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
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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.

EFFECTS OF INITIAL ADHESION EVENTS ON THE PHYSIOLOGY OF *PSEUDOMONAS AERUGINOSA*

by

Elinor deLancey Pulcini

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APPROVAL

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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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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).

<u>*Pseudomonas aeruginosa*</u>

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)

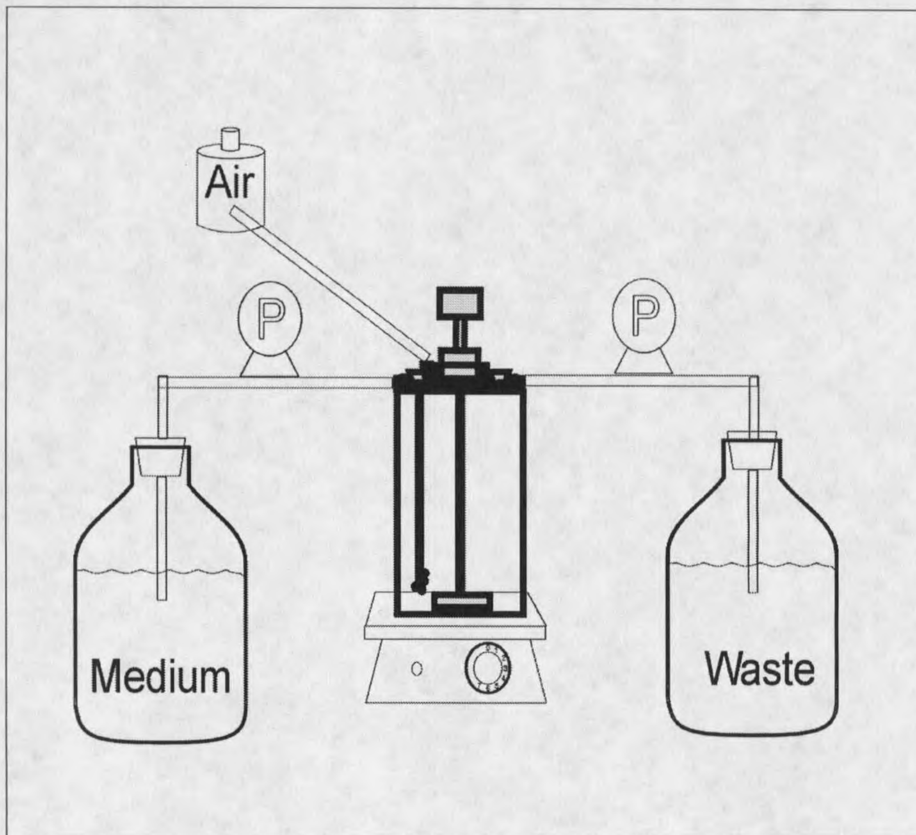


Figure 3.1 Schematic of the chemostat system used to grow planktonic cells.

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).

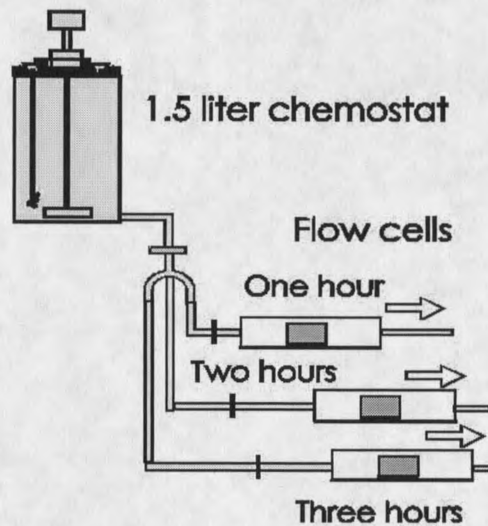


Figure 3.2. Schematic of the chemostat and flow cell design for adhesion experiments. At the end of the specified adhesion time, the flow cell would be taken off line, opened, and the cells processed as described.

Each flow cell contained a 1.0 x 1.0 cm silicon chip (Harrick Scientific Corp.) (Figure 3). Continuous inoculation from the chemosat through the flow cells was maintained at 2.0 ml min^{-1} for 10 minutes.

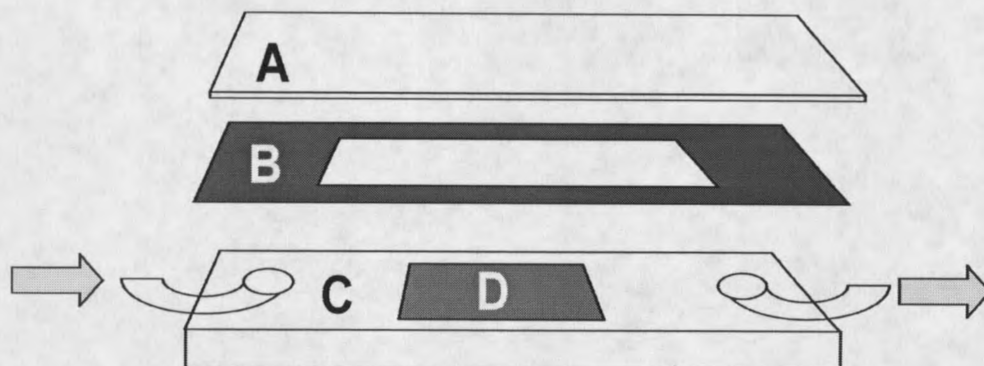


Figure 3.3. Schematic of the flow cell used for adhesion experiments: (A) #2 glass coverslip, (B) latex gasket, (C) clear polycarbonate base, (D) silicon chip. The entire set up was screwed down and held together by means of a stainless steel cover with a hole in the center for use under the microscope.

Fresh medium was then introduced into the flow cells at the same rate of flow (2.0 ml min^{-1}). The flow cells were kept upside down with the silicon chip surface at the top of the cell. This was done to ensure that cells on the surface of the chip had actually attached to the surface and had not simply fallen out of solution and landed on the surface. Flow was maintained for one, two and three hour adhesion experiment times. At the end of the adhesion time, a sample of planktonic cells was taken from the chemostat. Flow was halted to the appropriate flow cell. The flow cell was opened and the surface of the silicon chip was scraped into sterile PBS solution. A total of 3 replicates was performed.

Total direct counts

Total direct counts were performed immediately on each chemostat sample and on each of the attached samples. A 0.05 ml volume of sample was vortexed for 30 seconds in an acid washed, sterile glass vial with 1.0 ml of 0.05% acridine orange (AO) stock solution (Sigma), 1.0 ml of 5.0% formalin, and 2.95 ml of filter sterilized distilled water. This procedure resulted in a 1:100 dilution of the sample. The sample was filtered onto a 25 mm, 0.2 um polycarbonate filter membrane (Nucleopore) in a three manifold vacuum chimney filtration apparatus. Membranes were transferred to a microscope slide and allowed to briefly air dry. A drop of immersion oil was placed on the membrane and covered with a glass cover slip. Twenty random fields were counted using the 100x objective with a 10x ocular on an Olympus BH-2 Epi-Illumination UV microscope. The count was converted to cells per ml as follows:

$$\text{Cells/ml} = \frac{\text{(average cells per field)x(fields per filter)}}{\text{(dilution factor)x (volume filtered of 1.0 ml)}}$$

Total RNA

Chemostat and attached samples were analyzed for total RNA using TRIzol (Life Technologies) following recommended manufacturer protocols. Care was taken to minimize RNase contamination. All tubes and pipette tips were pretreated with RNase ZAP (Invitrogen) and sterilized. Prior to performing the isolation, the lab bench surfaces and disposable gloves were also treated with RNase ZAP. Planktonic samples (20.0 ml chemostat aliquots) and attached samples (scraped from flow cells)

were collected as described above and immediately iced. 1.0 ml of TRIzol reagent was added to each sample and tubes were shaken by hand for 15 seconds and allowed to sit on the benchtop for 3 minutes. Samples were centrifuged at 11,000 rpm for 15 minutes. The clear, RNA containing, aqueous phase was transferred to a clean, treated tube. RNA was precipitated using 0.5 ml of 75% isopropyl alcohol. Samples were mixed and then allowed to sit at room temperature for 10 minutes. Samples were then centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The sample was vortexed and then centrifuged at 7,500 rpm for 5 minutes. The ethanol supernatant was removed and the RNA pellet was allowed to air dry for 10 minutes. The pellet was dissolved in sterile nanopure water. Samples were analyzed on the Spectronic 601 (Milton Roy) at 260 and 280 nm. The A260/280 ratio was calculated for each sample. A ratio of less than 1.6 is an indication that the RNA may not have been completely dissolved or that the aqueous phase was contaminated with the phenol phase. Samples with a ratio of less than 1.6 were allowed to sit longer to allow for complete dissolution of the RNA. If the ratio was still low, then the samples were not used.

Analysis of protein expression

P. aeruginosa ERC1 was grown to steady state in chemostat as described above. Chemostat grown cells (500 ml) were used to inoculate vessels containing plugs of Teflon™ mesh (Tetko Inc.). The plugs were made of layers of 61% open area Teflon™ mesh tightly rolled to form an approximately 9 cm high by 7 cm wide cylinder secured with a cable tie. A nutrient feed was maintained at the same dilution

rate as the chemostat cultures and the vessels were aerated and stirred to minimize carbon or oxygen limiting conditions. The adhesion experiments lasted for 3 hours and were repeated at least 3 times. At the end of the adhesion test period, the Teflon™ plug was rinsed within the vessel to remove any loosely attached cells and placed in ice cold buffer solution (medium without any additional carbon). Cells were removed from the Teflon™ mesh plug by sonicating in the ice-cold buffer 6 times with 10 second bursts interspersed with 10 second non-sonication/rest periods. During this process, the plug was kept constantly moving up and down in the ice-cold buffer to help flush cells from the plug into the buffer. The buffer solution containing the cells was immediately centrifuged (10,000 rpm for 20 minutes at 4° C). The pelleted cells were placed on ice for processing for SDS PAGE.

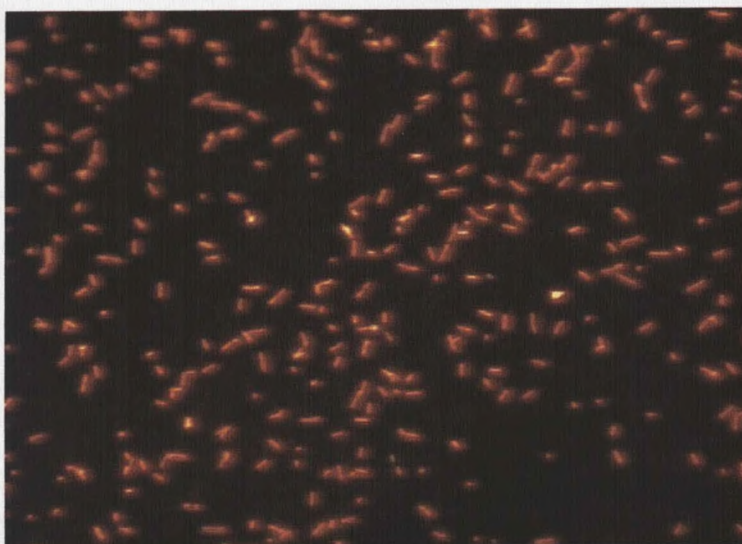
Prior to SDS PAGE, samples were assayed for total protein using a modified Lowry method (Sigma Kit 690) so that standardized loading of gels could be accomplished. The samples were sonicated on ice for 1 minute and were then immediately diluted 1:4 (v:v) in sodium dodecyl sulfate (SDS) sample buffer according to BioRad protocols. The SDS sample buffer contained 3.8 ml distilled water 1.0 ml 0.5 M Tris-HCl (pH 6.8), 0.8 ml glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-beta-mercaptoethanol, 0.05% (w/v) bromophenol blue. Eppendorf tubes containing SDS buffer solution and the protein sample were incubated at 95 °C for 5 minutes. Samples were then either immediately loaded into SDS PAGE gels or were frozen at –70° C for later use.

SDS PAGE

SDS polyacrylamide gel electrophoresis (PAGE) was performed based on the procedure described by Laemmli (Laemmli, 1970). The resolving gel of 5% to 20% gradient acrylamide was overlaid by a 4% acrylamide stacking gel. Approximately 10 μg of protein sample was loaded in each lane. SDS PAGE gels were run in the BioRad Protean II xi vertical system at 15 mAmps. Proteins were visualized using a modified silver stain method as described by Blum et al., (1987).

