% PVP
12.5% PVP
15% PVP
BF

Figure 8. Transmission electron micrographs (45 000X) of sectioned *P. aeruginosa* PAO1 grown A) as planktonic bacteria, B) in viscous solution (10% PVP), C) as biofilm bacteria. Bar = 0.1 μm.

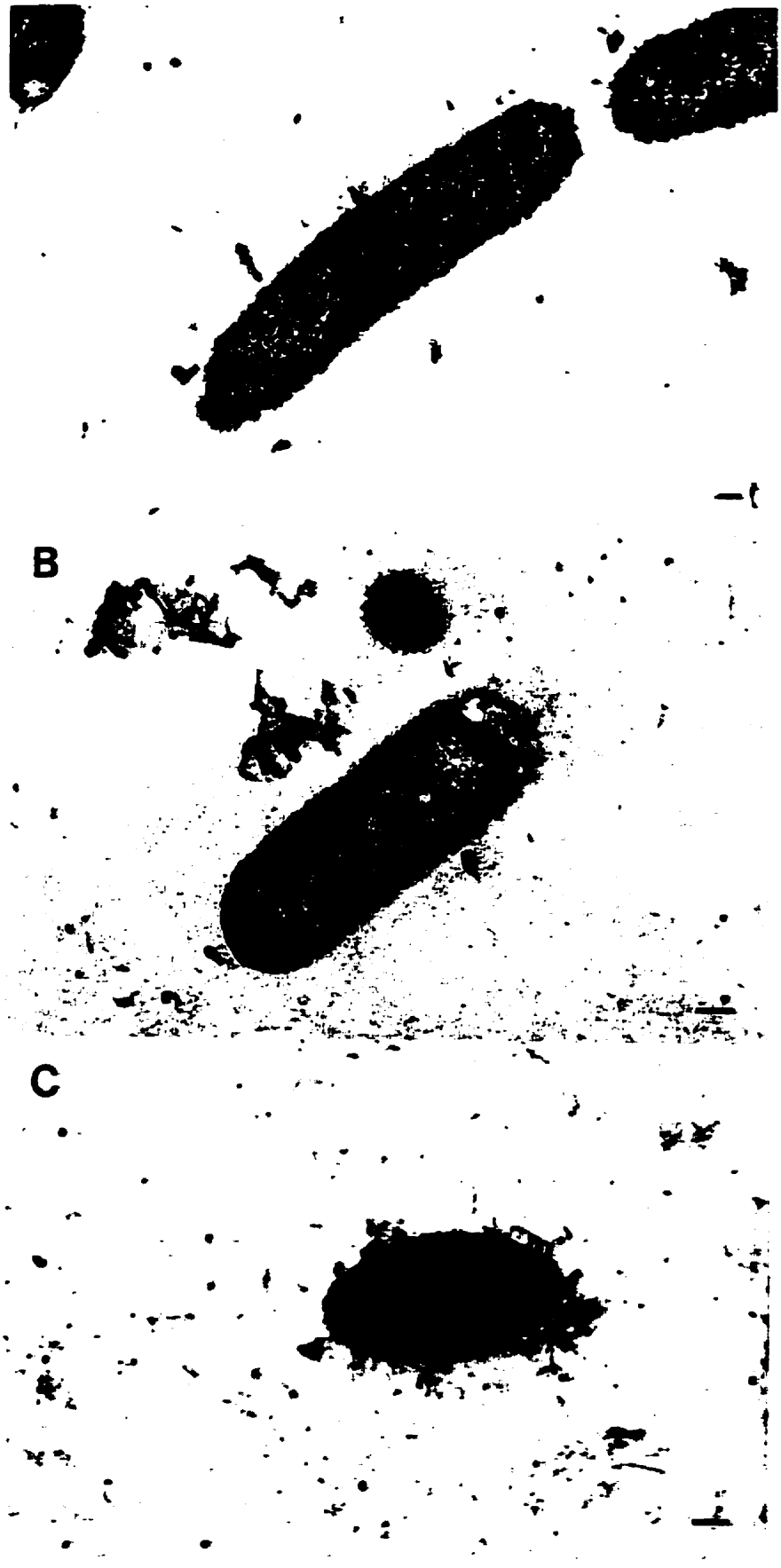

B
C

Figure 9. Light micrographs (1000X) of *C. albicans* ATCC 14053 grown in MHB with A) 0% PVP, B) 10% PVP, and C) 15% PVP. Bar = 10.0 μm.

