Effects of initial adhesion events on the physiology of Pseudomonas aeruginosa
by Elinor deLancey Pulcini

A dissertation submitted in partial fulfillment Of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University

Abstract:
Bacteria in biofilms have been shown to be metabolically and physiologically different from planktonic bacteria. Most studies have been conducted on mature biofilms that can be days or more old. Previous experiments suggest that wholesale changes in protein expression do occur during the first few hours of attachment, indicating a general change in physiology. An understanding of the physiologic changes that occur in a bacterial cell during initial biofilm development is crucial for the eventual control of biofilm formation.

. The goal of this research project was to elucidate the changes in physiology and metabolism that occur in Pseudomonas aeruginosa during biofilm formation with particular interest paid to the processes of initial adhesion, defined as less than or equal to 3 hours. Chemostat grown cultures were used to inoculate flow cells and to inoculate small vessels of Teflon™ mesh for 1, 2 and 3 hours. RNA analysis showed a significant increase in the concentration of total RNA per cell during attachment. SDS PAGE analysis indicated the presence of protein bands at three hours not present in the planktonic samples, suggesting de novo protein synthesis.

Two dimensional (2 D) gel electrophoresis showed changes in protein expression throughout the 3 hour experimental period with a total of 55 proteins found to be differentially expressed. Eight proteins not visualized in planktonic samples were up expressed in as little as 10 minutes of attachment Twenty five proteins were selected for analysis using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Proteins involved in LPS and alginate production, virulence factor expression and antibiotic resistance were found to be up regulated during initial adhesion. A comparison of differential protein expression during stress conditions and during attachment was made in order to assess the involvement of global regulatory mechanisms induced during biofilm development. Results suggest that various signal transduction pathways were up regulated.

The relationship of global stress response expression and the induction of signal transduction pathways in the development of biofilms may provide valuable information for future biofilm control methodologies.

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APPROVAL

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Elinor deLancey Pulcini

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission the College of Graduate Studies.

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Finally, I dedicate this work in memory of my parents.

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ABSTRACT

Bacteria in biofilms have been shown to be metabolically and physiologically different from planktonic bacteria. Most studies have been conducted on mature biofilms that can be days or more old. Previous experiments suggest that wholesale changes in protein expression do occur during the first few hours of attachment, indicating a general change in physiology. An understanding of the physiologic changes that occur in a bacterial cell during initial biofilm development is crucial for the eventual control of biofilm formation.

The goal of this research project was to elucidate the changes in physiology and metabolism that occur in *Pseudomonas aeruginosa* during biofilm formation with particular interest paid to the processes of initial adhesion, defined as less than or equal to 3 hours. Chemostat grown cultures were used to inoculate flow cells and to inoculate small vessels of Teflon™ mesh for 1, 2 and 3 hours. RNA analysis showed a significant increase in the concentration of total RNA per cell during attachment. SDS PAGE analysis indicated the presence of protein bands at three hours not present in the planktonic samples, suggesting *de novo* protein synthesis.

Two dimensional (2 D) gel electrophoresis showed changes in protein expression throughout the 3 hour experimental period with a total of 55 proteins found to be differentially expressed. Eight proteins not visualized in planktonic samples were up expressed in as little as 10 minutes of attachment

Twenty five proteins were selected for analysis using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Proteins involved in LPS and alginate production, virulence factor expression and antibiotic resistance were found to be up regulated during initial adhesion. A comparison of differential protein expression during stress conditions and during attachment was made in order to assess the involvement of global regulatory mechanisms induced during biofilm development. Results suggest that various signal transduction pathways were up regulated .

The relationship of global stress response expression and the induction of signal transduction pathways in the development of biofilms may provide valuable information for future biofilm control methodologies.

CHAPTER 1

INTRODUCTION

A biofilm can be defined as a community of microorganisms embedded within an exopolysaccharide matrix. The majority of bacteria in the environment are found attached to surfaces rather than suspended as unicellular, freely suspended planktonic cells. Biofilms are found in almost every environmental system studied and in nearly every industrial and medical setting where microbial contamination is a problem.