Results

Previous research has shown that staining with AO can be used as an index of physiological activity in pure cultures of bacterial cells (McFeters et al., 1991; Wentland et al., 1996). This dye fluoresces orange when bound to single strand nucleic acids and green when bound to double strand nucleic acids (Wentland et al., 1996). Therefore, the RNA to DNA ratio within the cell, as visualized by AO staining, gives an indicator of the rate of growth. Cells in log phase, with a higher RNA to DNA ratio, are visualized as red orange in color while cells in stationary phase, with a smaller RNA to DNA ratio, appear green (Wentland et al., 1996). Chemostat grown planktonic cells appeared orange in color following staining with AO, an indication that the planktonic cells are in log phase growth (Figure 4). In the three replicate experiments, the majority of the planktonic (chemostat grown) cells appeared orange with occasional (less than 5% of the total cell count for any given period) green fluorescent cells. However, upon attachment to a surface, the ratio of



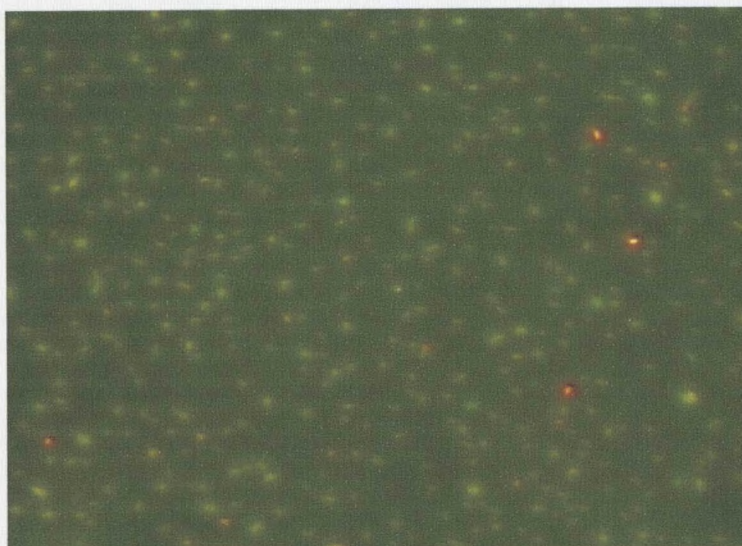


Figure 3.4. Microscope images (40X) of *P. aeruginosa* cells stained with acridine orange in planktonic culture (upper photo) and during attachment (lower photo). Change in color from orange to green is indicative of the change in growth phase from exponential to lag phase growth during attachment.

orange to green cells began to change with a higher percentage of green fluorescing cells (Figure 4). This change in fluorescence from orange to green is an indication of reduced RNA synthesis.

Steady state within the chemostat maintained cell counts at approximately 5.0 x 10^6 cells ml^{-1}. During chemostat growth, planktonic cells exhibited a baseline concentration of total RNA averaging at approximately 0.3 ug per ml (Figure 5).

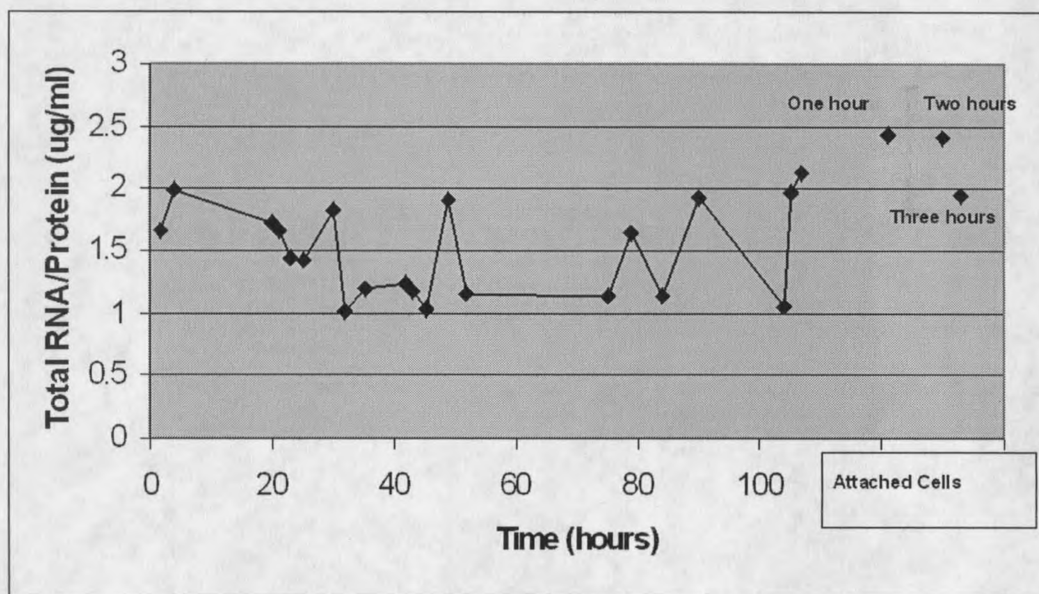


Figure 3.5. Variation in total RNA during initial adhesion. Chemostat grown planktonic cells were used to inoculate flow cells for one, two and three hours.

Total RNA was measured as absorbance at 260 nm and was normalized to the number of cells. During attachment, total RNA concentration increased in comparison to baseline planktonic total RNA expression.

Figure 6 shows a representative silver stained SDS PAGE gel comparing attached and planktonic samples. Lane 3 of this gel contains proteins from cells attached to Teflon™ mesh for 3 hours. Lanes 2 and 4 contain planktonic chemostat grown cells. In comparing attached samples to planktonic samples, at least 3 protein bands were *de novo* expressed during the 3 hour period of attachment.

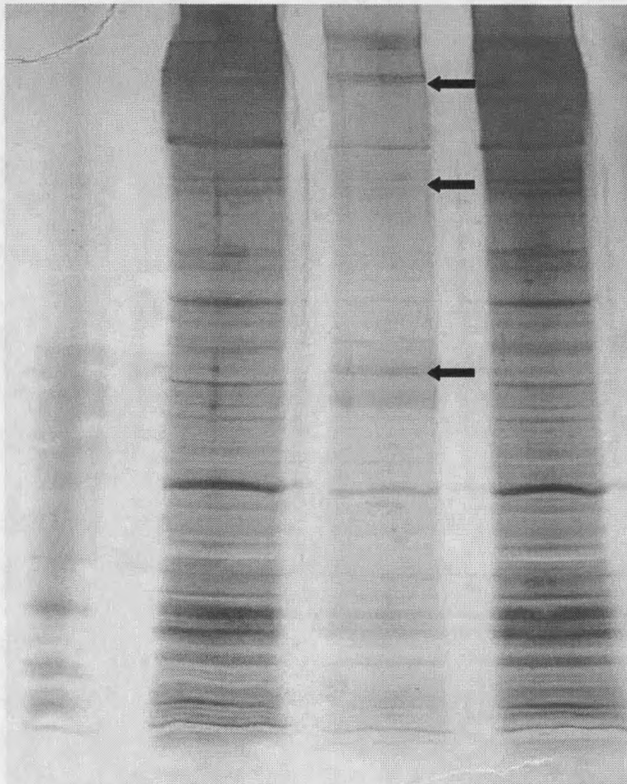


Figure 3.6. Representative silver stained SDS PAGE gel of planktonic (Lanes 2 and 4) and 3 hour attached samples (Lane 3) of *P.aeruginosa*. Arrows indicate protein bands up expressed during attachment.

Conclusion

These preliminary experiments were designed to indicate whether changes in physiology could be detected in the first three hours of attachment in *P. aeruginosa* ERC1. Very little work had been done on cells during initial adhesion; most biofilm research has utilized mature biofilms of several hours to days old. Since there are so few cells attaching to a surface during initial adhesion, it was uncertain that changes in physiology in these attaching cells could be detected on a molecular level.

Physiologic changes were detected in initial attaching cells during the three hours of experiment time. Prior to attachment, chemostat grown cells were shown to be in log phase growth. Since fast growing cells have a greater RNA to DNA ratio, AO is binding to mostly single strand nucleic acids and thus the cells stained orange. Within the first hour of initial adhesion on the flow cell, the attached bacterial cells were visualized as green after staining with AO indicating the change in the ratio of RNA to DNA that is characteristic of lag phase. These results were later corroborated by work done by Rice et al. (2000) indicating that log phase cells of *P. aeruginosa* grown in the same medium will surface undergo a significant lag phase period (up to 12 hours) upon attachment. This lag phase may be an indicator that changes are taking place within the cell to up-regulate various metabolic pathways in order to develop the biofilm phenotype.

In these experiments, attaching cells showed changes in total RNA concentrations within the first 3 hours. The concentration of the stable RNAs, both tRNA and rRNA, has been shown to be directly related to the growth rate and the

increased rate of protein synthesis in exponentially growing cells (Emilsson and Nilsson, 1995). However, since attaching cells are not replicating, an expected result would be a corresponding decrease in RNA concentration within the cell. These experiments show just the opposite; that non-dividing, initially attaching cells are experiencing an increase in RNA production. This obvious change in the rate of protein synthesis with no accompanying cell division indicates that these cells are undergoing physiologic and metabolic changes during initial attachment.

During initial adhesion, *P. aeruginosa* differentially expresses at least 3 proteins as compared to planktonically grown cells. A similar study by Brozel et al. (1995) comparing SDS PAGE protein profiles of planktonic and attached cells showed the up regulation of 5 proteins during attachment. A number of proteins may also have been down regulated during attachment. Unfortunately, due to variations in the protein load concentrations, it is uncertain that disappearing bands are the result of a change in expression.

This series of experiments provided crucial data for the basis of the remainder of this project. Since the majority of research at this time was on older, more mature biofilms this work provided the confirmation that discernible and measurable changes do occur in less than 3 hours within attaching cells. This then allowed for the development of experimental procedures to specifically identify the changes that occur within attaching cells during initial adhesion which is the basis of the remainder of this dissertation.

CHAPTER 4

VARIATIONS IN PROTEIN EXPRESSION IN *PSEUDOMONAS AERUGINOSA* DURING INITIAL ADHESION

Introduction

Biofilm formation by bacteria has been well documented since ZoBell's work in the 1940's (Zobell, 1943). Current research indicates that 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 (deBeer et al., 1994) and allow for the circulation of nutrients and oxygen deep within the biofilm matrix layer (Davies and Geesey, 1995; Davies 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).

Historically, the methodologies utilized in biofilm studies were heavily influenced by the traditional microbiology view of bacteria as planktonic organisms. More recently, research has indicated that there are clear differences between planktonic and biofilm bacteria, specifically with respect to changes in the physiology and metabolism of bacteria as they become attached organisms. Bacterial cells within a biofilm have been shown to have greater resistance to antibiotics and biocides than freely suspended cells (Gorg et al., 2000; Humphery-Smith et al., 1997). The process

of attaching to a surface induces alginate production in *Pseudomonas aeruginosa* (Davies and Geesey, 1995), and induces the production of exopolysaccharides in both *Staphylococcus epidermidis* and in *Vibrio cholerae* El Tor (Pratt and Kolter, 1999).

There are at least two major approaches to determining changes in physiology. One involves examination of the genes and their level of expression. The second is to directly investigate the induction or repression of proteins. The examination of changes in protein expression provides a snapshot of the condition of an organism at a particular point in time. This approach, now termed proteomics, has many advantages over genomic studies due to the fact that the DNA sequence cannot provide information concerning the translation of gene products, if they occur and the extent of post-translational modifications (Humphery-Smith et al., 1997). The information gathered from proteomic studies can provide crucial data for the basis of genetics studies. Differential protein expression patterns can be indicative of the up regulation of specific genes and operons. The potential success of proteomics has been greatly enhanced with improvements in protocols for two-dimensional (2 D) gel electrophoresis. The gels produced utilizing immobilized pH gradients for the isoelectric focusing step are able to provide a level of standardization that allows for a more accurate comparison of results between replicate gels and between those from different populations (i.e., planktonic vs. biofilm organisms) (Gorg et al., 2000).

The majority of biofilm studies have been conducted on mature biofilms that are several days old. This approach is obviously inadequate if the intent is to identify the nature and time point at which cells transition from the planktonic to the 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.

Materials and Methods

Organism and growth conditions

Pseudomonas aeruginosa ERC1 (ATCC # 700888) was grown to steady state in chemostat with a residence time of 5 hours using a minimal salts glucose medium (Anonymous,1986). Chemostat grown cells (500 ml) were used to inoculate vessels containing plugs of Teflon™ mesh (Tetko Inc.). The plugs were made of layers of 61% open area Teflon™ mesh tightly rolled to form an approximately 9 cm high by 7 cm wide cylinder secured with a cable tie. A nutrient feed was maintained at the same dilution rate as the chemostat cultures and the vessels were aerated and stirred to minimize carbon or oxygen limiting conditions. Adhesion tests were performed at 10 minutes, 1 hour, 2 hours, and 3 hours with a minimum of three replicates performed for each of the adhesion tests. At the end of the adhesion test period, the Teflon™ plug was rinsed within the vessel to remove any loosely attached cells and placed in ice cold buffer solution (medium without any additional carbon) containing 1.5M Tris pH 8.8 to prevent proteolysis. Cells were removed from the Teflon™ mesh plug by sonicating in the ice-cold buffer with 6 times 10 second bursts interspersed with 10 second non-sonication/rest periods. During this process, the plug was kept

constantly moving up and down in the ice-cold buffer to help flush cells from the plug into the buffer. The buffer solution containing the cells was immediately centrifuged (10,000 rpm for 20 minutes at 4° C). The pelleted cells were removed and immediately iced. Planktonic control samples (20.0 ml) were taken from the chemostat and immediately centrifuged and processed as described above.

Protein extraction

Samples were assayed for total protein using a modified Lowry method (Sigma Kit 690). The cells were lysed with a probe sonicator (3 times 6 second burst, 10 second rest) on ice in lysis buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), and 40 mM Tris base. Proteins were precipitated using ice cold 10% trichloroacetic acid (TCA) in acetone. Proteins were collected by centrifugation (10,000 rpm for 20 min at 4° C) and the pellet was allowed to air dry to remove residual acetone.

Protein solubilization

Proteins were solubilized in a buffer solution containing 8M urea, 2% CHAPS, 2% pH 3-10 nonlinear Immobilized pH Gradient (IPG) buffer and a trace of bromophenol blue. Immobiline Dry Strips pH 3-10 nonlinear (Amersham Pharmacia) were rehydrated in the Multiphor II (Amersham Pharmacia) reswelling tray following Amersham Pharmacia protocols.