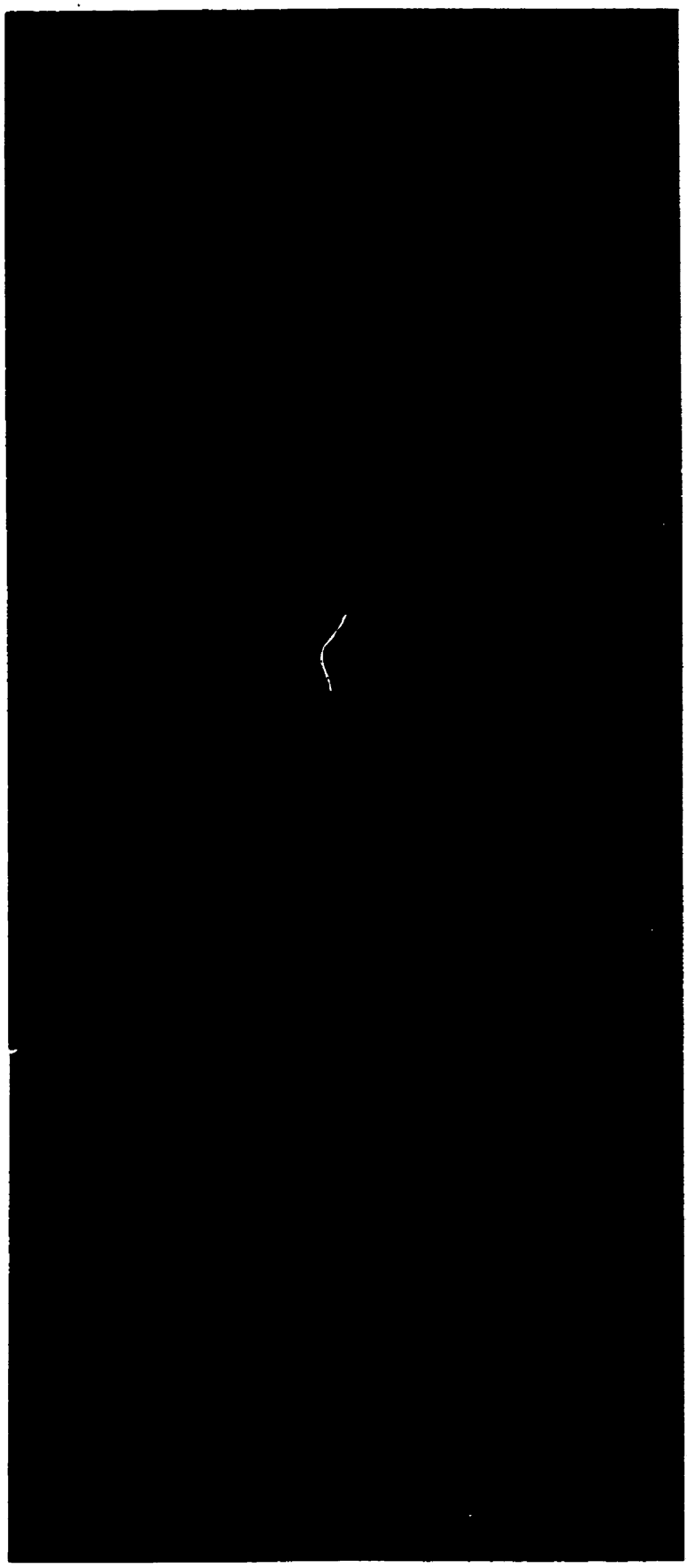


Figure 10. Morphometrical measurements of *C. albicans* ATCC 14053 grown in MHB with 0% PVP (w/v), 7.5% PVP, 10% PVP, 12.5% PVP, and 15% PVP solutions. (n=25) *P<0.05 compared with 0% PVP.