Biofilms are complex heterogeneous systems replete with channels, streamers and other forms and shapes that allow bacteria within the biofilm to exist under various conditions of flow (Costerton and Lewandowski, 1995) and allow for the circulation of nutrients and oxygen deep within the biofilm matrix layer (Stewart et al.,1993; Xu et al., 1998). The presence of communication/signaling molecules within the biofilm matrix appears to determine the architecture of the matrix and, in some cases, may induce detachment of parts of the biofilm (Davies et al., 1998). As the bacterial cell attaches to a surface and begins the process of biofilm formation, clear differences with respect to changes in the physiology and metabolism between planktonic and biofilm bacteria occur. For example, bacterial cells within a biofilm have been shown to have greater resistance to antibiotics and biocides than freely suspended cells (Cochran et al., 2000; Costerton et al., 1999)

Biofilm studies usually have been conducted on mature biofilms that are several hours to days old. This approach is inadequate if the intent is to identify the

nature and time point at which cells transition from a planktonic to a biofilm phenotype. The aim of this study has been to determine changes in the physiology of *Pseudomonas aeruginosa* during initial adhesion, defined as less than or equal to 3 hours of adhesion time, by means of the examination of differential protein expression.

The overall goal of this project was to determine the changes in the physiology of bacterial cells during initial adhesion. These changes in physiology were assessed by the examination of differential protein expression patterns. The examination of proteins, also known as products of gene transcription, can provide information as to the genes that are being transcribed during attachment. The specific objectives of this project were:

(1) To demonstrate that discernible changes occur in bacterial cells during the first 3 hours of attachment.

Rationale: Prior to the beginning of this project, very little work was done on cells in the early stages of surface attachment.

(2) To demonstrate that attachment to a surface changes the protein expression profile of bacterial cells.

Rationale: The determination of the changes in protein expression would provide information regarding the existence of a biofilm phenotype.

(3) To analyze the proteins expressed during initial adhesion.

Rationale: The determination of the differentially expressed proteins will provide information regarding the up regulation signal transduction pathways crucial for initial attachment and eventual biofilm growth.

The dynamics of biofilm development are reviewed in Chapter 2 entitled "A Review of Physiologic and Metabolic Changes in the Bacterial Cell during Biofilm Development". An abbreviated and condensed version of this chapter has been published as an article entitled "Biofilms: Sensing and Signaling" in the California Dental Association Journal May 2001. At the inception of this project, it was unknown if it would be possible to detect molecular level differences occurring in bacterial cells during initial adhesion since the majority of research projects at that time focused on mature biofilms. Chapter 3 summarizes the work done to assess preliminary differences in these cells and the results contained therein satisfy objective 1. In order to assess accurately changes in protein expression on a global scale, a complete protein profile must be made. Two dimensional (2 D) gel electrophoresis is the method of choice for this type of analysis. Results from differential protein expression profiles during initial adhesion appear in Chapter 4. This chapter entitled "Variations in Protein expression in *Pseudomonas aeruginosa* during Initial Adhesion" was submitted to FEMS Microbiological Letters. The results from chapters 3 and 4 satisfy objective 2. The determination of the actual proteins using the mass spectrometric method MALDI-TOF is described in Chapter 5 entitled "MADLI-TOF Analysis of Differential Protein Expression in *Pseudomonas aeruginosa* during Initial Adhesion. Chapters 4 and 5 were combined into one paper

entitled "Analysis of Differential Protein Expression in *Pseudomonas aeruginosa* during Initial Adhesion using 2 D Gel Electrophoresis and MALDI-TOF" submitted to Microbiology. Global networks called signal transduction pathways often regulate the control of protein expression in bacteria. The assessment of the role of signal transduction pathways during initial adhesion is described in Chapter 6. This chapter entitled "Evidence for Global Regulation of the Biofilm Phenotype in *Pseudomonas aeruginosa*" was submitted to Molecular Microbiology. This chapter specifically addresses objective 3. The integration of all results is summarized in the final chapter of this dissertation.