Protein electrophoresis

Two-dimensional protein electrophoresis was performed based on the procedure described first by O'Farrell (1975) and modified as described by Gorg

et.al. (2000). The first-dimension focusing procedure was carried out using the Multiphor II system following Amersham Pharmacia protocols using 20 ug of protein for analytical gels. A strip of blotter paper, saturated with 6.0 % dithiothreitol (DTT) was placed over the IPG strips at the cathode end. Strips were focused at 3500 mv for 20 hours. Strips were then either used immediately for the second dimension or stored at –70°C for later use.

Prior to the second dimension run, the strips were equilibrated in 2.5 ml of equilibration buffer (50 mM of 1.5 M Tris-Cl pH 8.8, 6M urea, 30% glycerol, 2% lauryl sulfate, sodium salt (SDS), and a trace of bromophenol blue) containing 25.0 mg of DTT for 10 minutes and then in 2.5 ml of equilibration buffer containing 62.5 mg of iodoadacetamide for 10 minutes. Strips were placed on paper towels and rinsed with sterile nanopure water prior to placement on SDS PAGE gels.

The second dimension run was performed using 12.5% acrylamide gels in the BioRad Protean II xi vertical system. Strips were overlaid on 12.5% SDS PAGE gels (16cm by 20cm by 1mm) and were sealed in place with hot 1.0% agarose. Gels were run at 8.5 mAmps per gel for 10 hours. Proteins were visualized using silver stain following the protocol as described by Blum et al. (1987).

In all cases, the comparison of protein profiles was made using the planktonic sample from the same run as the attached sample. All samples from a single experiment were processed simultaneously using the same reagents. This minimized changes in protein expression that could have been due to subtle differences in such variables as medium constituent concentrations, dilution rate, or protein processing.

Each time point in the experiment was repeated at least 3 times and gels for time point were done at least in triplicate.

Results

The comparison of planktonic and attached samples showed changes in protein expression throughout the progression of the attachment from 10 minutes to 3 hours. Figure 1a shows a representative 2D PAGE protein gel for planktonic, chemostat grown *P. aeruginosa* ERC1.

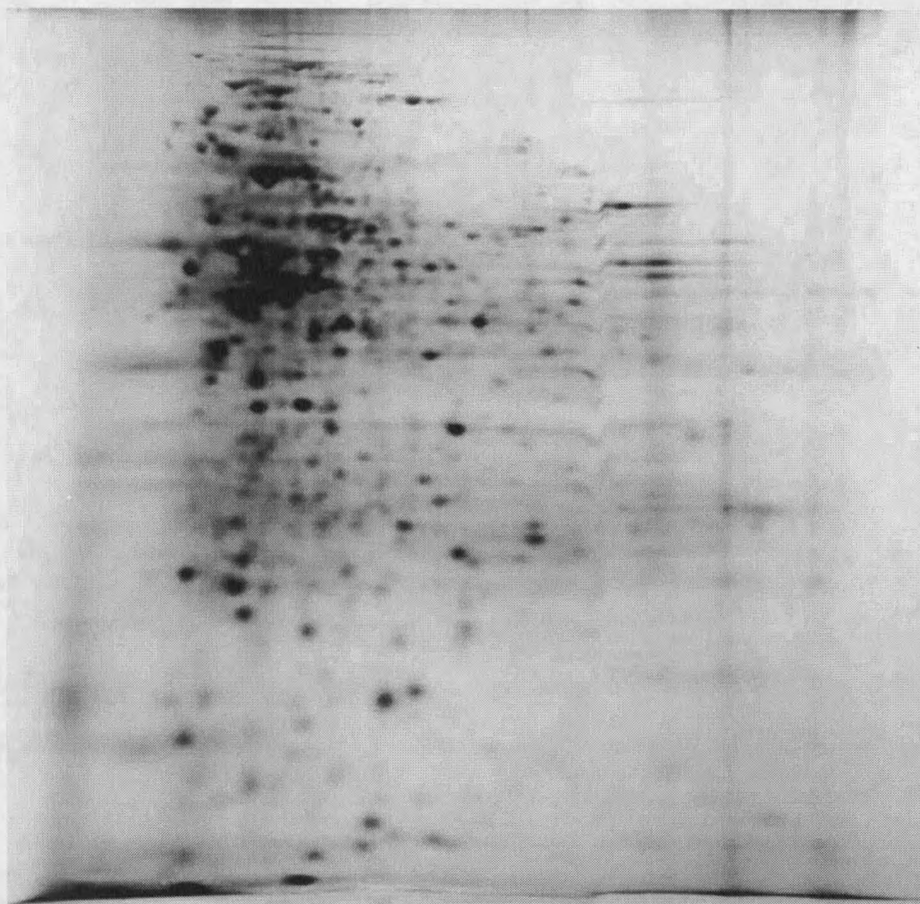


Figure 4.1 a Representative 2 D gel of a planktonic sample of *P. aeruginosa*.

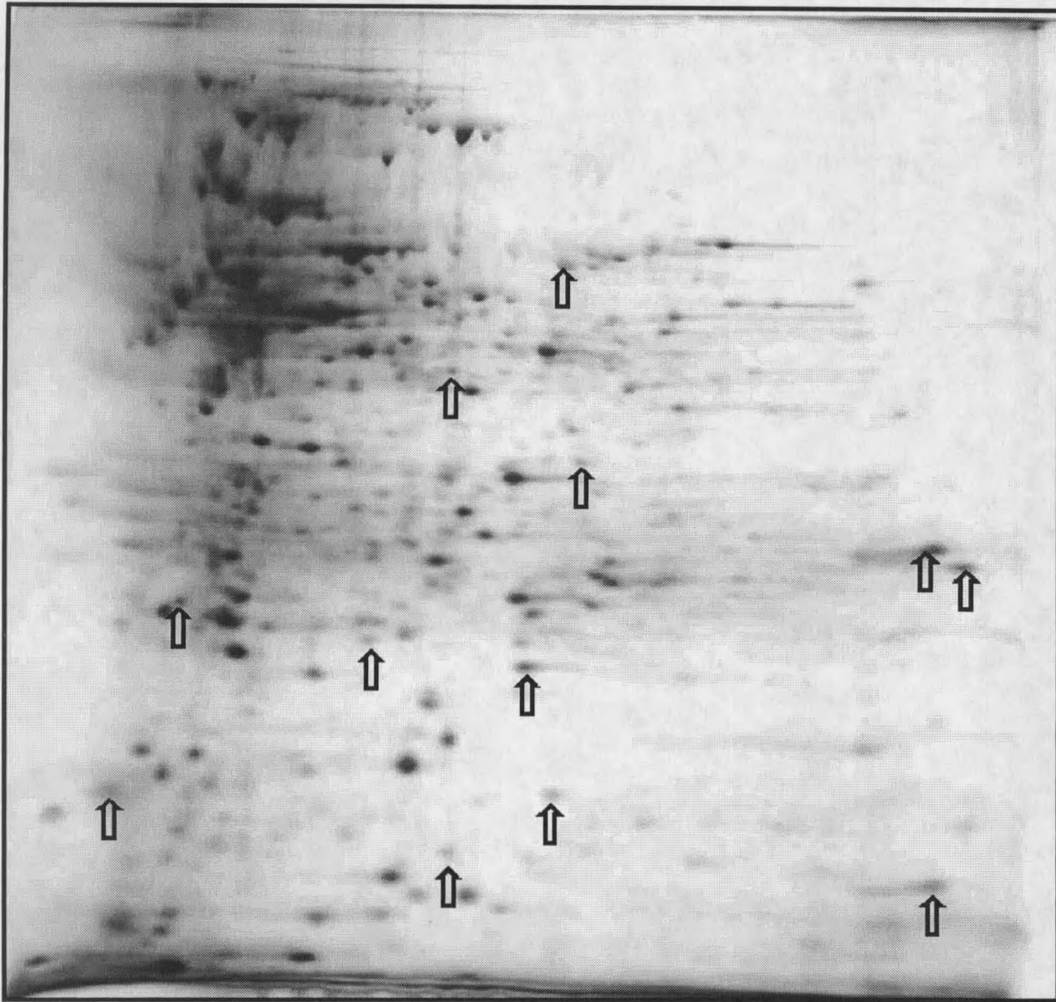


Figure 4.1 b
2 D gel of a three hour attached sample of *P.aeruginosa*.
Arrows indicate proteins up expressed in comparison to planktonic samples.

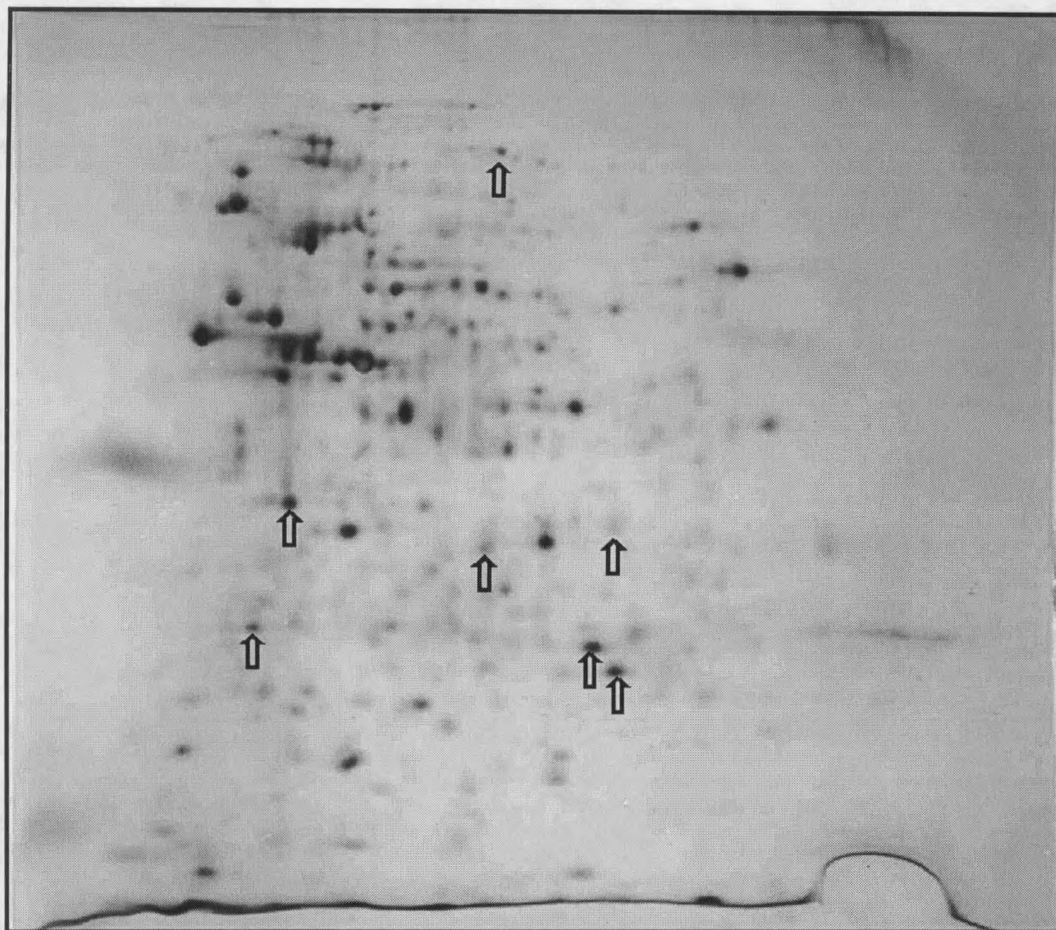


Figure 4.1 c

2 D gel of a two hour attached sample of *P. aeruginosa*. Arrows indicate proteins up expressed in comparison to planktonic samples.

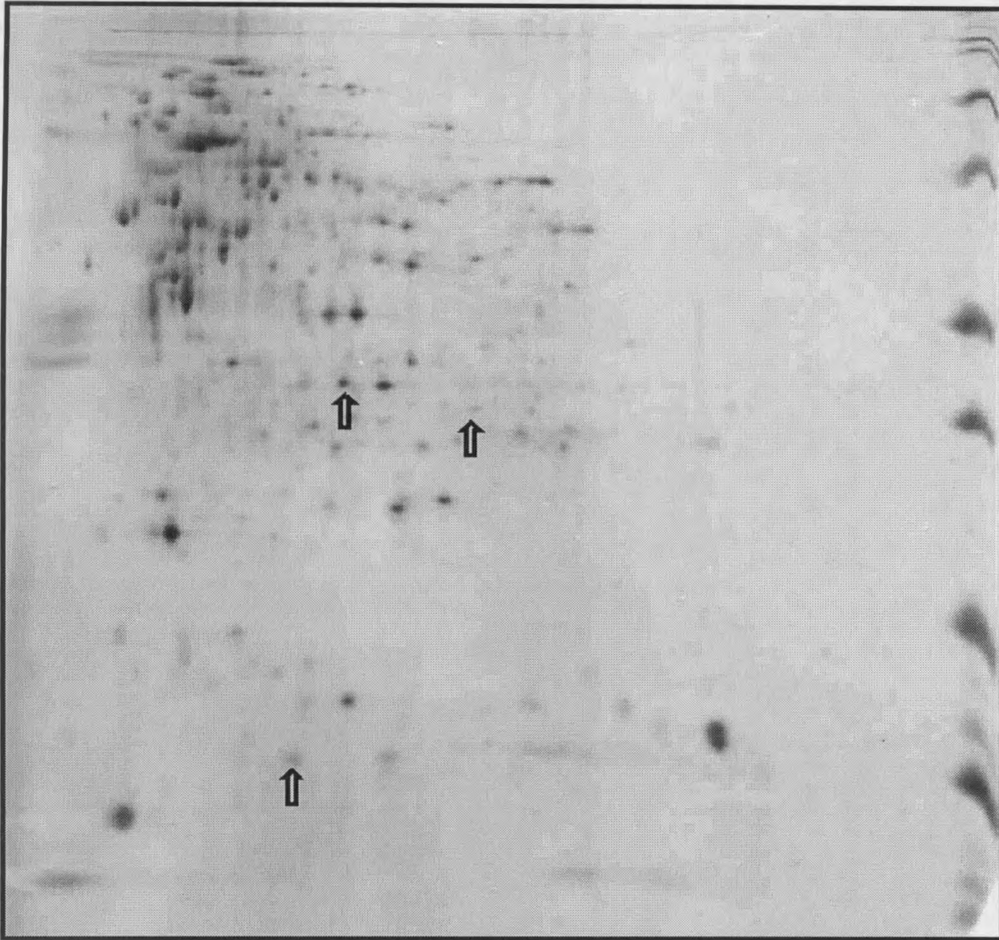


Figure 4.1 d

2 D gel of a one hour attached sample of *P. aeruginosa*. Arrows indicate proteins up expressed in comparison to planktonic samples.