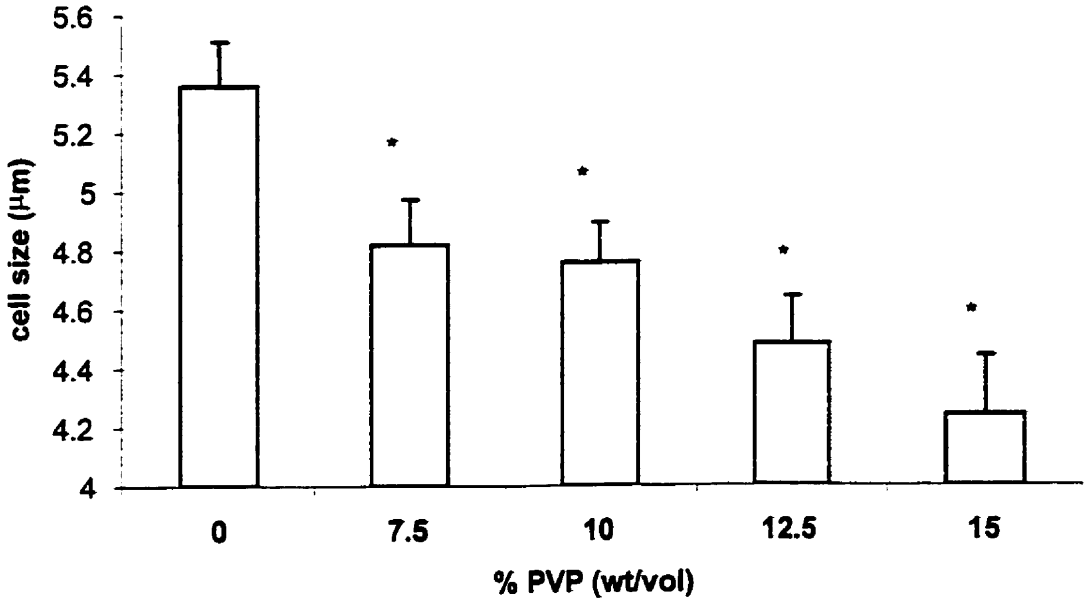

5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
cell size (μm)
*
*
*
*
0
7.5
10
12.5
15
% PVP (wt/vol)

Table 1. Geometric mean of antibiotic susceptibility of *P. aeruginosa* PAO1 as a planktonic population (MIC/MBC) (n=8) and as a biofilm population (MBEC) (n=3) as derived by the NCCLS assay and an assay with CBD.

Antibiotic	**MIC (μg/ml)**	**MBC (μg/ml)**	**MBEC (μg/ml)**
Gentamicin	3.1	5.2	16.0
Piperacillin	5.7	45.3	2048.0

Table 2. Amphotericin B susceptibility of *C. albicans* ATCC 14053 as a planktonic population (MIC/MFC) and as a biofilm population as derived by the NCCLS assay and an assay with the CBD.

Antibiotic	MIC (μg/ml)	MFC (μg/ml)	MBEC (μg/ml)
Amphotericin B	0.094	0.024	16

* this work was done by Darin Fogg, Biofilm Research Group, University of Calgary, Calgary, Alberta.

Table 3. Geometric mean of gentamicin susceptibility of *P. aeruginosa* PAO1 incubated in viscous solutions containing 0% PVP (w/v), 7.5 % PVP, 10% PVP, 12.5% PVP and 15% PVP for 6.5 hours prior to adding antibiotic.

% PVP (w/v)	MIC (μg/ml)	P-value*	MBC (μg/ml)	P-value*
0	2.6	n/a	6.1	n/a
7.5	3.0	0.5796	5.3	0.7328
10	7.0	0.1328	21.1	0.0667
12.5	9.2	0.0486	16.0	0.0729
15	10.6	0.0582	36.8	0.01

* The p-value was obtained by comparing the susceptibility in viscous solution to the control (0% PVP) using an unpaired t-test.

Table 4. Geometric mean of piperacillin susceptibility of *P. aeruginosa* PAO1 incubated in viscous solutions containing 0% PVP (w/v), 7.5 % PVP, 10% PVP, 12.5% PVP and 15% PVP for 6.5 hours prior to adding antibiotic.

% PVP (w/v)	MIC (µg/ml)	P-value*	MBC (µg/ml)	P-value*
0	3.5	n/a	128.0	n/a
7.5	8.0	0.2268	891.4	0.0081
10	8.0	0.172	776.0	0.0126
12.5	10.6	0.0922	1448.2	<0.0001
15	12.1	0.079	891.4	0.0004

* The p-value was obtained by comparing the susceptibility in viscous solution to the control (0% PVP) using an unpaired t-test.

Table 5. Geometric mean of gentamicin susceptibility of *P. aeruginosa* PAO1 incubated in viscous solutions containing 0% PVP (w/v), 7.5 % PVP, 10% PVP, 12.5% PVP and 15% PVP O/N followed by a 1:100 dilution and 3 hour incubation prior to adding antibiotic.

% PVP (w/v)	MIC (μg/ml)	P-value*	MBC (μg/ml)	P-value*
0	2.2	n/a	3.2	n/a
7.5	1.2	0.0761	1.6	0.1832
10	1.4	0.1774	5.7	0.3989
12.5	1.6	0.3605	4.0	0.6703
15	2.0	0.5995	4.5	0.2094

* The p-value was obtained by comparing the susceptibility in viscous solution to the control (0% PVP) using an unpaired t-test.

Table 6. Geometric mean of piperacillin susceptibility of *P. aeruginosa* PAO1 incubated in viscous solutions containing 0% PVP (w/v), 7.5 % PVP, 10% PVP, 12.5% PVP and 15% PVP O/N followed by 1:100 dilution and 3 hour incubation prior to adding antibiotic.