CHAPTER 2

A REVIEW OF PHYSIOLOGIC AND METABOLIC CHANGES IN THE BACTERIAL CELL DURING BIOFILM DEVELOPMENT

Introduction

Biofilms exist. People have been aware of biofilms for hundreds if not thousands of years. The fouling of ships' hulls, dental plaque, and slimy seaside rocks are all examples of biofilms. Despite the long history that man has had with biofilms, it has only been in the last 15 years that the concept of a biofilm has come to the forefront of scientific research. The awareness that bacteria do not usually live in the environment in suspensions of single cells, but instead form complex biofilm habitats (Costerton and Lewandowski, 1995; Stickler, 1999), has significant ramifications for the relevance of how we study most bacterial species. Biofilms are found in almost every environmental system studied and in nearly every industrial and medical setting where microbial contamination is a problem (Potera, 1996; Costerton et al., 1999). Anywhere there is water or moisture and a surface, biofilms will grow. This leads to the clogging of industrial water supplies and the contamination of source waters for food processing and other manufacturing (Costerton et al., 1999). The development of these biofilms can be a great cause for concern. In the drinking water industry, for example, biofilms form within the piping of the treatment plant and throughout the water distribution system. The potential for

the existence of pathogens sequestered within these biofilms has been the focus of regulatory concerns (Camper, 2000).

The problems associated with bacterial contamination is an excellent illustration of a basic precept in biofilm science: **biofilms are the preferred mode of growth for most bacteria**. Existence as a biofilm provides bacteria with a protective environment that effectively prevents attack by antimicrobials, biocides, and even by immunologic factors. Biofilms are costly for industry due to their biofouling potential which can cause pressure drop or product degradation (Costerton and Lewandowski, 1995). The detachment of biofilms has been implicated in the contamination of food and household products during manufacture and processing. Biofilms are also associated with public health issues. For example, biofilms in drinking water systems may act as a reservoir for potential pathogens (Stickler, 1999). In the human body, there is a direct relationship between the presence and severity of dental plaque, a biofilm, and the increased potential of suffering a heart attack or stroke (Potera, 1996). Despite the growing body of research into biofilm formation, relatively little is known about the metabolism and physiology of biofilm bacteria (Costerton et al., 1999).

Attached bacteria produce an exopolysaccharide matrix that can act as a protective polymer for the cells embedded within. As the biofilm grows and thickens, it begins to develop into a heterogeneous matrix interspersed with channels that allow nutrients and oxygen to penetrate into the depths of even the thickest biofilms. Researchers have shown that the cells within the biofilm matrix exhibit differences in

physiology depending on their location. This concept of spatial heterogeneity within a biofilm has been applied to oxygen limitations (from aerobic to anaerobic), pH, nutrients, and rates of growth (Hasset et al., 1999; Xu et al., 1998). Within a thick biofilm, there exist various microniches that allow for numerous types of metabolic processes to take place. Dental plaque is an excellent example of the complexity of microorganisms that can exist within a biofilm with a range of metabolic capabilities (Whittaker et al., 1996).

The development of a biofilm appears to be a very effective survival strategy for bacteria. The cells within the biofilm exhibit an increased resistance to biocides and antimicrobials in comparison to planktonic cells. A number of hypotheses have been put forth to attempt to explain this phenomenon. In some cases, there is a limitation to the penetration of the antimicrobials into the biofilm matrix. Since cells within the matrix are living at different physiologic states, the rate of uptake of the antimicrobial into the cell can be affected. The exopolysaccharide of the biofilm matrix may provide a physical barrier to the penetration of antimicrobials (Stewart et al., 1998). The differences in bacterial cell physiology within the biofilm will reduce the susceptibility of cells to some antimicrobials such as growth-dependent antibiotics (De Kievit et al., 2001). However, diffusion and growth limitations alone may not account for the entire decrease in susceptibility to antimicrobials seen in biofilm cells. A study of the effects of antibiotics on *Klebsiella pneumoniae* biofilms grown on microporous polycarbonate membranes showed that ampicillin, unable to penetrate the biofilm matrix, cannot kill *K. pneumoniae* biofilm cells. In contrast, ciprofloxacin

was shown to be able to diffuse through the *K. pneumoniae* biofilm in as little as 20 minutes. However, *K. pneumoniae* cells were resistant to ciprofloxacin at even 10 times the established MIC (Anderl et al., 2000). This suggests the physiologic changes that the planktonic bacterium undergoes as it becomes a biofilm cell may somehow also affect its susceptibility to various antimicrobials.