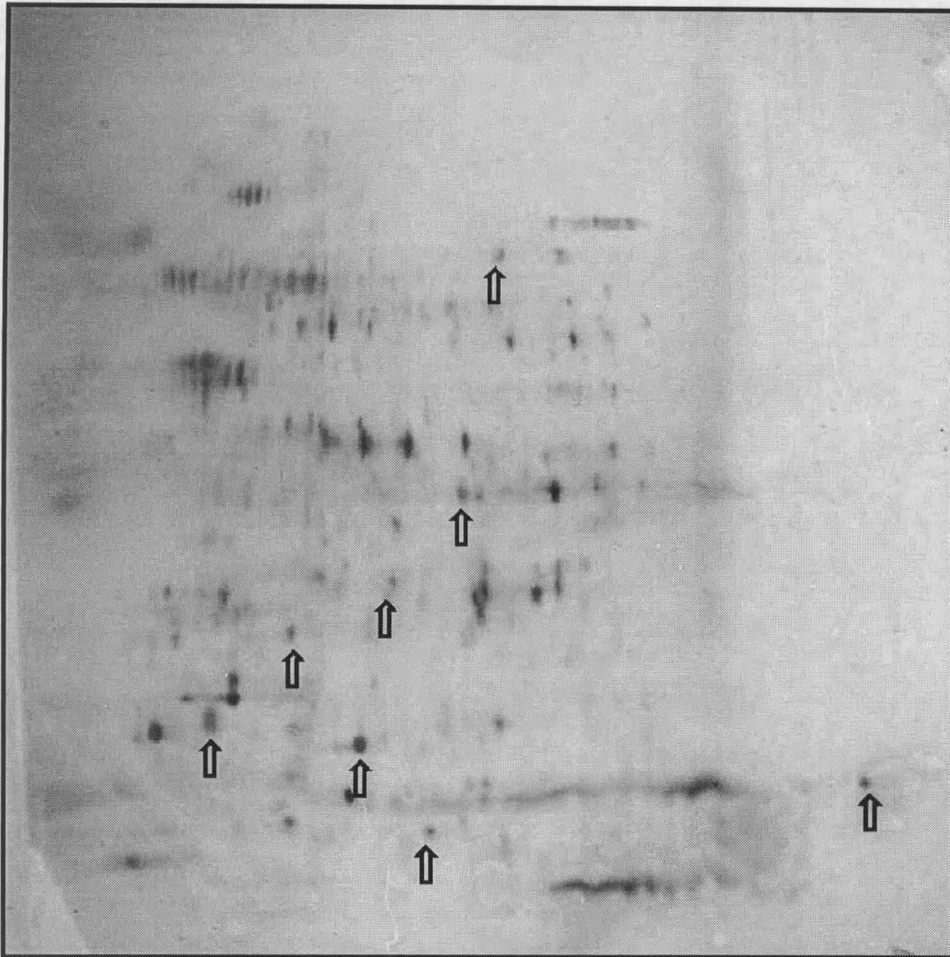


Figure 4.1e

2 D gel of a ten minute attached sample of *P. aeruginosa*. Arrows indicate proteins up expressed in comparison to planktonic samples.

Figures 1 b, 1 c, 1 d, and 1 e show 2D PAGE gels for 3 hours, 2 hours, 1 hour and 10 minutes of attachment time respectively. Arrows on the gels indicate proteins that were up expressed during period of adhesion as compared to the planktonic gel.

Conclusion

The differences in the protein expression profiles of attached and planktonic

Pseuodomonas aeruginosa ERC1 cells show a rapid physiologic change during the initial establishment of biofilm. These changes occur within minutes and vary over the 3 hour initial attachment duration of these experiments. This study shows changes occur in the bacterial cell prior to the development of any kind of complex biofilm. This indicates that these physiologic changes are most probably due to the processes of attaching to the surface.

The process of bacterial attachment to a surface involves metabolic and physiologic changes within the cell. The induction of alginate genes was shown to occur in as little as 15 minutes in *P. aeruginosa* (Davies et al., 1993). Attached cells of *Staphylococcus aureus* exhibited a shortened exponential growth rate, smaller size and less protein content in comparison to planktonic grown cells (Williams et al., 1999). In comparison, *P. aeruginosa* cells have been shown to exhibit a longer lag phase during initial adhesion (Rice et al., 2000). The hydrophobicity or hydrophilicity of the surface has been shown to affect colony morphology in sessile marine bacteria (Dalton et al., 1994). There is some conjecture that the physiologic differences may be such that a biofilm phenotype could exist (Costerton et al., 1999).

Biofilms are costly for industry due to their biofouling potential and due to their potential to cause product degradation (Stoodley et al., 1994). Biofilms in drinking water systems may act as reservoirs for potential pathogens (Camper, 2000). In the human body, there is a direct relationship between the presence and severity of dental plaque (a biofilm) and the increase of the potential of suffering a heart attack (Costerton et al., 1999). The determination of specific physiological changes between

planktonic and biofilm cells could identify potential targets for biofilm control in a wide variety of applications, including human medicine, household product development, and product manufacture/quality assurance.

CHAPTER 5

MADLI-TOF ANALYSIS OF DIFFERENTIAL PROTEIN EXPRESSION IN *PSEUDOMONAS AERUGINOSA* DURING INITIAL ADHESION

Introduction

Biofilm cells have been shown to be physiologically different from their planktonic counterparts. Attached or attaching bacteria have been shown to increase the production of alginate, adopt a mucoid appearance, and are able to withstand log increases in antibiotics or biocides (Davies et al., 1993; Hoyle et al., 1993; Lam et al., 1980; Costerton et al., 1999). Variations in expression of quorum-sensing genes *las* and *rhlI* occur during the course of biofilm development (De Kievit et al., 2001). *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 lag phase in the biofilm despite the available nutrients (Xu et al., 2001). Given the growing body of evidence that shows differences 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 (Loo et al., 2000; Costerton, 2000; McLean et al., 2001, Schembri and Klemm, 2001; Nesper et al., 2001).

There are a number of methodologies that may be undertaken to determine what proteins are uniquely expressed during initial adhesion including genomics and proteomics. Genomic studies hace been facilitated with the recent sequencing of the complete *P. aeruginosa* genome (Stover et al., 2000). The approximately 6000 open reading frames (ORFs) within the total genomic DNA of approximately 6.4 Mb, have been annotated by the *Pseudomonas* Community Annotation Project (http://www.pseudomonas.com/index.html). However, to date, 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). This large number of ORFs illustrates a limitation to genomic studies, gene function must be related to the level of response to a particular environmental cue (VanBogelen et al., 1996). Another limitation is that 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).

In contrast, proteomics, the determination of expressed proteins, can provide complementary information to genomics-based studies. The proteome yields information about the specific proteins expressed under a set of conditions and can give information about the relative concentrations of gene-products (proteins) and the extent of post-translation modifications (Wasinger and Humphery-Smith, 1998). The identification of the proteins can then give information about the specific ORFs that were activated (Nouwens et al., 2000), (VanBogelen et al., 1996). The success of proteomics has been greatly enhanced with improvements in protocols for two-

dimensional gel electrophoresis (2 D). The gels produced utilizing immobilized pH gradient strips for the isoelectric focusing step are able to provide a level of standardization that allows for a more accurate comparison of results between replicate gels and between those from different populations (i.e., planktonic vs. biofilm organisms). Proteins, visualized as spots within the gels, can be analyzed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. MALDI has been shown to be a sensitive method for the mass mapping of peptides (Jensen et al., 2001; Shevchenko et al., 1996). 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.

This study was undertaken to specifically address changes in the physiology of *P. aeruginosa* during initial adhesion, defined as less than 3 hours of adhesion time. The analysis of differential protein expression patterns during attachment will provide the data necessary to determine if a specific set of proteins exist that could be used as indicators of the point in the progression from the planktonic to biofilm mode of existence.

Materials and Methods

Organism and growth conditions

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

salts medium (Manual of Industrial Methods). Glucose at 0.1g/L was used as the carbon source. Chemostat grown cells at approximately 10^6 cells/ml were used to inoculate vessels (500 ml) containing plugs of Teflon™ mesh (Tetko Inc.). The plugs were made of layers of 61% open area Teflon™ mesh tightly rolled to form an approximately 9 cm high by 7 cm wide cylinder secured with a cable tie. A nutrient feed was maintained at the same flow rate as the chemostat cultures and the vessels were aerated and stirred to minimize carbon or oxygen limiting conditions. Adhesion tests were performed at room temperature for 3 hours with a minimum of three replicates performed. At the end of the adhesion test period, the Teflon™ plug was rinsed by holding the mesh plug with sterile tongs and moving the plug up and down within the vessel to remove any loosely attached cells. The mesh plug was placed in ice cold buffer solution (medium without any additional carbon) containing 1.5M Tris pH 8.8 to prevent proteolysis. Cells were removed from the mesh plug by sonicating in the ice-cold buffer with 6 times 10 second bursts interspersed with 10 second non-sonication/rest periods using a probe sonicator (Tekmar Inc.) following manufacturer's directions for tuning the amplitude. During this process, the plug was kept constantly moving up and down in the ice-cold buffer to help flush cells from the plug into the buffer. The buffer solution containing the cells was immediately centrifuged (10,000 rpm for 20 minutes at 4° C). The pelleted cells were removed and immediately iced. Planktonic control samples (20.0 ml) were taken from the chemostat and immediately centrifuged and processed as described above.

Protein extraction

Samples were assayed for total protein using a modified Lowry method (Sigma Kit 690). The cells were lysed with a probe sonicator (3 times 6 second burst, 10 second rest) on ice in lysis buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), and 40 mM Tris base. Proteins were precipitated using ice cold 10% trichloroacetic acid (TCA) in acetone. Proteins were collected by centrifugation (10,000 rpm for 20 min at 4° C) and the pellet was allowed to air dry to remove residual acetone.

Protein solubilization

Proteins were solubilized in a buffer solution containing 8M urea, 2% CHAPS, 2% pH 3-10 nonlinear Immobilized pH Gradient (IPG) buffer and a trace of bromophenol blue. Immobiline Dry Strips pH 3-10 nonlinear (Amersham Pharmacia) were rehydrated in the Multiphor II (Amersham Pharmacia) reswelling tray following Amersham Pharmacia protocols.

Protein electrophoresis

Two-dimensional protein electrophoresis was performed based on the procedure described first by O'Farrell (1975)and modified as described by Gorg et al. (2000). The first-dimension focusing procedure was carried out using the Multiphor II system following Amersham Pharmacia protocols using 500 ug of protein for analytical gels. A strip of blotter paper, saturated with 6.0 % dithiothreitol (DTT) was placed over the IPG strips at the cathode end. Strips were focused at 3500 mv for 20

hours. Strips were then either used immediately for the second dimension or stored at −70°C for later use.

Prior to the second dimension run, the strips were equilibrated in 2.5 ml of equilibration buffer (50 mM of 1.5 M Tris-Cl pH 8.8, 6M urea, 30% glycerol, 2% lauryl sulfate, sodium salt (SDS), and a trace of bromophenol blue) containing 25.0 mg of DTT for 10 minutes and then in 2.5 ml of equilibration buffer containing 62.5 mg of iodoadacetamide for 10 minutes. Strips were placed on paper towels and rinsed with sterile nanopure water prior to placement on SDS PAGE gels.

The second dimension run was performed using 12.5% acrylamide gels in the BioRad Protean II xi vertical system. Strips were overlaid on 12.5% SDS PAGE gels (16cm by 20cm by 1mm) and were sealed in place with hot 1.0% agarose. Gels were run at 8.5 mAmps per gel for 10 hours. Proteins were visualized using Gel Code Coomassie stain (Pierce) following the recommended protocols.

In all cases, the comparison of protein profiles was made using the planktonic sample from the same experimental run as the attached sample. All samples from a single experiment were processed simultaneously using the same reagents. This minimized changes in protein expression due to subtle differences in such variables as medium constituent concentrations, flow rate, or protein processing.