% PVP (w/v)	MIC (μg/ml)	P-value*	MBC (μg/ml)	P-value*
0	2.5	n/a	3.6	n/a
7.5	2.2	0.549	3.2	0.7805
10	2.5	1	6.1	0.302
12.5	3.6	0.4105	7.1	0.1086
15	4.0	0.3409	7.1	0.245

* The p-value was obtained by comparing the susceptibility in viscous solution to the control (0% PVP) using an unpaired t-test.

Table 7. Geometric mean of amphotericin B susceptibility of *C. albicans* ATCC 14053 incubated in viscous solutions containing 0% PVP (w/v), 7.5 % PVP, 10% PVP, 12.5% PVP and 15% PVP for 24 hours prior to adding antifungal.

% PVP (w/v)	MIC (μg/ml)	P-value*	MFC (μg/ml)	P-value*
0	0.063	n/a	0.35	n/a
7.5	0.063	1.000	1.0	0.0910
10	0.088	0.3879	2.0	0.0367
12.5	0.088	0.3879	2.0	0.0367
15	0.125	0.0050	4.0	0.0454

* The p-value was obtained by comparing the susceptibility in viscous solution to the control (0% PVP) using an unpaired t-test.

CHAPTER 4: DISCUSSION

Numerous studies have shown that adhesion to a solid surface triggers biofilm formation (Ceri et al, 1999; Costerton et al, 1995; Davies et al, 1998; Kumon et al, 1995; O'Toole and Kolter, 1998; Qian et al, 1997). Within the biofilm, the expression of a unique sigma factor is believed to alter the microbial phenotype (Costerton et al, 1995). What regulates this change in phenotypic expression remains the next question. It is thought that within an adherent biofilm, bacteria become sessile. In this study, the effect of restricting movement of bacteria within a viscous environment on the expression of a biofilm phenotype as measured by antibiotic resistance is explored. The hypothesis, that a viscous environment can model a biofilm due to the reduced random movement of bacteria resulting from the increased viscosity was tested. Decreased antibiotic susceptibility in a viscous medium, comparable to that seen in biofilms, is supportive of this hypothesis.

There are few published reports on viscosity and bacteria. Many studies explored the effects of a viscous environment on bacterial motility (Berg and Turner, 1979; Ferrero and Lee, 1988; Greenberg and Canale-Parola, 1977; Kimsey and Spielman, 1990; Mitchell et al, 1991; Pietrantino, 1987; Schneider and Doetsch, 1974; Shigematsu et al 1998; Shoesmith, 1960). Studies found that the velocity of bacteria increased initially with an increase in the viscosity of the environment. Further increases in viscosity resulted in a rapid decrease in bacterial velocity (Greenberg and Canale-Parola, 1977; Pietrantino et al, 1987; Schneider and Doetsch, 1974; Shigematsu, 1998; Shoesmith, 1960). The viscosity of the medium in which an organism swims maximally is dependent on the structure of the organism. Spirochaetes have a helical body shape,

which allows them to better navigate through high viscosity than polar flagellated rod-shaped bacteria like *Pseudomonas*. For example, *Campylobacter jejuni* has a maximum velocity at 40 cP (Shigematsu et al, 1998) compared with *P. aeruginosa* at 2 cP (Greenberg and Canale-Parola, 1977). At a certain viscosity, known as the minimum immobilizing viscosity (MIV), bacteria lose translational motility as well as other types of movement (Greenberg and Canale-Parola, 1977; Lawrence et al, 1992, Szymanski et al, 1995). The MIV is also organism specific. The MIV of *P. aeruginosa* was 60 cP (Greenberg and Canale-Parola, 1977). From these reports, it can be concluded that viscosity is a possible model for bacterial movement restriction.

Polyvinylpyrrolidone (K-360), a high molecular weight polymer, was used in this study because the addition of PVP to Mueller-Hinton broth strongly increased the viscosity (Figure 1) and had negligible effects on osmolality (Petrino and Doetsch, 1978) (Figure 2). It has been used in numerous bacterial viscosity experiments (Kawagashi et al, 1996; Lawrence et al, 1992; Nakamura et al, 1988; Petrino and Doetsch, 1979; Schneider and Doetsch, 1974; Shigematsu et al, 1988). One study chose PVP as the viscosity-conferring compound because it mimics the ecological niche of *Helicobacter pylori*, the gastric mucous layer (Nakamura et al, 1988). The viscosities obtained from the addition of PVP to solution (Figure 1) are consistent with literature (Nakamura et al, 1988; Petrino and Doetsch, 1979; Schneider and Doetsch, 1974). However, one study stated that the viscosity of a 20% (w/v) PVP-360 was between 165-200 cP (Lawrence et al, 1992), which is dramatically different from the viscosities obtained in literature and in this study. The maximum concentration of PVP-360 used in this study was a 15% PVP which had a viscosity of approximately 1600 cP. According to Rubinstein (1977), a

linear relation between shear rate and shear stress occurs at all PVP concentrations, therefore even at very high concentrations, PVP solutions behave as perfect Newtonian solutions. A Newtonian fluid is characterized by a viscosity which is independent of the shear rate at which it is measured (Howard, 1991).