As the biofilm develops, bacterial cells within the matrix will release chemical signals. These signal molecules may enable the bacterial colonies to develop the characteristics of a more mature biofilm. A number of bacterial species, both gram-positive and gram-negative, use these chemical signal molecules to coordinate activity (Davies et al. 1998). The action of these signal molecules relies on a process called quorum sensing. In quorum sensing, the ability of the molecule to cause an action is dependent on its concentration within the environment. That concentration can increase only when there is a sufficient number of bacterial cells producing that particular signal. Probably some of the best known quorum sensing systems are found in marine bacteria of the genus *Vibrio*. Species of this bacterial genus symbiotically colonize the light organs of certain fish or squid and will emit luminosity only when the population density has reached sufficient quorum density numbers (Ruby and McFall-Ngai, 1999). The cell-to-cell signaling systems of *P. aeruginosa* have been extensively studied as a model for quorum sensing during biofilm development by gram-negative bacteria and will be discussed in more detail later in this chapter.

Research into the cell-to-cell signaling capabilities of gram-positive biofilm forming bacteria has also been on-going. Mutants of *Streptococcus gordonii*, a gram-positive bacterium that initiates the formation of dental plaque, were assayed for defective biofilm formation. In this particular study, there were nine mutants shown to have defects in genes of known function that could not form biofilms. One of the genes identified, *comD*, is a known component of the cell-to-cell signaling system in gram-positive bacteria (Loo et al. 2000).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative organism found in a wide range of environments including water, soil, and plants and is also an opportunistic human pathogen (Deziel et al. 2001). It has been implicated in nosocomial infections, infections in burn patients and immunocompromised patients, is commonly found in biofilms on indwelling medical devices and is the cause of chronic infection in cystic fibrosis (Van Delden and Iglewski 1998; Costerton et al., 1999; Donlan 2001; Singh et al., 2000).

The complete genome of *P. aeruginosa* PAO1 has been sequenced (Stover et al., 2000). The genome contains 6.3 million base pairs (Mbp) with 5,570 predicted open reading frames (ORFs) (Stover et al., 2000) which have been annotated by the *Pseudomonas* Community Annotation Project (http://www.pseudomonas.com/index.html). Approximately 20 to 40% of the ORFs have no sequence similarity to other known genes indicating they may be unique to *P. aeruginosa* (Nouwens et al. 2000). The size of the genome and genetic complexity of

this organism may illustrate its adaptability to such a diversity of environments. Approximately 8.4 % of the genome contains regulatory genes, the greatest percentage seen in any bacterial genome to date (Stover et al., 2000). This great regulatory capacity accounts for the ability of *P. aeruginosa* to respond to changes in environmental cues with a global type of regulatory response.

P. aeruginosa possesses an intrinsic resistance to antimicrobial agents. The low permeability of its outer membrane, the presence of multidrug efflux pumps, and its ability to produce beta lactamases all combine to make this organism extremely efficient in its ability to withstand antibiotic challenge (Hancock, 1998; Rocchetta and Lam, 1997).

A number of physiologic factors have been shown to be crucial for the development of biofilms by *P. aeruginosa*. Upon attachment to a surface, there is an increase in the production of alginate and an eventual progression to a mucoid appearance which also enables the attached cells to withstand log increases in antimicrobials (Costerton et al., 1999; Stewart, 1994; Davies et al. 1993; Hoyle et al. 1993). The quorum sensing genes *las/rhl* play a role in the development and maintenance of the biofilm (Davies et al., 1998; De Kievit et al., 2001). Mutant strains of *P. aeruginosa* deficient in the LasR quorum sensing system have been shown to produce biofilms that lack the towers and channels often seen in *P. aeruginosa* biofilms. In addition, these mutant biofilms lack the resistance to treatment by SDS seen in wild type biofilms (Davies et al., 1999). Recently, researchers have isolated quorum sensing molecules produced by *P. aeruginosa* from

the sputum of cystic fibrosis patients suggesting that this is a biofilm disease of the lungs (Singh et al., 2000).