<u>Peptide mass fingerprinting</u>

Planktonic and 3 hour attached 2 D gels were compared and protein spots that were up regulated in each were selected for analysis. Proteins were excised from the gel and care was taken to minimize the amount of excess acrylamide surrounding the

actual protein spot. Excised gel pieces were placed in siliconized Eppendorf tubes previously treated with 50% acetonitrile. The excised gel pieces were washed twice with approximately 100 ul (or enough to cover the gel fragments) of a 1:1 solution of 25 mM NH_4HCO_3 and 50% acetonitrile. For each wash, the Eppendorf tubes were placed on a shaker at room temperature for 30 minutes. Each time the wash solution supernatant was removed and discarded. Gel pieces were then dehydrated in a Speed Vac lyophilizer for approximately 20 minutes to dry the pellets. In gel tryptic digestion was carried out overnight at 37 °C by the addition of 20 ul trypsin solution (12.5 ng/ul trypsin (sequencing grade, Promega) in 25 mM NH_4HCO_3). As the gel pieces rehydrated, additional 25 mM NH_4HCO_3 was added to cover the gel pieces. Following digestion, the Eppendorf tubes were briefly centrifuged and 20 ul 0.1% trifluoracetic acid (TFA) was added. Samples were centrifuged again and the supernatants were transferred to new Eppendorf tubes. The samples were purified using ZipTip C18 columns following manufacturer guidelines (Millipore). A 5.0 ul aliquot of the peptide solution was combined with 5.0 ul of a saturated solution of α cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1 % TFA. In addition, 1.0 ul of 10.0 picomole/ul angiotensin II was added as an internal standard. 2.0 ul of the solution was spotted on the template and allowed to air dry (Gevaert et al., 1998; Gevaert and Vandekerckhove, 2000; Jensen et al., 2001; Shevchenko et al., 1996).

Peptides were analyzed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using a Bruker Biflex III system in reflectron mode with acceleration voltage set at 25kV. All samples were randomly

labeled to prevent bias during the analysis process. The spectra were calibrated using angiotensin II at 1047.18 Da [M+H]+. Analysis of peptide masses was done using MS-Fit using the Protein Prospector database (http://prospector.ucsf.edu/). Parameters selected for the search were as follows: trypsin digest and peptide mass set at +/- 30 ppm. Genpept 7.2.2001 was used as the reference database for the identification of proteins. If proteins were not found that matched the molecular weight and pI of the excised gel spot, then the peptide mass tolerance was increased as noted in Table 1.

Results

The purpose of these experiments was to delineate differential protein expression during initial adhesion. Protein profiles (2 D gels) of attached cell samples were compared to planktonic cells from the same experimental run. A total of 25 spots showed differential expression as the result of attachment. Of the 25, 13 were selected for MALDI-TOF analysis. The remaining 12, visualized as lightly Coomassie stained spots, were deemed to be possibly problematic either due to low protein concentration or weak expression of the spot within the gel. Of the 13 spots, 9 proteins that were up expressed at 3 hours of attachment were and 4 proteins in the planktonic gels that were down regulated (no longer present) in the 3 hour gels were selected for peptide analysis.

The identified proteins are listed in Table 1. In all cases, the mass spectra analysis were matched to a minimum of 3 peptides to establish the identity of the

Protein Name	Gene Name	Confidence Level	Gene Designation	Peptide Mass Tolerance	Function
Proteins up regulated at 3 hours					
Glycosyl transferase	*migA*	3	PA1014	50 ppm	hydrolyze glycosidic bonds LPS synthesis
Glycosyl hydrolase		3	PA2162	30 ppm	polysaccharide hydrolysis
UDP-glucose pyrophosphorylase	*galU*	2	PA2023	115 ppm	LPS production virulence factor
Heat shock protein	*hscA*	2	PA3810	50 ppm	Chaperone virulence factor
Aliphatic amidase	*amiE*	1	PA3366	30 ppm	carbon regulation ABC transport
Hypothetical protein AE004751		4		100 ppm	unknown
Hypothetical protein AE004444		4		100 ppm	unknown
2,4-dienoly-CoA reductase	*fadH1*	2	PA3092	30 ppm	fatty acid & phospholipid metabolism/ virulence factor
Fe(III)-pyochelin receptor precursor	*fptA*	1	PA4221	30 ppm	siderophore formation virulence factor ABC transport
Proteins up regulated in planktonic					
Biotin carboxylase	*accC*	1	PA4848	30 ppm	fatty acid biosynthesis growth state indicator
Transcriptional regulator	*ptxR*	3	PA1312	100 ppm	exotoxin A production, pyoverdine production
TonB receptor	*optS*	3	PA2466	100 ppm	siderophore recognition & transport
Hypothetical protein AE004539		4		100 ppm	unknown

Table 5.1. Proteins differentially expressed by *P. aeruginosa* at 3 hours of attachment. Functions listed for proteins having confidence levels of 2 or 3 are considered to be putative.

protein and the majority of the proteins were matched at either 30 or 50 ppm. Confidence levels for the proteins are included in Table 1. The confidence level indicates the type of evidence on which the gene or protein name is based. There are

four categories of confidence levels described in Table 2. Confidence level 1 means that the function of the gene is experimentally demonstrated in *P. aeruginosa* while confidence level 4 means that there are no homologs to any known sequences. Functions described for proteins with confidence levels of 2 and 3 are considered to be putative.

Confidence Level	Description
1	Function demonstrated in *P. aeruginosa*.
2	Function of homologous gene demonstrated in another organism.
3	Function based on conserved amino acid sequence or sequence homology.
4	No homology to any sequences reported to date.

Table 5.2. Description of confidence level designation for protein and gene function. The confidence level indicates the type of evidence on which the gene or protein name is based

Replicate gels indicate there are more proteins up regulated than down regulated during attachment to a surface; out of the 25 differentially expressed proteins, only 4 were down regulated. In the attached sample, proteins linked to LPS production were up regulated as well as proteins involved with siderophore formation, polysaccharide hydrolysis, ABC type amide transport and poly-hydroxyl

alkanoate (PHA) synthesis. Down regulated at 3 hours were proteins for transcriptional regulation, a Ton-B receptor and fatty acid biosynthesis.

Results indicate an increase in LPS and alginate production associated with attachment and biofilm development. In addition, these results suggest attaching cells exhibit increased nutrient requirements and thereby up-regulate proteins such as those for siderophore formation and polysaccharide hydrolysis that enable the cells to process nutrients more efficiently. The down-regulation of a protein for fatty acid biosynthesis and a transcriptional regulator in attached cells may be indicative of a change in growth state from exponential growth in the planktonic state to lag phase in the attached state.

Discussion

During the first 3 hours of initial adhesion, bacterial cells attach to a surface but the development of a differentiated biofilm has not yet begun. Research has shown that bacterial cells undergo metabolic and physiologic changes during biofilm development (Bollinger et al., 2001; Boucher et al., 2000; Keith and Bender, 1999; Costerton, 2000; Davies et al., 1993). However, it is during the time period of initial adhesion that, presumably, cells are progressing from a physiologic state characteristic of planktonic cells to one characteristic of a biofilm state. The understanding of the changes that take place within attached but not yet biofilm-forming cells may provide crucial insight into the processes that these cells undergo to formulate a biofilm phenotype (Costerton, 2000; Lam et al., 1980). This study

endeavored to analyze differential protein expression in *P. aeruginosa* during initial adhesion.

Proteins up regulated during initial adhesion

2 D gels of planktonic control samples were compared to 3 hour attached samples and protein spots that were present only in the 3 hour gels were designated up regulated. Up regulated during initial adhesion were glycosyl transferase (Level 3), glycosyl hydrolase (Level 3), UDP-glucose pyrophosphorylase (Level 2), heat shock protein HscA (Level 2), aliphatic amidase (Level 1), 2,4-dienoly-CoA reductase (Level 2), Fe(III)-pyochelin receptor/precursor (Level 1), and 2 unknown proteins (Level 4).

In this study, two proteins in the alginate and LPS biosynthetic pathways (glycosyl transferase and UDP-glucose pyrophosphorylase) were up regulated during initial adhesion. *P.aeruginosa* up regulates alginate production when attaching to a surface (Costerton, 2001; Davies et al., 1993). The glycosyl transferase that was identified in this study shows a 70% homology to the MigA mucus inducible protein in *P. aeruginosa*. The *migA* gene, highly inducible in cystic fibrosis patients, has been shown to be regulated by the *rhl* quorum sensing regulatory system (Yang et al., 2000). The *rhl* system has been shown to play a crucial role in the export of pilin and assembly of pili and is important in biofilm development (Glessner et al., 1999).

The second up regulated protein, UDP-glucose pyrophosphorylase (*galU*) converts glucose 1-phosphate derived from glucose 6-phosphate to UDP-glucose, an

intermediate in the formation of LPS (Boels et al., 2001). In *Bacillus subtilis*, sigma factor B, which controls a stationary phase regulon, transcribes *gtaB* which in turn encodes UDP-glucose pyrophosphorylase (Varon et al., 1993). When exponentially dividing *P. aeruginosa* cells attach to a surface they enter a lag phase for 12 to 24 hours (Rice et al. 2000). During this time, presumably the cells are undergoing the metabolic changes necessary to enable them to stay attached to that surface. In *E. coli*, *galU* mutants are adhesion defective and show reduced type 1 fimbriae production and a corresponding lack of motility (Genevaux et al., 1999).

Glycosyl hydrolase enables the bacterial cell to hydrolyze polysaccharides. *P. flurorescens* subsp. *cellulosa* has been shown to use a glycosoyl hydrolase for the hydrolysis of plant cell wall polysaccharides (Hazlewood and Gilbert, 1998). There is little information available as to the function of glycosyl hydrolase in *P. aeruginosa*.

The up regulation of 2,4-dienoyl-CoA reductase enables the cell to metabolize fatty acids and phospholipids. Rhamnolipid production, a quorum sensing regulated virulence factor in *P. aeruginosa* (Fuqua et al., 1996), is produced in the presence of acetyl-CoA via the fatty acid biosynthetic pathway (Campos-Garcia et al., 1998). *P. aeruginosa* knockout mutants in the polyphosphate kinase (ppk) gene are unable to form thick biofilms and show severely reduced levels of production of both rhamnolipid and elastase (another virulence factor) (Rashid et al., 2000).

The heat shock protein HscA is a member of the DnaK family of molecular chaperones (Campos-Garcia et al., 2000). In *E. coli*, the *hsc* gene, heat shock cognate, lacks a consensus heat shock promoter sequence and its expression is not

induced by temperature elevation (Seaton and Vickery, 1994), suggesting that the expression of this protein may have more to do with factors in cell metabolism not related to heat shock. In *Azotobacter vinelandii*, co-expression of *hscB* and *hscA* apparently function in the production and maturation of iron-sulfur cluster containing proteins (Zheng et al., 1998). The previously described protein, 2,4-dienoyl-CoA reductase is an iron-sulfur flavoprotein (Liang et al., 2000), thus implicating HscA in the formation of a biofilm associated virulence factor.

P. aeruginosa has been shown to react to iron limiting conditions by producing the siderophores pyoverdin and pyochelin. The compounds are released outside the cell, complex with iron and deliver to the cell through membrane receptors (Reimmann et al., 2001). Both of these siderophores have been shown to play an important role in the expression of virulence in *P. aeruginosa* infections (Takase et al., 2000). The up regulation of the Fe(III)-pyochelin receptor during initial adhesion may not only indicate the need for increased nutrients but also the up expression of virulence factors associated with attachment and biofilm formation (Davey and O'Toole, 2000). In addition, the *pchR* regulatory gene in *P. aeruginosa* contains the biosynthetic genes required for pyochelin siderophore formation (Reimmann et al., 1997). A putative operon located downstream which also appears to function in pyochelin biosynthesis has also been shown to resemble ABC (ATP binding casette) transporters. ABC transporters function in the active efflux of antibiotics out of the bacterial cell (Putman et al., 2000) and this up expression during initial adhesion may be indicative of one mechanism for the increase in antibiotic

resistance associated with biofilm formation (Costerton et al., 1999; Costerton and Stewart, 2000; Xu et al.,1998).

The catabolite repression control (Crc) protein not only regulates carbon metabolism but also the formation of biofilms in *P. aeruginosa* (O'Toole et al., 2000). Crc also functions in the repression of the aliphatic amidase *amiE*, a structural gene of the amidase operon (Collier et al., 2001). The up regulation of this aliphatic amidase may indicate that Crc has not yet been activated and indeed may even be indicative of the fact that the attaching cells have not yet achieved the full protein expression pattern typical of the biofilm phenotype. In addition, the amidase operon contains two genes, *amiB* and *amiS*, that share similarities to ABC transporters (Wilson et al., 1996). The presence of these two separate systems, pyochelin and aliphatic amidase, that may also function in the up regulation an active antiobiotic efflux system may indicate the presence of a crucial step in the development of the antibiotic resistance so characteristic of biofilm cells.

<u>Proteins down regulated during initial adhesion</u>

Protein spots that disappeared in 2 D gels of 3 hour samples in comparison to the planktonic control samples were designated as down regulated during initial adhesion. Those proteins down regulated were biotin carboxylase (Level 1), a transcriptional regulator (Level 3), a TonB-dependent receptor (Level 3), and one unknown protein (Level 4).