Comparison between a more viscous solution to a less viscous solution can be problematic because more of the viscosity-conferring compound (PVP) must be used. This will result in the displacement of molecules such as water. Loss of available water will affect bacterial growth as well as motility. However, one study (Schneider and Doetsch, 1974) found that a solution of 10% PVP (w/v) became turbid with bacteria after 8 hours at room temperature and the bacteria, upon dilution of the 10% PVP, were motile. In these investigations, a maximum PVP concentration of 15% (w/v) was used. Bacterial growth was not inhibited at this viscosity. PVP molecules aggregate to form spheres resulting in a minimum interparticulate contact area. As the concentration of PVP increases, the size of the spherical aggregates increase but the number remains the same (Rubinstein, 1977). This is a possible explanation for the lack of bacterial growth inhibition with an increase in viscosity. PVP is a good choice as a viscosity-conferring compound for these investigations.

The growth curves demonstrated that viscosity did not have a negative effect on growth of either *P. aeruginosa* or *C. albicans* (Figures 4 and 5). The final cell yields were significantly higher in the viscous solutions than the non-viscous media (0% PVP). Several studies found that the viscosity of the growth medium did not affect the growth rates of any of the microorganisms studied. (Ballesteros et al, 1993; Lawrence et al, 1992). Stecchini et al (1998) reported that the presence of PVP-30 in broth media did not

affect cell yield of *B. cereus* and the final counts were similar to those obtained in unsupplemented media, however a decrease in growth rate occurred. High viscosity can greatly interfere with the growth of microorganisms because transport processes between cells and the aqueous environment eventually slow down (Gould and Christian, 1988). As mentioned earlier, PVP maintains a minimum interparticulate contact area. This is a possible explanation why the growth of *P. aeruginosa* and *C. albicans* was not inhibited, however it is not an explanation for the increased growth of organisms in PVP solutions. Lawrence et al (1992) found that *Proteus mirabilis* swarmer cells (found on solid surfaces) had increased growth rate as compared to swimmer cells. The organisms grown in the viscous solutions could be expressing a biofilm phenotype resulting in a higher cell yield than the planktonic population. Figure 4 demonstrates that the growth of the planktonic *P. aeruginosa* population (0% PVP) leveled off at a concentration of 10^7 CFU/ml at hour 10. A growth curve of a *P. aeruginosa* biofilm using the CBD found that a concentration of 10^7 CFU/peg was reached at hour 6 (unpublished data). It is difficult to compare the growth of organisms in liquid media to biofilm growth because cell counts are expressed in different units. Organisms in liquid media are usually expressed as CFU per ml whereas often times biofilm cell counts are expressed as the number of colony forming units per surface adhered to such as CFU/peg in the Calgary Biofilm Device (Ceri et al, 1999) or CFU/cell desk (Shigeta, 1997) or CFU/catheter surface area (cm^2) (Anwar et al, 1989) used with the modified Robbin's device. A previous study reported that biofilms at concentrations of 10^8 CFU/ml could be produced in a closed system (Prosser et al, 1987). Biofilm bacteria have been shown to be more metabolically active than their planktonic counterparts (Nickel et al, 1985). However, Anwar et al

(1992) report biofilm cells as slow growing organisms, which are less metabolically active than planktonic cells. Reports by Foley and Gilbert (1997) and Lawrence et al (1992) state that biofilm growth rate is slow relative to batch culture (planktonic). The findings in this study are not supportive of this fact. Cell yields greater than the planktonic culture were observed in viscous solutions (Figures 4 and 5). This is the first known report of increased growth of microorganisms in a viscous environment.