P. aeruginosa 3 day old biofilms cultured under conditions that continually replenished nutrients, have been shown to exhibit higher *rpoS* expression, indicating that the cells may be in stationary phase in the biofilm despite the available nutrients (Xu et al., 2001). Given the growing body of evidence that shows changes in physiology during biofilm development, it has been postulated that these changes are significant enough to result in the development of what is now termed the biofilm phenotype (Costerton, 2000). Crucial to the understanding of the biofilm lifestyle, then, would be the determination of those proteins that are expressed during attachment.

Another factor crucial to the development of *P. aeruginosa* biofilms is motility. Mutants defective in flagellar mediated motility were unable to establish initial adhesion; mutants defective in type IV pilus biogenesis were unable to develop microcolonies or to establish a biofilm structure commonly seen in wild type organisms (O'Toole and Kolter, 1998; Pratt and Kolter, 1999).

A number of factors crucial to the stages of biofilm development in *P. aeruginosa* are also very potent virulence factors. The expression of virulence factors by pathogenic bacteria are tightly regulated and linked to environmental cues (Finlay and Falkow, 1997; Gauthier and Finlay, 1998). A single cue may activate global regulators which in turn can control the expression of a number of unrelated genes (Finlay and Cossart, 1997). Included in the list of regulators identified as virulence

factors are LasR (Tang et al., 1998; Rashid et al., 2000; Singh et al., 2000), alginate (Costerton, 2000), and type IV pili (O'Toole and Kolter, 1998).

Physiologic and Metabolic Changes in Attaching Cells

The biofilm matrix provides sessile bacteria a level of protection that planktonic bacteria do not have. Sessile bacteria protected by a polymeric matrix are better able to survive stresses found in the environment such as temperature fluctuations, low nutrient conditions and protection from UV irradiation (Xu et al. 1998; Elasri and Miller, 1999; Sutherland, 2001). Attached bacteria have been shown to be resistant to antibiotics and biocides when compared to planktonic cells (Costerton et al., 1999; Xu et al., 2000). A number of explanations have been posited as to why biofilms afford this type of protection. The exopolysaccharide matrix can, in some cases, block the transport of the antimicrobial through the biofilm. This however, is not true for all biofilms and for all antimicrobials. Studies have shown that certain antimicrobials are capable of penetrating throughout the matrix (Anderl et al., 2000). The diminished effect of the antimicrobial still exists in these cases as well. Bacteria within the biofilm may live at different states of physiology due to differences of oxygen and nutrients within the biofilm which could then affect uptake mechanisms of the antimicrobial into the cell (Xu et al., 2000).

That bacteria are able to withstand log increases of antimicrobials, even when the specific antimicrobial can penetrate the matrix, indicates that these cells are different in some way from planktonic cells. Recent evidence suggests that biofilm growth results in a different phenotype compared with cells growing in planktonic

suspensions. Not only do attached cells withstand higher levels of biocides for longer periods of time than their planktonic counterparts (Huang et al., 1995; Xu et al., 2000), but research indicates that attached cells of *P. aeruginosa* express different molecules than the sessile cells. Such differential expression results in differences in alginate synthesis, outer membrane proteins, and in signaling molecules such as homoserine lactones between sessile and planktonic cells (Keith and Bender, 1999), (Davies et al., 1999; Stover et al., 2000). The new tools of genetic and molecular techniques have identified regulatory mechanisms involved in biofilm formation indicating that bacteria within the biofilm matrix are very different metabolically and physiologically from their planktonic counterparts.