The FabG protein, a biotin carboxylase, functions in carbon and energy storage specifically in the formation of medium chain length poly-3-hydroxyalkanoates (mcl-PHAs) (Ren et al., 2000). PHAs are synthesized by the polymerization of coenzyme A (CoA) linked fatty acids and can occur through a number of different pathways (Madison and Huisman, 1999). Research has shown a direct correlation between the transcription of the *acc* genes that encode the carboxylase subunits and cell growth (Li and Cronan, 1993). The up regulation of the biotin carboxylase (also known as acetyl-CoA carboxylase) found in the fatty acid biosynthetic pathway is indicative of an exponential (planktonic) growth phase. Its corresponding down regulation in attached cells may be indicative of the change in growth state to lag phase in sessile bacteria (Li and Cronan, 1993).

The TonB protein has been shown to be crucial in the ability of *P. aeruginosa* to acquire iron using either pyoverdin, salicylate, or pyochelin siderphores (Takase et al., 2000). TonB receptors are located in the periplasm with their N-termini anchored to the cytoplasmic membrane (Braun and Killmann, 1999). The *P. aeruginosa* database lists 15 probable TonB dependent receptors but little is known about the specific function of each of these. The down regulation of a probable TonB dependent receptor is somewhat puzzling given the up regulation of the Fe(III)-pyochelin receptor during attachment. The specific transcriptional regulator that is down expressed at 3 hours may be linked to this TonB receptor. The transcriptional regulator identified in *E. coli* shares a 57% homology to PtxR in *P. aeruginosa.* The *ptxR* gene is a regulatory gene for exotoxin A production (Colmer and Hamood,

1999) and has been linked to the expression of the *pvcABCD* operon and therefore appears to play a role in pyoverdine production (Stintzi et al. 1999). Pyoverdine production, a virulence factor associated with biofilm formation (Deziel et al. 2001), is down regulated at 3 hours of attachment. This may be indicative of the complexity of the global regulation involved in the progression to the biofilm phenotype.

Conclusion

The development of a biofilm involves more than the mere sticking of a cell to a surface. Instead there are a variety of changes in the physiology of the attaching cell when compared to their planktonic counterparts. The results obtained from this proteomic based study allow for the examination of the changes that take place within a wild type organism and reflect what is actually taking place within a biofilm. At 3 hours of attachment, a point at which a biofilm has not yet developed, the up regulation of proteins similar to those involved with alginate production, antibiotic resistance mechanisms and virulence factors is seen. Crucial to the understanding of biofilm formation is an understanding of the point at which the cell has undergone its transition from the free-floating planktonic state to the attached state. The delineation of specific proteins involved in the regulatory mechanisms involved in biofilm formation may provide insight as to potential biofilm control methodologies.

CHAPTER 6

THE EXPRESSION OF STRESS RESPONSE PROTEINS DURING INITIAL ADHESION BY *PSEUDOMONAS AERUGINOSA*

Introduction

Bacterial cells subjected to stress such as temperature or osmotic changes will produce proteins as a transient response to changes in the environment. Despite the association with stress, it has been determined that these proteins also can play a role in processes not induced by stress (Hueck and Hillen, 1995; Giard et al., 2000). For example, GroEL and DnaK, both heat shock proteins, function as chaperones in the process of protein folding. These chaperones have been shown to be important not only in stress response, but have also been shown to be essential for normal growth (Karow et al.,1991).

The expression of particular set of proteins that enables cells to attach to a surface and to eventually form biofilms may also include pathways that are involved in stress response regulation. However, the role of signal transduction pathways associated with the expression of stress response proteins in biofilms is not well understood. It has been shown that proteins associated with the physical aspect of bacterial attachment to a surface are up regulated during biofilm formation. These include, but are not limited to, the synthesis of alginate (Davies and Geesey, 1995), pili formation (O'Toole et al., 2000) and the conversion to mucoid phenotypes (Hoyle

et al.,1993; Singh et al., 2000).

The conversion to a mucoid phenotype has been shown to be crucial for biofilm formation in *Pseudomonas aeruginosa* and in environmental pseudomonads such as in *P. fluorescens* and *P. syringae* (Costerton, 2000; Davies et al., 1999; Singh et al., 2000). This conversion to mucoidy involves the expression of a number of genes not directly involved in the synthesis of alginate. *P. aeruginosa* PAO1 strain *mucA22* has been shown to express a number of proteins under the control of sigma factor 22, a heat stress response regulator (Rashid et al., 2000), including AlgA and AlgD, both part of the alginate biosynthetic pathway (Deighton et al., 1996). In *P. syringae*, *algT*, which controls alginate biosynthesis, has also been shown to encode sigma factor 22 as well. Studies indicate that *algT* expression in *P. syringae* is activated by heat shock (similar to *P. aeruginosa*); however, *algT* expression was also induced by osmotic stress, exposure to H_2O_2, copper sulfate and paraquat (Keith and Bender, 1999). The implication, then, is that the conversion to the mucoid phenotype so important for biofilm development may be regulated at the global level.

There are a number of proteins expressed during biofilm formation that are not directly involved in the actual attachment process but instead appear to be part of larger signal transduction pathways. How these additional proteins function in the development of a biofilm remains to be elucidated. The initial attachment to a surface and the eventual formation of a biofilm may stress the bacterial cell due to nutritional and/or oxygen deficits that develop around the attaching cells. This may be due in part to changes in fluid flow parameters around the cells as they form a biofilm

and may also be due to the fact that closely packed clumps of cells are now competing for carbon and oxygen. Cells attached to a surface now only have a portion of their membrane available for the absorption of oxygen and carbon with that portion of the membrane directly attached to the surface now literally unavailable.

These metabolic changes that occur within biofilm forming cells may have direct implications for how we treat and control biofilm growth. For example, *P. aeruginosa* Paer1, isolated from a contact lens, when grown either under nitrogen or carbon limited conditions, showed increased adhesion to contact lenses (Cowell et al., 1998). The *P. aeruginosa* sigma factor AlgU is required for this organism's ability to effectively resist oxidative stress and extreme temperatures and also functions to control the conversion to a mucoid state (Boucher et al., 2000). It is crucial to delineate those global regulatory pathways that may be instrumental in biofilm formation.

Working with a mucosal *Staphylococcus aureus* isolate, Rachid et al. (2000) showed that the alternative transcription factor sigma B, a global stress response regulator in *S. aureus*, may also play a role in the development of biofilms during osmotic stress. Sigma B also appears to function in biofilm formation in *S. epidermidis*. Knobloch et al. (2001), using the biofilm forming *S. epidermidis* strain 1457, was able to show that the inactivation of *rsbU*, a positive regulator of sigma B resulted in a biofilm deficient phenotype. Further, these researchers showed that the synthesis of polysaccharide intercellular adhesin (PIA), which is essential for biofilm development, was significantly increased under stress conditions of high osmolarity

and in the presence of ethanol (Knobloch et al., 2001).

The role of signal transduction pathways in initial attachment and eventual biofilm formation could provide insight as to the complex physiologic mechanism required for a sessile mode of growth. The possibility that, in order to attach to a surface, bacterial cells activate global regulatory networks as opposed to the up expression of a few dozen unrelated proteins holds implications for the future ability to control biofilm formation in a number of settings. The goal of this study was to analyze similarities in protein expression during initial cell attachment prior to biofilm formation and stress conditions using a comparison of differential protein expression profiles in *P. aeruginosa* during stress in planktonic cultures and during attachment to a surface.

Materials and Methods

Organism and growth conditions

Pseudomonas aeruginosa ERC1 (ATCC # 700888) was grown to steady state at room temperature in a chemostat with a residence time of 5 hours using a minimal salts medium (Manual of Industrial Methods). Glucose at 0.1g/L was used as the carbon source. Chemostat grown cells at approximately 10^6 cells/ml were used to inoculate vessels (500 ml) containing plugs of Teflon™ mesh (Tetko Inc.). The plugs were made of layers of 61% open area Teflon™ mesh tightly rolled to form an approximately 9 cm high by 7 cm wide cylinder secured with a cable tie. A nutrient feed was maintained at the same flow rate as the chemostat cultures and the vessels were aerated and stirred to minimize nutrient gradients within the reactor or oxygen

limiting conditions. Adhesion tests were performed at room temperature for 3 hours with a minimum of three replicates performed. At the end of the adhesion test period, the Teflon™ plug was rinsed by holding the mesh plug with sterile tongs and moving the plug up and down within the vessel to remove any loosely attached cells. The mesh plug was placed in ice cold buffer solution (medium without any additional carbon) containing 1.5M Tris pH 8.8 to prevent proteolysis. Cells were removed from the mesh plug by sonicating in the ice-cold buffer with 6 times 10 second bursts interspersed with 10 second non-sonication/rest periods using a probe sonicator (Tekmar Inc.) following manufacturer's directions for tuning the amplitude. During this process, the plug was kept constantly moving up and down in the ice-cold buffer to help flush cells from the plug into the buffer. The buffer solution containing the detached cells was immediately centrifuged (10,000 rpm for 20 minutes at 4° C). The pelleted cells were removed and immediately iced.

Planktonic control samples (20.0 ml) were taken from the chemostat and immediately centrifuged and processed as described above.

Stress experiments

P. aeruginosa ERC1 was grown to steady state as described above but in 500 ml capacity chemostats without Teflon™ mesh plugs. A chemostat culture was then subjected to heat by placing the chemostat on top of a stirring hot plate while nutrient feed and oxygen input remained unchanged. The temperature was gradually increased to 40°C (from 30°C) and held at 40°C for 3 hours to coincide with the maximum adhesion experiment time. Nutrient feed and oxygen input was maintained

at the same rate as planktonic non-stressed and attachment experiments. For starvation conditions, chemostat grown cells were maintained with oxygen input and mixing rates but with no additional nutrient feed for 3 hours. A minimum of three replicates was performed for each of the stress tests. At the end of each stress experiment, cells were collected by centrifugation and processed for 2 D gel electrophoresis.

Protein extraction

Concentrated biofilm and planktonic cell preparations were assayed for total protein using a modified Lowry method (Sigma Kit 690). The cells were lysed with the probe sonicator (3 times 6 second burst, 10 second rest) on ice in lysis buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), and 40 mM Tris base. Proteins were precipitated using ice cold 10% trichloroacetic acid (TCA) in acetone. Proteins were collected by centrifugation (10,000 rpm for 20 minutes at 4° C) and the pellet was allowed to air dry to remove residual acetone.

Protein Solubilization

Proteins were solubilized in a buffer solution containing 8M urea, 2% CHAPS, 2% pH 3-10 nonlinear Immobilized pH Gradient (IPG) buffer and a trace of bromophenol blue. Immobiline Dry Strips pH 3-10 nonlinear (Amersham Pharmacia) were rehydrated in the Multiphor II (Amersham Pharmacia) reswelling tray following Amersham Pharmacia protocols.

Protein electrophoresis

Two-dimensional (2 D) protein electrophoresis was performed based on the procedure described first by O'Farrell (1975) and modified as described by Gorg et al. (2000). The first-dimension focusing procedure was carried out using the Multiphor II system following Amersham Pharmacia protocols using 500 μg of protein for analytical gels. A strip of blotter paper, saturated with 6.0 % dithiothreitol (DTT) was placed over immoblized pH gradient (IPG) strips at the cathode end (Amersham Pharmacia). Strips were focused at 3500 mV for 20 hours. Strips were then either used immediately for the second dimension or stored at –70°C for later use.

Prior to the running second dimension, each focused IPG strip was equilibrated in 2.5 ml of equilibration buffer (50 mM of 1.5 M Tris-Cl pH 8.8, 6M urea, 30% glycerol, 2% lauryl sulfate, sodium salt (SDS), and a trace of bromophenol blue) containing 25.0 mg of DTT for 10 minutes and then in 2.5 ml of equilibration buffer containing 62.5 mg of ioadacetamide for 10 minutes. Strips were placed on paper towels and rinsed with sterile nanopure water prior to placement on SDS PAGE gels.

The second dimension run was performed using 12.5% acrylamide gels in the BioRad Protean II xi vertical system. Strips were overlaid on 12.5% SDS PAGE gels (16.0 cm by 20.0 cm by 1.0 mm) and were sealed in place with hot 1.0% agarose. Gels were run at 8.5 mAmps per gel for 10 hours. Proteins were visualized using Gel Code Coomassie stain (Pierce).

In all cases, the comparison of protein profiles was made using the control planktonic sample from the same experiment as the attached or stressed sample. All samples from a single experiment were processed simultaneously using the same reagents and same protein load (500 μg). This minimized experimental variability due to subtle differences in medium constituent concentrations, flow rate, or protein processing. Each time point in the experiment was repeated at least three times and gels for each time point were done at least in triplicate.

Peptide mass fingerprinting

2 D gels from planktonic control, stressed, and 3 hour attached cells were compared. Protein spots that were up regulated during adhesion (as determined by the comparison of planktonic control gels and 3 hour attached gels) that were also up regulated during stress conditions were selected for analysis. Proteins were excised from the gel and care was taken to minimize the amount of excess acrylamide surrounding the actual protein spot. Excised gel pieces were placed in siliconized Eppendorf tubes previously treated with 50% acetonitrile. The excised gel pieces were washed twice with approximately 100 μl (or enough to cover the gel fragments) of a 1:1 solution of 25 mM NH_4HCO_3 and 50% acetonitrile. For each wash, the Eppendorf tubes were placed on a shaker at room temperature for 30 minutes. Each time the wash solution supernatant was removed and discarded. Gel pieces were lyophilized for approximately 20 minutes. In gel tryptic digestion was carried out overnight at 37 °C by the addition of 20 μl trypsin solution (12.5 ng/ul trypsin (sequencing grade, Promega) in 25 mM NH_4HCO_3). As the gel pieces rehydrated,

additional 25 mM NH_4HCO_3 was added to cover the gel pieces. Following digestion, the Eppendorf tubes were briefly centrifuged and 20 µl 0.1% trifluoracetic acid (TFA) was added. Samples were centrifuged again and the supernatants were transferred to new Eppendorf tubes. The samples were purified by means of reverse phase C18 column chromatography using ZipTip C18 columns following manufacturer guidelines (Millipore). A 5.0 µl aliquot of the peptide solution was combined with 5.0 µl of a saturated solution of α cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1 % TFA. In addition, 1.0 µl of 10.0 picomole/ul angiotensin II was added as an internal standard. 2.0 µl of the solution was spotted on the template and allowed to air dry (Shevchenko et al. 1996; Gevaert and Vandekerckhove, 2000; Jensen et al. 1999).