Many bacteria modulate their gene expression to cope with various changes in environment such as nutrient, temperature, cell density which can result in dramatic changes in cell morphology (Kawagashi et al, 1996). Morphological differences were observed in *P. aeruginosa* and *C. albicans* with changes in the viscosity of the growth media. The cell size of planktonic and biofilm *P. aeruginosa*, and the bacteria grown in viscous solutions containing 7.5% PVP, 10% PVP, 12.5% PVP, and 15% PVP was determined (Figure 7). Anwar et al (1992) report that embedded biofilm cells are smaller than surface biofilm cells which resemble the planktonic organisms. This is not supported by observations in this study. A significant cell size increase was observed when comparing the *P. aeruginosa* biofilm population to the planktonic population (Figure 7). The adhesion of bacteria to surfaces and subsequent biofilm formation appears to result in an increase in cell size. Wright et al (1988) reported filamentous growth in a mucoid strain of *Pseudomonas aeruginosa* under conditions of magnesium depletion in planktonic and biofilm cells. Non-mucoid strains did not demonstrate this type of growth. These observations suggest that cellular morphology may be affected by growth conditions. In this study, *P. aeruginosa* was not observed as filamentous when grown in viscous solutions or under biofilm conditions. This could be due to the fact that

P. aeruginosa PAO1, the strain used in this study, is non-mucoid. Swarming bacteria such as *Vibrio* and *Proteus* have exhibited cell elongation upon exposure to solid surfaces (Atsumi et al, 1996; Kawagashi et al, 1996, Lawrence et al, 1992; Sar et al, 1990). The organisms grown in the viscous solutions were also larger than the planktonic cells and of comparable size to the biofilm cells (Figure 7). This further supports the theory that bacteria in viscous environments express a biofilm phenotype. Contrary to the observations of *P. aeruginosa*, a reduction in size of *C. albicans* cells was seen as the viscosity increased (Figure 10). This could be related to the increase in osmolality with increasing viscosity. Water loss to the environment is more likely to occur in *C. albicans* than in *P. aeruginosa* due to the structure of the cell wall. Fungal cell walls consist of chitin and/or cellulose as opposed to peptidoglycan found in bacteria (Creager et al, 1990). There are no published reports on the effect of viscosity on cell size, however Stecchini et al (1998) found that when the viscosity of the system increased, a significant reduction in the colony size of *B. cereus* occurred.

Flagella expression is dependent on environmental factors in some organisms. *Vibrio sp.* possess two types of flagella: a single polar flagellum and lateral flagella. The polar flagella are constitutively expressed in both swimming cells found in liquid media and swarming cells found on solid surfaces. The numerous lateral flagella are expressed only in swarmer cells. Experiments have shown that a high viscosity environment induces the expression of lateral flagella (Atsumi et al, 1996; Kawagashi, 1996; Lawrence et al, 1992; Sar et al, 1990). It was postulated that *P. aeruginosa* would not express flagella under biofilm conditions because the organisms were immobile thus not requiring a flagella. In addition, the organisms grown at the various viscosities would not

present flagella because the viscous environment would trigger a biofilm phenotype. This was not the case in these investigations. A polar flagella was present in all planktonic bacteria (0% PVP) as well as the organisms exposed to the various solutions of PVP (Figure 6A-E). Surprisingly, flagella were shown on the biofilm bacteria as well (Figure 6F). Currently, there are no published reports on the presence of flagella in biofilms. However, it has been reported that flagella and pili are necessary in biofilm formation of *P. aeruginosa* and *E.coli* (O'Toole and Kolter, 1998; Pratt and Kolter, 1998). This was determined by following biofilm formation in strains mutant in flagella and pili production. It was found that *P. aeruginosa* without flagella were not able to initiate biofilm formation, whereas pili deficient organisms formed a monolayer of cells but were not able to form a dense biofilm matrix. Pili are responsible for surface associated movement known as twitching motility (Bradley, 1980). For *E. coli* biofilm formation, flagella were required for initial surface contact and pili were required for adhesion. Bacteria use different approaches to initiate biofilm formation but motility appears to be the common denominator for biofilm formation for these two organisms. In this study, *C. albicans,* a non-motile fungus easily formed a biofilm. Other non-motile microorganisms such as Gram positive *Staphylococcus epidermidis* and *Staphylococcus aureus* have been reported to exist as biofilms (McDermid et al, 1993; Power et al, 1990). Further studies must be done to elucidate the role of motility on biofilm formation. The author is unaware of any literature on flagella expression in *P. aeruginosa* in response to a viscous environment.

The results obtained from the antibiotic testing of gentamicin and piperacillin for *P. aeruginosa* and amphotericin B for *C. albicans* in this study (Tables 1-2) clearly

demonstrates antibiotic resistance of organisms in biofilm mode. By using an established *in vitro* model of colonization (Ceri et al, 1999), *P. aeruginosa* PAO1 was found to be resistant to piperacillin and showed some resistance to gentamicin. *C. albicans* ATCC 14053 demonstrated some resistance to amphotericin B but the MBEC was still within an achievable drug concentration. These findings further support that antimicrobial activities of various agents used for treatment of adherent populations are better tested using an *in vitro* model of biofilm formation as opposed to an *in vitro* susceptibility model of planktonic organisms.