Cellular attachment to a surface is now viewed as a complex process which may involve flagellar-mediated motility, type IV-mediated twitching motility, outer membrane protein adhesins, the expression of alginate or exopolysaccharides, cell to cell signaling or other still to be elucidated pathways. In *P. aeruginosa*, both flagellar mediated movement and twitching motility are important for initial surface attachment (Deziel et al., 2001). Up-expression of the *P. aeruginosa algC* gene, needed for alginate synthesis, occurs shortly after cell attachment to a surface (Davies and Geesey, 1995). *Vibrio cholerae* 01 El Tor possesses different colony morphologies, smooth and rugose (Watnick et al., 2001). The rugose colony variant produces exopolysaccharide, is capable of biofilm formation and is resistant to chlorine. *In vitro* experiments indicate that switching between the smooth and rugose

phenotypes does occur, indicating a possible genetic link between organisms not able to form biofilms with those that can (Yildiz and Schoolnik, 1999).

The development of advanced genetic and molecular techniques have enabled biofilm researchers to characterize physiologic changes of cells as they transition from a planktonic to a biofilm existence. Analysis of *Streptococcus gordonii* biofilm defective mutants indicates that some of the genes associated with biofilm formation are also involved in signal transduction, peptidoglycan synthesis, quorum sensing, and osmoadaptation (Loo et al., 2000). *P. aeruginosa* mutants for catabolite repression control (Crc), which regulates carbon metabolism, were found to produce a flat, dispersed monolayer of cells in contrast to the characteristic mushroom shaped microcolonies separated by voids (O'Toole et al., 2000). The type of LPS produced has an effect as well. *P. aeruginosa* produces two types of lipopolysaccharides (A band and B band). A band mutants showed minor variation in attachment while B band mutants showed a reduced ability to attach to hydrophilic surfaces and an increased attachment to hydrophobic surfaces (Makin and Beveridge, 1996). In addition, the regulatory pathways appear to be different due to a variety of environmental cues such as nutrient limitation, type of carbon source, pH, or the type of substratum. *P. aeruginosa* and *P. fluorescens* are able to develop biofilms under a variety of growth conditions while *E. coli* K-12 and *E. coli* 0157:H7 have been shown to be more selective as to the effects of media and supplementation on biofilm growth (Dewanti and Wong, 1995; O'Toole et al., 2000). K-12 will not form biofilms in minimal media without amino acid supplementation (Pratt and Kolter, 1999) while

0157 will only form a biofilm under low nutrient conditions (Dewanti and Wong, 1995).

The question must then be asked at which point in biofilm development does the progression into different physiologic states begin? Exponentially growing cells of *P. aeruginosa* have been shown to undergo a surface associated transitional phase during initial attachment with cell division not occurring for 12 hours post adhesion (Rice et al. 2000). It is entirely probable that during this transitional phase, the cell is undergoing regulatory changes that enable it to become a biofilm cell.

Proteomics

The large number of ORFs and the 20 to 40% of these ORFs that have no sequence similarity to other known genes within the *P. aeruginosa* genome illustrates potential limitations to genomic studies. Gene function must be related to the level of response to a particular environmental cue (VanBogelen et al., 1996). The DNA sequence alone cannot provide information concerning the translation of gene products, if they occurred and the extent of post-translational modifications (Humphery-Smith et al., 1997; Gorg et al., 2000).

The proteomic strategy, on the other hand, involves analysis of differentially expressed (induced or repressed) proteins that take a snapshot of the organism at a particular time. A proteomic approach has the advantage of yielding information on *in situ* protein expression that includes post-translational modifications that are often overlooked in a genetic approach (Nouwens et al., 2000). The protemic approach relies heavily on the methodology called two dimensional (2 D) gel electrophoresis.

The process of 2 D gel electrophoresis involves a surface charge fractionation in the first dimension by isoelectric focusing (IEF) and mass driven separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension (O'Farrell, 1975; Gorg et al., 2000). The success of proteomics has been greatly enhanced by improvements in protocols for 2 D gel electrophoresis. These protocols utilize immobilized pH gradient strips for IEF and provide a level of standardization that allows better comparisons to be made between replicate gels and from comparative populations (Bjellqvist et al., 1993; Humphery-Smith et al., 1997; Gorg et al., 2000).