Peptides were analyzed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using a Bruker Biflex III system in reflectron mode with an acceleration voltage of 25kV. All samples were randomly labeled to prevent bias during the analysis process. The spectra were calibrated using angiotensin II at 1047.18 Da [M+H]+. Analysis of peptide masses was performed using the MS-Fit Program within the Protein Prospector database (http://prospector.ucsf.edu/). Parameters selected for the search were as follows: trypsin digest and peptide mass tolerance set at +/- 30 ppm. Genpept 7.2.2001 was used as the reference database for the identification of proteins. All protein samples were randomly labeled to avoid any selection bias during this analysis process. The

peptide mass tolerance was increased as noted in Table 1 if no proteins were found to match the molecular weight and pI of the excised gel spot.

Results

Protein profiles for attached *P. aeruginosa* cells were compared to profiles for control and planktonic stressed cells. The proteins up regulated during attachment and also present in the stress profiles were selected for analysis (Figure 1). A total of 12 protein spots up regulated both during 3 hours of attachment and during the respective stress condition were analyzed.

Evaluation of Coomassie stained protein spots of similar molecular weights can be both quantitative as well as qualitative with the larger and darker the protein spot on a gel representing more protein (Neuhoff et al., 1990; Neumann et al.,1992). In these experiments, the original sample load was always the same (500 μg of protein loaded on the IPG strip) so the change in protein spot intensity at a specific molecular weight can been interpreted as a relative change in expression during these experiments. Four of the proteins were strongly expressed (visualized as very dark spots) during 3 hour attachment and were also highly expressed under stress (two from 40 °C and two from starvation stress). Eight proteins were weakly expressed (faint spots) during 3 hour attachment but showed enhanced expression (darker stained spots) under stress (five from 40 °C and three from starvation stress). Of the 12 spots selected for analysis, 11 were identified.

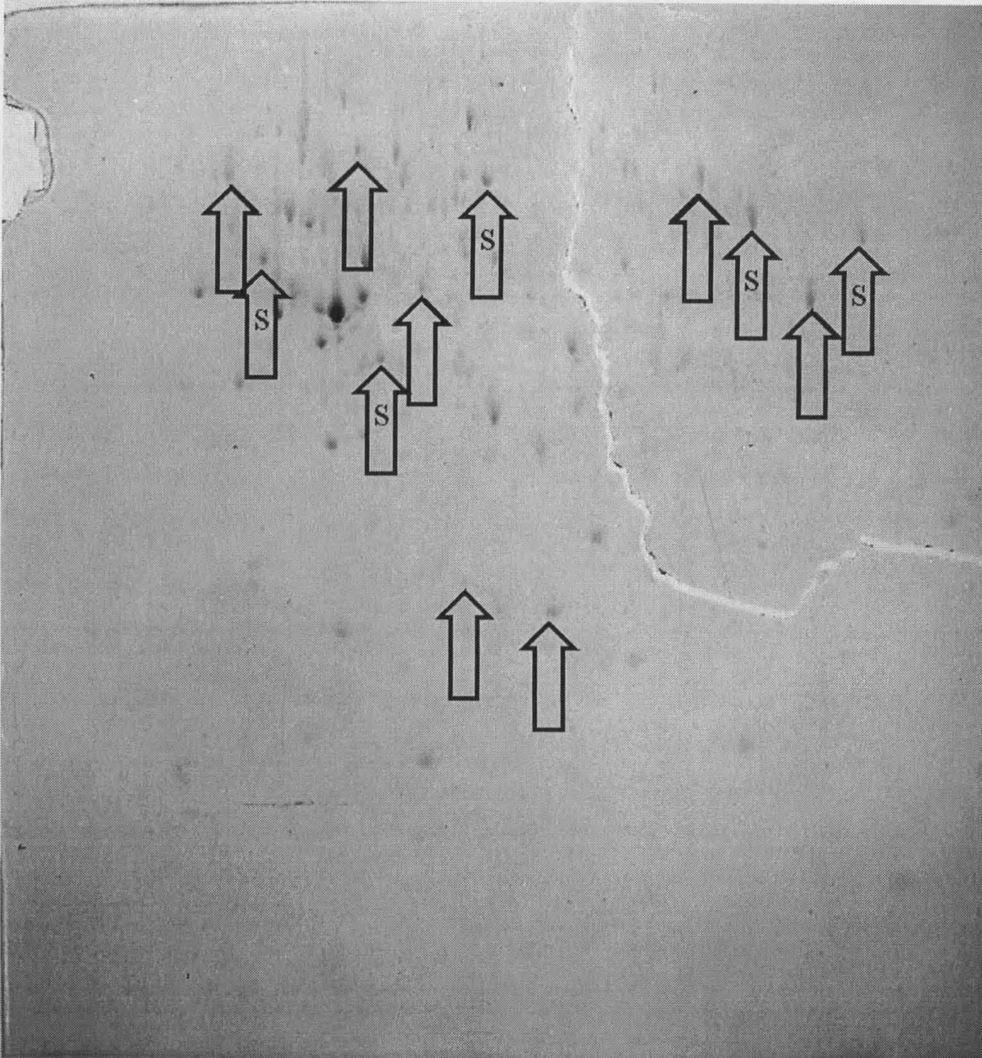


Figure 6.1: Protein profile (2 D gel) of *P. aeruginosa* cells at 3 hours of attachment to a surface. Arrows indicate proteins that were selected for MALDI-TOF analysis. Open arrows indicate proteins expressed both during attachment and heat stress. Arrows designated with 'S' indicate proteins expressed both during attachment and starvation stress.

The identified proteins are listed in Table 1. In all cases, the mass spectra analysis were matched to a minimum of 3 peptides to establish the identity of the

Protein Name	Expression	Gene Name	Confidence Level	Gene Designation	Peptide Mass Tolerance	Function
Strong expression during stress weak expression during attachment						
Glutamate ammonia ligase adenylyltransferase	heat	*glnE*	2	PA5014	100 ppm	nitrogen assimilation protein production
Ribonuclease E	heat	*rne*	2	PA2976	30 ppm	RNA processing and degradation
3'-phosphoadenosine-5'-phosphosulfate reductase	heat	*cysH*	2	PA1756	50 ppm	Sulfate assimilation protein production
Exoribonuclease RNase R	heat	*rnr*	2	PA4937	30 ppm	RNA processing and degradation
XcpX	heat	*xcpX*	1	PA3097	50 ppm	Type II secretion type IV pili
DNA gyrase subunit A	starve	*gyrA*	1	PA3168	50 ppm	DNA repair
Exodeoxyribonuclease V beta chain	starve	*recB*	2	PA4284	100 ppm	DNA repair
ExbB2	starve	*exbB2*	2	PA0693	50 ppm	siderophore production virulence factor
Strong expression both during stress and attachment						
Hypothetical protein AE004444	3h/heat		4		100 ppm	unknown
2,4-dienoyl-CoA reductase	3 hr/heat	*fadH1*	2	PA3092	30 ppm	fatty acid & phospholipid metabolism virulence factor
Aliphatic amidase	3 hr/starve	*amiE*	1	PA3366	30 ppm	carbon regulation antibiotic resistance
UDP-glucose pyrophosphorylase	3 hr/starve	*galU*	2	PA2023	115 ppm	LPS formation virulence factor lag phase induction

Table 6.1. Summary of the proteins expressed both during conditions of stress and during initial adhesion in *P. aeruginosa*. Protein spots were selected from 2 D gels and subsequently analyzed by MADLI-TOF mass spectrometry. Functions listed for proteins having confidence levels of 2 or 3 are considered to be putative.

protein and the majority of the proteins were matched at either +/- 30 or +/-50 ppm for peptide mass tolerance. Confidence levels for protein function, as previously described in Chapter 5, are noted.

Proteins which showed relatively weak expression during attachment and relatively strong expression during heat shock were RNA processing and degradation proteins (ribonuclease E and exoribonuclease R), a Type II secretion protein (XcpX), glutamate ammonia ligase adenylyltransferase for nitrogen assimilation and CysH for sulfate assimilation. Proteins strongly expressed during both attachment and heat shock were 2,4-dienoyl CoA reductase $FadH_2$ and a protein identified in the *Pseudomonas* database of unknown function.

Proteins which showed enhanced expression during starvation stress were DNA gyrase subunit A and exodeoxyribonuclease V beta chain, both involved in DNA repair, and ExbB, which functions in siderophore production for enhanced iron uptake. Proteins that were strongly expressed under both conditions of starvation and attachment were alipahtic amidase for carbon regulation and UDP-glucose pyrophosphorylase for the production of LPS.

Discussion

The purpose of this study was the analysis of stress expression as it relates to attachment processes. Protein profiles (2 D gels) of attached cells were compared to planktonic control profiles from the same experimental run and up regulated proteins were noted. Protein profiles for the stress experiments were then compared to protein

profiles for attached cells. Proteins up regulated during cell attachment that were also present in the stress profiles were selected for analysis. A total of 12 proteins were selected for analysis. Eight of the proteins were more strongly expressed under stress as compared to attachment. The variation in relative expression of these proteins may be indicative of the transient nature of *de novo* protein synthesis during attachment. The 3 hour attachment time may represent the time at which these proteins are in the process of up regulation rather than the time point at which they are maximally expressed.

Heat shock and attachment protein expression

Proteins expressed during both attachment and heat shock were 2,4-dienoyl CoA reductase (Level 2), ribonuclease E (Level 2), exoribonuclease R (Level 2), XcpX (Level 1), glutamate ammonia ligase adenylyltransferase (Level 2) and CysH (Level 2).

Attaching bacterial cells must synthesize proteins that enable them to adapt to a sessile growth state. Changes in protein expression require the up regulated production of proteins that function in RNA processing and degradation. Endoribonuclease E (RNase E) has been shown to be essential in the processing and degradation of RNA in *E. coli* (Walsh et al., 2001). GroEL, a heat shock protein, has been shown to posses RNase E like activity and, in fact, RNase E can be precipitated with an antibody against GroEL (Sohlberg et al., 1993). RNase E is part of a ribonucleolytic complex that also includes polynucleotide phosphorylase (PNPase),

RhlB RNA helicase, enolase, DnaK (a chaperonin), GroEL and polynucleotide phosphate kinase (PPK) (Liou et al., 2001).

Also up regulated during heat shock and attachment was exoribonuclease R. Originally identified in *E. coli*, this enzyme processes and degrades RNA and may have a putative connection to virulence expression (Cheng et al., 1998). The *vacB* which encodes the RNase R homolog in both *Shigella flexneri* and *E. coli* has been shown to be required for virulence expression in these species (Cheng et al., 1998).

The manufacture and processing of *de novo* proteins during attachment may require an increase in nutrient assimilation in order to provide for the necessary molecular components. Glutamate-ammonia-ligase adenylyltransferase, which functions in the nitrogen assimilation cascade (Jaggi et al., 1997), was up regulated during heat shock and attachment. Adenylyltansferases have been shown to regulate, along with uridylyl transferases, the enzymatic activity of glutamine synthetase, a key enzyme in nitrogen assimilation (Jaggi et al., 1997). In *E. coli*, the folding of glutamine synthetase is facilitated by GroEL and GroES (Fisher, 1992). At present, there are no studies to indicate the relationship of glutamine and heat shock protein production in bacteria..

Another protein expressed during heat shock that functions in nutrient assimilation is 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase. In *P. aeruginosa*, this protein catalyzes 5'-adenylylsulfate reductase (APS) which is required for sulfate assimilation (Bick et al., 2000). PAPS reductase is encoded by *cysH* and is part of a putative operon preceded by *cysB*, a transcriptional regulator,

which is transcribed in the opposite direction (Neumann et al., 2000; Haverkamp and Schwenn, 1999). In *P. aeruginosa, cysB* has been shown to activate the transcription of *algD* (Delic-Attree et al., 1997). The initiation of alginate biosynthesis, crucial for biofilm formation in *P. aeruginosa*, begins with the transcription of the *algD* promoter (Chitnis and Ohman, 1993; Keith and Bender, 1999). *P. aeruginosa* alginate functions as a virulence factor (Engels et al., 1985; Keith and Bender, 1999) and also functions to increase the ability of the microorganism to survive stress conditions (Boucher et al., 2000).

An additional nutrient assimilation protein expressed during heat shock and attached growth conditions is 2,4-dienoyl-CoA reductase. This iron-sulfur flavoprotein is required for the oxidation of unsaturated fatty acids (Liang et al., 2000) and is required for the production of rhamnolipids via the fatty acid biosynthetic pathway (Campos-Garcia et al., 1998). Rhamnolipid production is a virulence associated trait and has been shown to be regulated in a quorum sensing manner in *P. aeruginosa* (Fuqua et al., 1996). *P. aeruginosa* knockout mutants in the polyphosphate kinase (ppk) gene are unable to form thick biofilms and show severely reduced levels of production of both rhamnolipid and elastase (another virulence factor) (Rashid et al., 2000).