It has been shown that the restriction of bacterial movement by adhesion to surfaces is a signal for the expression of a biofilm phenotype. As demonstrated above, biofilm formation results in antibiotic resistance. In these investigations, bacteria and fungi were grown in viscous environments to determine if restriction of movement is a signal for biofilm phenotypic expression. Decreased antibiotic susceptibility could be an indication of the expression of biofilm phenotype. The minimum bactericidal concentration (MBC) of gentamicin (36.76 μg/ml) (Table 3) and piperacillin (1448.15 μg/ml) (Table 4) on *P. aeruginosa* in viscous solutions was similar to the MBEC of the biofilm population (gent- 16 μg/ml; pip- 2048 μg/ml) (Table 1). *C. albicans* had an MFC in 15% PVP (4 μg/ml) (Table 9) which was significantly higher than in the planktonic population (0.0935 μg/ml) (Table 2) but was not as high as the MBEC of the biofilm bacteria (16 μg/ml) (Table 2). An explanation for why *P. aeruginosa* presented an antibiotic resistance comparable to the biofilm whereas *C. albicans* was significantly lower could be related to the vibrational frequencies of the organisms (Szymanski et al, 1995). The rationale behind the expression of a biofilm phenotype in organisms grown in

viscous environments is that the immobilization of the organism acts as a signal. The organisms become immobile because it loses its swimming ability but moreover the frequency of their vibrations is affected. Whether they completely stop vibrating or alter their frequency is unknown. Organisms vibrate with a characteristic frequency that applies a strain to the viscous solution. With any gel-like material, the apparent viscosity varies with the strain placed on the solution (King, 1980). Since organisms vibrate at different frequencies, they may behave as if they were in solutions of quite different viscosity (Szymanski et al, 1995). Previous experiments found that the viscosity of reconstituted intestinal mucus ranged between 178 and 316 cP at 16 Hz. However, when exposed to a frequency of 30 Hz, the viscosity was estimated to be approximately 140 cP (Bell and Allen, 1984; Mantle et al, 1990; Sellers et al, 1983). It is not certain the actual viscosity experienced by *P. aeruginosa* and *C. albicans* in this experiment.

Two *P. aeruginosa* inocula were used in this study: 6.5 hour, and 20 hour followed by 1:100 dilution and 3 hour incubation. The resulting antibiotic susceptibilities in viscous solutions were drastically different. The antibiotic susceptibilities are similar in the planktonic populations (0% PVP) and are comparable to the results obtained following NCCLS protocol. Antibiotic resistance increased with increasing viscosity. The MBC of gentamicin and piperacillin in some of the viscous solutions using the 6.5 hour inoculum were comparable to the MBEC obtained from the biofilm population. Further studies should include the kinetics of biofilm expression in viscous solutions in order to determine when organisms in viscous environments behave like biofilms. The differences in antibiotic susceptibility could also be attributed to inoculum size. The antibiotic susceptibilities obtained using the 20-hour inoculum followed by 1:100 dilution

and further incubation vastly differed from the 6.5-hour inoculum. These discrepancies could be due to inoculum concentration. The concentration (5×10^6 to 5×10^7CFU/ml) was approximately one log smaller than the 6.5-hour inoculum concentration (5.0×10^7 to 5.0×10^8 CFU/ml). The difference in antibiotic susceptibility could possibly be attributed to growth rate. The 20-hour inoculum may be still in lag phase as opposed to the 6.5-hour inoculum that is in log phase.

Numerous studies report that growth rate affects the susceptibilities of biofilm bacteria to antibiotics (Anwar et al, 1990; Anwar et al, 1992; Brown et al, 1990; Evans et al, 1991; Gilbert et al, 1990; Shigeta et al, 1997). Growth rate alterations affect the physiology of bacteria such as cell envelope composition, which in turn can cause dramatic changes of the cell's susceptibility to antimicrobial agents (Brown et al, 1990). It has been reported that the susceptibility of β-lactam antibiotics increase with growth rate because the expression of penicillin binding proteins are growth rate dependent (Brown et al, 1988; Gilbert et al, 1990). Antimicrobial agents that target cell wall activity are highly dependent on growth rate. In a study by Shigeta et al (1997), *P. aeruginosa* biofilm cells grew exponentially until day 3 after which cultures remained stationary. Piperacillin had little effect on 3 and 5-day-old biofilms, whereas 1-day-old biofilms were affected. Another study reported *P. aeruginosa* biofilms grew exponentially until day 5 (Anwar, 1989). Low doses of tobramycin (5 μg/ml) eradicated 2-day-old biofilms, whereas 7-day-old biofilms were not killed after exposure to 20 μg/ml. These studies suggested that the resistant biofilms were in stationary phase and thus exhibiting a slow rate of growth, which resulted in antibiotic resistance. They concluded the biofilm expression system was extremely sensitive to growth rate. Studies by Ceri et al (1999)

and Nickel et al (1985) contradict the aforementioned findings. It was demonstrated that an antibiotic resistant *P. aeruginosa* biofilm could be produced in 4 hours (Ceri et al, 1999). Nickel et al (1985), found that an 8-hour *P. aeruginosa* biofilm was resistant to tobramycin.