Proteins, visualized as spots within the gels, can be analyzed using one of two methods. Protein sequencing, most commonly N-terminal Edman degradation, provides conclusive information as to the identity of the protein (Humphery-Smith et al., 1997). Unfortunately, the N-terminus can be blocked, usually by formylation or acetylation, and is considered a major limitation to this methodology (Humphery-Smith et al., 1997). Peptide mass fingerprinting by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry has been shown to be a sensitive and reliable method (Jensen et al., 1999; Shevchenko et al., 1996). In this process, the protein of interest is enzymatically cleaved and the resultant peptide mix is analyzed. The peptide mass fingerprint is compared to peptide mass fingerprints in databases.

The analysis of differentially expressed proteins has been further enhanced by the continuing completion of genome sequences, the comparison to and listing of

gene products (proteins) and the availability of this information on world wide web databases. For example, the Protein Prospector website (http://prospector.ucsf.edu/) links to nine different protein databases, enabling the researcher to perform relatively fast and accurate analyses of either peptide mass fingerprinting data from MALDI-TOF or direct sequence data from N-terminal sequencing.

The overview presented in this paper illustrates that a variety of physiologic changes take place within bacterial cells as they attach and form biofilms. An understanding of the physiologic changes that occur in bacterial cells during biofilm development is crucial for the eventual control of biofilm formation.

CHAPTER 3

DETECTION OF CHANGES IN PHYSIOLOGY OF BACTERIAL CELLS DURING INITIAL ADHESION

Introduction

Pervasive in early biofilm research were two assumptions; first, that biofilm bacteria and planktonic or free-floating bacteria were phenotypically the same, and second, that biofilms were relatively simple systems of homogeneous slime. In addition, the traditional microbiological view of bacteria as planktonic organisms influenced biofilm studies (Costerton et al., 1999). Traditional microbiologic techniques such as plating and broth culturing of bacteria are usually performed on freely suspended organisms. These more traditional methodologies have, until recently, warped our view of how bacteria really live and survive in the environment (Anderl et al., 2000; Cochran et al., 2000; Costerton and Stewart, 2000). Only recently has research begun to elucidate the complexity of biofilm formation, specifically with respect to changes in physiology and metabolism of bacteria as they become attached organisms (Huang et al., 1995; Xu et al., 2001; Xu et al., 1998).

When this research project first began in 1995, the majority of biofilm studies had been conducted on mature biofilms that were at least several hours if not days old. A PubMed literature search of papers published prior to 1996 lists 20 citations

for initial adherence and biofilms and of those, only 6 citations deal specifically with initial microbial attachment processes (Busscher et al., 1995; Schumacher-Perdreau et al., 1994; McAllister et al., 1993; Prince, 1992; Lauwers et al., 1990; Marshall and Goodman, 1994). This emphasis on mature biofilms is inadequate if the intent is to identify the nature and time point at which cells transition from a planktonic to a biofilm state. The list of differences between biofilm bacteria and their planktonic counterparts has been growing and now includes a wide variety of characteristics ranging from increased antibiotic resistance to up regulation of genes for alginate production (Bollinger et al., 2001; Costerton, 2000; Hoyle et al., 1993; Lam et al., 1980; Xu et al., 2001; Xu et al., 1998). The extent of these differences has been great enough to cause researchers to postulate the existence of a biofilm phenotype (Costerton, 2000; Loo et al., 2000; McLean et al., 2001; Nesper et al., 2001; Schembri and Klemm, 2001).

A research plan was developed to specifically address changes in the physiology of *Pseudomonas aeruginosa* during initial adhesion, defined as less than or equal to 3 hours of adhesion time. The main objective of this study was to determine if physiologic changes in the bacterial cells could be detected in as little as three hours of adhesion time.

Materials and Methods

Organism and culture conditions

P. aeruginosa ERC1 (ATCC # 700888) was grown to steady state in a chemostat with a residence time of 5 hours at room temperature using a minimal salts

medium as described in the Manual of Industrial Methods (Anonymous, 1986). Glucose at 0.1g/L was used as the carbon source. The chemostat is a 3 liter total volume constantly stirred tank reacter (CSTR). 15.0 ml of *P. aeruginosa* overnight batch culture was used to inoculate the chemostat which was then run in batch mode (no nutrient influent) for 24 hours prior to nutrient inflow. The schematic of the chemostat system and set-up is shown in Figure 1. Samples (total volume of 20 ml)