XcpX was also up regulated during heat stress and attachment. Xcp proteins aid in the secretion of proteins across the outer membrane of gram-negative bacteria via the general secretory pathway (GSP), a type II system. It is crucial that the transported proteins remain in an unfolded state during translocation across the

bacterial inner membrane through the periplasm to the outer membrane (Filloux et al., 1998). SecB and GroEL or DnaK (in SecB independent translocation) act as the chaperones for this process. Both GroEL and DnaK are considered to be heat shock produced proteins in a number of organisms including *E. coli*, *Bacillus subtilis*, and *P. aeruginosa* (Michel et al., 1998; Mogk et al., 1997; Allan et al., 1988). In addition, the XcpX protein, along with four other Xcp proteins (XcpT, XcpU, XcpV and XcpW) shows sequence homology to type IV pili to the extent that these five Xcp proteins are now designated as pseudopilins (Bleves et al., 1996). The presence of type IV pili has been shown to be crucial in the development of *P. aeruginosa* biofilms (O'Toole and Kolter, 1998). Therefore, the up regulation of XcpX may be an important prerequisite in the initial attachment phase of biofilm formation.

The expression of heat shock proteins during biofilm formation at room temperature is probably an indicator of a more global type response mechanism within the bacterial cell. The global response enables that bacterial cell to act in a more efficient manner with respect to the metabolic and physiologic changes required for adhesion to a surface.

Starvation and attachment protein expression

Proteins expressed during starvation and attachment were DNA gyrase subunit A (Level 1), exodeoxyribonuclease V beta chain (Level 2), ExbB (Level 2), aliphatic amidase (Level 1) and UDP-glucose pyrophosphorylase (Level 2).

The results of starvation stress experiments and attachment experiments show

the up regulation of proteins enabling nutrient depleted cells to change and/or more efficiently metabolize nutrients. TonB functions in the active transport of vitamin B12 and the Fe (III) siderophores such as pyoverdin and pyochelin by using the proton motive force to move these compounds into the periplasmic space (Ahmer et al., 1995). Auxiliary proteins ExbB and ExbD are required to stabilize TonB in its energy-dependent conformation (Higgs et al., 1998) with *exbB* acting as the promoter of *exbD* transcription (Ahmer et al., 1995). The expression of ExbB then, would be indicative of the up regulation of the iron acquisition process. The pyoverdin and pyochelin siderophores are considered to be required for bacterial growth and virulence expression in *P. aeruginosa* (Takase et al., 2000).

UDP-glucose pyrophosphorylase (*galU*) an enzyme in the glycolytic pathway has been shown to produce precursors for the biosynthesis membrane-derived oligosaccharides and for LPS (Bohringer et al., 1995). The stationary phase transcription factor sigma B, in *B. subtilis*, has been shown to control the transcription of *gtaB* which in turn encodes UDP-glucose pyrophosphorylase (Varon et al., 1993). In *P. aeruginosa*, exponentially dividing planktonic cells enter stationary phase for 12 to 24 hours upon attachment to a surface (Rice et al., 2000). The up expression of UDP-glucose pyrophosphorylase is a possible indicator of the increase in production of LPS, a virulence factor in *P. aeruginosa* (Engels et al., 1985; Keith and Bender, 1999), as well as an indicator of the change in growth stage status.

Aliphatic amidase is encoded by *amiE*, a structural gene of the amidase operon (Collier et al., 2001). Other amidases within the operon, AmiA and AmiD,

play a role in the cleavage and reprocessing of cell wall peptidoglycan to muropeptides in the periplasmic space (Kraft et al., 1999). The increase in the concentration of murein turnover products has been shown to induce the expression of beta lactamases which are capable of inhibiting the action of beta lactam antibiotics (Kraft et al., 1999). Also within the amidase operon are two genes, *amiB* and *amiS*, that share similarities to ABC type multi-drug transport systems (Wilson et al., 1996). The up regulated expression of this aliphatic amidase during attachment may be indicative of the regulation of processes crucial for the development of the antibiotic/biocide resistance that is considered characteristic of biofilms (Anderl et al., 2000; Anwar et al.,1989; Costerton and Stewart, 2000). In addition, this particular aliphatic amidase is repressed by the catabolite repression control (Crc) protein which regulates carbon metabolism (Collier et al. 2001). In these experiments, the presence of aliphatic amidase indicates that the Crc protein has not yet been activated at the 3 hour attachment time. *P. aeruginosa* mutants deficient in the carbon metabolism regulator protein (Crc), have been shown to be defective in the ability to form biofilms (O'Toole et al., 2000). Crc mutants form monolayers of cells on a surface phenotypically similar to the monolayers formed by type IV pilus defective mutants. This implies that Crc may be part of a signal transduction pathway that is involved in the development of biofilms (O'Toole et al., 2000). This relationship between aliphatic amidase and Crc may be indicative of a state of progression in biofilm development.

The expression of exodeoxyribonuclease V beta chain, up regulated during

starvation and attachment, is encoded by the *recB* gene. SOS regulon stress response to DNA damage in *E. coli* activates the RecA protein which in turn initiates the RecBCD pathway for the homologous recombination of linear DNA (Keller et al., 1990). The hydroxyl radicals that can generate DNA double strand breaks (DSB) have been shown to be produced in cells starved for phosphate (Moreau et al., 2001). Therefore, the initiation of the RecBCD pathway in this case may be in response to low nutrient conditions.

DNA gyrase, composed of GyrA and GyrB, can introduce negative supercoils into DNA molecules and therefore functions in the processes of DNA replication and repair (Akasaka et al., 1999). GyrA acts to bind, cleave and rejoin DNA while GyrB contains the site for ATPase activity to provide energy for the supercoiling reaction (Akasaka et al., 1999). The quinolone class of antibiotics are gyrase inhibitors that target *gyrA* (Yonezawa et al. 1995). *P. aeruginosa* quinolone-resistant isolates show mutations in the *gyrA* gene (Yonezawa et al., 1995).

Conclusion

The processes of biofilm formation and development have been shown to require a number of diverse factors. The proteins expressed during stress conditions in planktonic cells appear to share many functions related to bacterial attachment to surfaces. Proteins involved in the assimilation of nutrients such as nitrogen and sulfate required for protein production are also expressed during heat stress. DNA replication and repair, expressed during starvation, and RNA processing, expressed

during heat stress are additional factors required for protein production. Factors determined by previous research as important for cell adhesion included the up expression of pathways involved in alginate and LPS production (Davies and Geesey, 1995), both induced during heat and starvation conditions, as well as an aspect of type IV pili formation (O"Toole and Kolter, 1998), activated during heat stress. An antibiotic resistance mechanism was up regulated during starvation conditions, and both heat stress and starvation up regulated proteins for virulence factor expression.

Biofilm formation is likely controlled by a number of environmental signals. How attaching bacteria respond on a global scale to these signals appears to determine not only the developmental characteristics of a biofilm but also the up regulation of other factors noted as ancillary characteristics of a biofilm. Biofilm bacteria, through the regulation of various signal transduction pathways, may also "automatically" up regulate antibiotic resistance mechanisms and virulence factors. This work shows that there are a number of proteins expressed during initial adhesion of *P. aeruginosa* ERC1 to a surface that are also expressed during conditions of stress on planktonic cells, although the 12 proteins analyzed in this study are by no means all inclusive. The synthesis of *de novo* proteins requires the processing of RNA and DNA as well as protein components such as nitrogen, sulfate, carbon and iron. The putative up regulation of virulence factors and antibiotic resistance mechanisms as well as the initiation of AlgD transcription by a pathway not normally associated with its biosynthesis illustrate the more global role that stress response expression signal transduction pathways may take in the development of biofilms.

CHAPTER 7

SUMMARY

The study of biofilm dynamics has been a growing field over the past decade driven, in part, by the ramifications of biofilm growth. Biofilm related issues in industry and health care include problems with microbially induced corrosion, biofouling, chronic recurring infections, and medical device related infections (Anwar et al., 1989; Costerton and Lewandowski, 1995; Costerton et al., 1999; Davey and O'Toole, 2000; Donlan, 2001). Biofilms can also play a positive role in the degradation of environmental pollutants and the degradation and recycling of organic matter and its chemical constituents (Davey and O'Toole, 2000). The development of a biofilm offers a protective haven for the bacterial cell buried within the exopolysaccharide matrix (Stewart et al.; 1998; Xu et al.; 2000). Beyond the physical protective aspects of the biofilm, biofilm bacteria develop antibiotic resistance mechanisms and express virulence factors not seen in their planktonic counterparts (Nicas and Iglewski, 1985; Ahmer et al., 1995; Davey and O'Toole, 2000; Costerton et al., 1999; Singh et al., 2000).

The overall goal of this study was to investigate the effects of initial adhesion on the physiology of *Pseduomonas aeruginosa*. Specifically, this research plan employed a proteomics approach through the analysis of differential protein expression during initial adhesion. The advantage of such an approach is that the

expressed proteins are a reflection of the genes that have been differentially regulated during attachment, thereby providing information about gene function and expression during early biofilm development.

Specifically, this research project endeavored to examine changes in the physiology of the bacterial cell during initial adhesion. During the 3 hour attachment maximum of these experiments, cells have adhered to the test surface, however the towers and channels considered to be characteristic of mature biofilms have not yet formed. Preliminary work confirmed that there were discernible and measurable changes taking place within bacterial cells during initial adhesion. When an exponentially growing bacterial cell first attaches to a surface, it ceases cell division. Acridine orange staining of planktonic and attached cells illustrated this phenomenon which is also confirmed by other research (Rice et al., 2000) and was later confirmed in this research project by the expression of certain proteins. One dimensional SDS PAGE gels comparing planktonic and attached samples showed limited differential protein expression during attachment.

To improve the detection of proteins, the decision was made to use 2D PAGE gel electrophoresis. 2 D gel electrophoresis can provide a more complete picture of protein expression enabling for the separation of well over 1000 proteins (Gorg et al., 2000; Laemmli, 1970; Bjellqvist et al., 1993). Using 2 D methodologies, it was possible to detect the up expression of 8 proteins not visualized in planktonic samples in as little as 10 minutes of attachment. These changes in protein expression continued throughout the 3 hour experimental period with the appearance and

disappearance of differentially expressed proteins through out the initial adhesion experimental time period. A total of 55 proteins were found to be differentially expressed during the 3 hours of initial adhesion.

MADLI-TOF analysis provides information for the identification of the specific protein spots within a 2 D gel. A total of 25 proteins that were differentially expressed during initial adhesion were selected for this type of analysis. Functions for the proteins were assigned according to the Confidence Level with only Level 1 proteins considered to be positively identified. All other protein functions are considered to be putative. Proteins involved in LPS, virulence factor expression and antibiotic resistance were up regulated during initial adhesion. Specifically, in the 3 hour attached samples, aliphatic amidase, involved in carbon regulation, and an Fe(III)-pyochelin receptor, involved in siderophore formation, were positively identified (Level 1). Proteins expressed at 3 hours with a putative function in *P. aeruginosa* were Level 2 proteins UDP-glucose pyrophosphorylase, HscA heat shock protein, and 2,4 dienoyl CoA reductase. Level 3 proteins were glycosyl transferase and glycosyl hydrolase. Both UDP-glucose pyrophosphorylase and glycosyl transferase were found to be up regulated at 10 minutes of attachment. Down regulated during attachment was biotin carboxylase (Level 1). Two other Level 3 proteins, a transcriptional regulator and a Ton-B receptor, were down regulated. A total of 3 proteins were unidentifiable.

Finally, 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. XcpX is a Level 1 protein expressed during both attachment and heat shock. Level 2 proteins expressed under these conditions were 2,4-dienoyl CoA reductase, ribonuclease E, exoribonuclease R, glutamate ammonia ligase adenylyl transferase and CysH. Level 1 proteins expressed during starvation and attachment were DNA gyrase subunit A and alipahtic amidase. Level 2 proteins were exodeoxyribonuclease V beta chain, ExbB, and UDP-glucose pyrophosphorylase. The induction of various signal transduction pathways in biofilm bacteria may also allow for the up regulation of proteins that enable attaching cells to develop those characteristics that presumably give biofilm cells a selective advantage such as antibiotic resistance mechanisms and virulence factor expression. This work shows that there are a number of proteins expressed during initial adhesion of *P. aeruginosa* to a surface that are also expressed during conditions of stress on planktonic cells. The synthesis of these *de novo* proteins suggests a global role for stress response expression and the induction of signal transduction pathways in the development of biofilms.

How the bacterial cell transitions from the planktonic to the attached state has profound implications for future control methodologies. It is obvious that we can no longer think of a sessile bacterial cell as just a planktonic cell stuck to a surface. Further, it is obvious that the differences between biofilm and planktonic cells transcend mere differences in the surrounding physical environment. Biofilm cells are different metabolically and physiologically from their planktonic counterparts. The existence of a biofilm phenotype has been frequently postulated by a number of

researchers (Loo et al., 2000; Costerton, 2000; Schembri and Klemm, 2001). Unfortunately, the confirmation of the existence of a single biofilm phenotype has yet to be confirmed. This research study shows that there may be, instead, a progression of characteristics toward the biofilm mode of existence. The direction of this progression involves a complex series of global signaling events most likely determined by numerous environmental cues.

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