It is unlikely that growth rate was the cause of the differences in antibiotic susceptibility in the viscous solutions among the different inocula. Increased resistance to antibiotics is exhibited by organisms which have a slow growth rate and are in stationary phase (Anwar et al, 1990; Brown et al, 1988; Shigeta et al, 1997). The slower growing population in this study (20-hour inoculum) was more susceptible to gentamicin and piperacillin than the log phase 6.5-hour inoculum. The inoculum size can be attributed to the changes in antibiotic susceptibility observed. Stringent control on growth rate and inoculum size must be made in order to achieve reproducible results.

The biofilm mode of growth confers on the organism an increased resistance to antibiotics and biocides (Anwar et al, 1989; Nickel et al, 1985; Raad et al, 1995; Wilson, 1996). The inherent resistance of biofilms to antimicrobial agents is proposed to occur via several mechanisms. One theory suggests that the antimicrobial agent is not able to penetrate the biofilm. It has been shown that antibiotics penetrate biofilms at different rates depending on the agent and the biofilm (Hoyle et al, 1992). A second hypothesis for biofilm resistance proposes that biofilms are under nutrient limitations resulting in slow-growing cells (Anwar et al, 1992). These cells have low metabolic activity resulting in decreased susceptibility to agents. A third model for biofilm resistance postulates that biofilm cells adopt a phenotype that is distinct from planktonic cells in response to growth on a solid surface.

The results in this study are not supportive of the reduced antibiotic penetration hypothesis for resistance. The negative stains of the planktonic and biofilm *P. aeruginosa* and of organisms grown in the different viscous solutions illustrated a glycocalyx layer surrounding the biofilm cells (Figure 6). No glycocalyx was observed around any of the organisms grown in the viscous solutions, however these organisms exhibited biofilm-like resistance to antibiotics. Transmission electron microscopy of ferritin-stabilized glycocalyx should be performed on the organisms grown in viscous solutions to confirm that glycocalyx is not present in these organisms. Further and more detailed examination of glycocalyx production is needed to prove that reduced penetration of agents is not a mechanism for resistance. Findings in this study contradict the theory that biofilm cells are slow growing and less metabolically active. *P. aeruginosa* grown in viscous solutions and biofilm populations were larger in size than planktonic cells (Figure 7). Anwar et al, 1992 indicated that slow growing cells were smaller in size than metabolically active cells. In addition, a greater cell yield was observed in *P. aeruginosa* and *C. albicans* grown in viscous solutions than in a planktonic environment (Figures 4 and 5). These observations suggest that biofilms are not slow growing populations. Results from these investigations are supportive of the third model proposed for biofilm antibiotic resistance, that biofilms adopt a distinct and protective phenotype. Indications of this unique biofilm phenotype were increased cell size, increased growth rate and antibiotic resistance of organisms in viscous solutions and in biofilm mode. The findings in this study suggest that growth on a solid surface is not necessarily the signal for biofilm expression. The expression of the biofilm

phenotype may be due to a decrease in movement or vibrational frequency caused by adherence to a solid surface or exposure to a viscous environment.

A biofilm phenotype was induced by the restriction of movement using a viscous environment. *P. aeruginosa* and *C. albicans* in viscous solutions had greater cell yields than the planktonic population. Morphological differences were observed with changes in the viscosity of the growth media. *P. aeruginosa* cells grown in viscous solutions and under biofilm conditions were larger than the planktonic cells. A reduction in the size of *C. albicans* was seen as the viscosity increased. Biofilm-like antibiotic resistance was observed under viscous conditions. An increase in resistance to the antimicrobial agents tested occurred with an increase in the viscosity of the environment. The MBC values for gentamicin and piperacillin grown at high viscosity were similar to the biofilm MBEC. A viscous environment may induce biofilm phenotypic expression. Further studies on the effect of viscosity on the expression of a biofilm phenotype should include the expression of biofilm genes in organisms grown in viscous solutions, the determination of vibrational frequencies of organisms in viscous environment, and the visualization of intact glycocalyx by TEM upon ferritin stabilization of the glycocalyx layer. This is the first known report of viscosity as a mechanism for biofilm phenotypic expression.

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