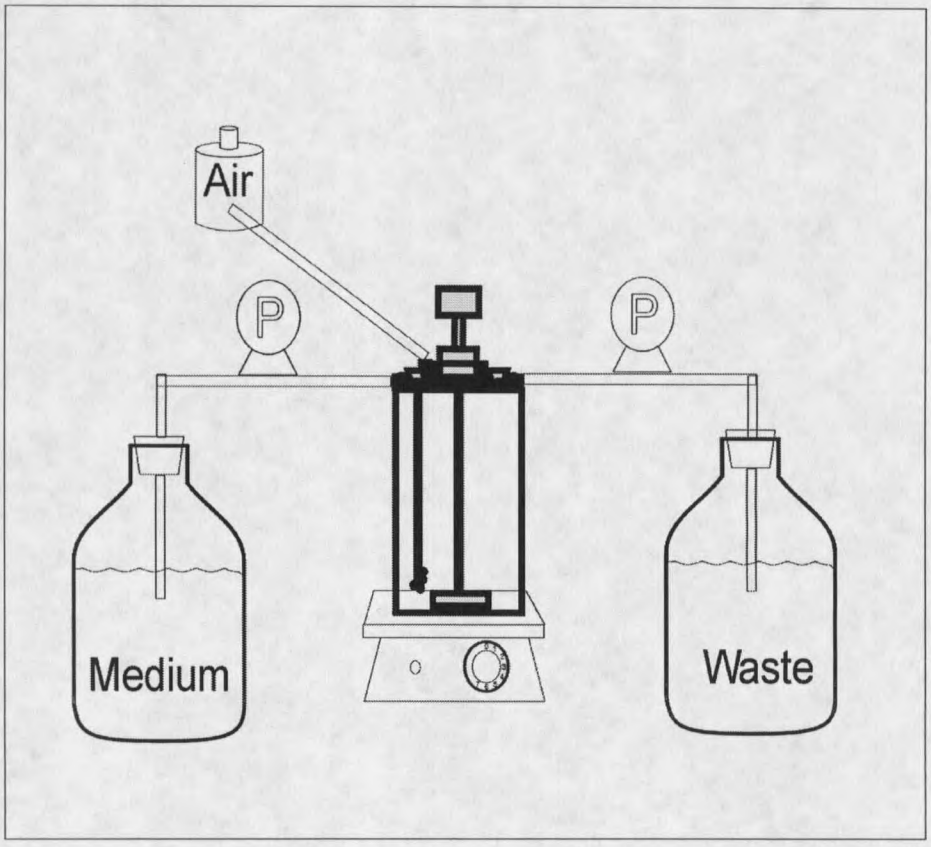

Figure 3.1 Schematic of the chemostat system used to grow planktonic cells.

were taken every 2 to 5 hours from the chemostat. An aliquot of each fresh sample was removed and used immediately for total direct counts by acridine orange staining. The remainder of the sample was frozen at -70 °C for later analysis.

When steady state within the chemostat was achieved, effluent from the chemostat was used to inoculate flow cells (Figure 2).

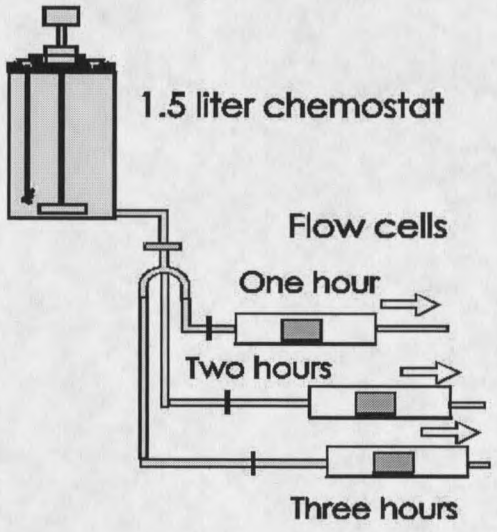

Figure 3.2. Schematic of the chemostat and flow cell design for adhesion experiments. At the end of the specified adhesion time, the flow cell would be taken off line, opened, and the cells processed as described.