Development of a selection strategy to identify genes of Pseudomonas aeruginosa that are induced during surface-associated growth
by Clayton Olaf Jarrett

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Microbiology
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Abstract:
Bacteria undergo physiological changes when exposed to different environmental conditions. There are well known responses to heat shock, oxidative stress and other conditions that induce phenotypic changes in bacterial cells. When bacteria attach to a surface and begin to grow they undergo many changes in cell physiology.

The most common mode of growth for bacteria in nature is as adhered or sessile cells. After adhering to a surface the bacteria often encase themselves in extracellular substances and spread over the surface, forming what is referred to as a biofilm. These biofilm bacteria are phenotypically different from planktonic cells suspended in a liquid medium. Many such changes in phenotype develop as a result of changes in the level of expression of various bacterial genes. Investigation into the phenotypic characteristics of attached bacteria should lead to the development of new control strategies for undesirable bacterial growth and may help enhance bacterial growth that is beneficial. Identification of genes that are induced within bacteria after they attach to a surface should further these goals considerably.

The present thesis describes work done to develop and test a genetic selection strategy for identifying genes of Pseudomonas aeruginosa that are induced following bacterial attachment to a surface. The model bacterium, P. aeruginosa, is ubiquitous in nature, a significant problem in the industrial setting, and a serious threat in the medical field. The system that was developed and tested in this work shows promise in furthering our understanding of the changes in gene expression that follow bacterial attachment to a surface.

DEVELOPMENT OF A SELECTION STRATEGY TO IDENTIFY GENES OF *PSEUDOMONAS AERUGINOSA* THAT ARE INDUCED DURING SURFACE-ASSOCIATED GROWTH

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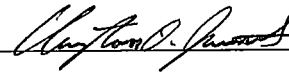
Signature 

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ABSTRACT

Bacteria undergo physiological changes when exposed to different environmental conditions. There are well known responses to heat shock, oxidative stress and other conditions that induce phenotypic changes in bacterial cells. When bacteria attach to a surface and begin to grow they undergo many changes in cell physiology.

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The present thesis describes work done to develop and test a genetic selection strategy for identifying genes of *Pseudomonas aeruginosa* that are induced following bacterial attachment to a surface. The model bacterium, *P. aeruginosa*, is ubiquitous in nature, a significant problem in the industrial setting, and a serious threat in the medical field. The system that was developed and tested in this work shows promise in furthering our understanding of the changes in gene expression that follow bacterial attachment to a surface.

INTRODUCTION

Environmental Significance of *Pseudomonas aeruginosa*

P. aeruginosa is ubiquitous in nature (Young 1977). The bacterium may persist in water (Hoadley 1977), in soil (Schroth 1977), and on plants (Kominos 1977). This widespread distribution of *P. aeruginosa* is fostered by many important phenotypic characteristics such as the bacterium's nutritional and genetic versatility (Stanier 1966). In addition to their ability to utilize a large number of naturally occurring compounds as carbon and energy sources, some strains of *P. aeruginosa* can fully degrade and utilize halogenated aromatics (Higson 1990). The species has also been found to contain strains with novel substrate ranges able to degrade even potentially toxic compounds such as polychlorinated biphenyls (PCBs) (Hickey 1990). Some strains can utilize other toxic compounds including toluene, and the genes necessary for the degradation of such compounds are often carried on transferrable plasmids (Moller 1998). Studies with *P. aeruginosa* have indicated that there is a significant potential for gene transfer among bacteria in freshwater environments (O'Morchoe 1988). This bacterium is seldom found in any great number in freshwater systems, but once a water source becomes contaminated *P. aeruginosa* is commonly isolated in large numbers (Hoadley 1977).

The transfer of plasmids just mentioned also eludes to another characteristic of *P. aeruginosa* that makes this bacterium both beneficial and problematic, the organism's genetic flexibility. The bacterium's ability to accept plasmids containing genes for the degradation of new compounds is quite beneficial when trying to engineer an organism that may be used to degrade contaminants. However, this same ability permits the possibility that *P. aeruginosa* may become a serious health threat by becoming resistant to multiple antibiotics through the exchange of plasmids. *P. aeruginosa* is resistant to many commonly

used antibiotics (Hentges 1985). Resistance plasmids can have a broad host range and therefore present a considerable problem in the clinical setting by being transferrable to *P. aeruginosa* from diverse bacterial reservoirs (Jacoby 1986).

Medical Significance of *Pseudomonas aeruginosa*

Given these characteristics of minimal growth requirements, nutritional versatility, and acquisition of resistance factors one can begin to appreciate why this bacterium is so ubiquitous and troublesome. The hospital environment is one of the places where this organism's abilities can be fully appreciated. *P. aeruginosa* has been found to contaminate liquids within the hospital such as eye drops, handcreams, and soaps (Lowbury 1975). Moist surfaces also provide an environment for the colonization and persistence of *P. aeruginosa*. Sink drains, humidifier surfaces, and ventilator tubing are all places where *P. aeruginosa* has been isolated repeatedly (Botzenhart 1987). In such hospital environments the bacterium can persist for long periods, increasing its chances of encountering other more specialized pathogens with which to exchange genetic information. Persistence on many hospital surfaces also increases the likelihood that it may be transferred to a new host. In one hospital study, 68% of individuals who were found to be colonized by *P. aeruginosa* did not test positive for the bacterium until some time after admission (Moody 1977). This statistic suggests that most of the patients were infected by strains which were encountered in the hospital.

The bacterium may also be introduced into the body by the use of hospital equipment that is contaminated. As an example, endoscopes were found to be the source of transmission for an outbreak of *P. aeruginosa* infections at a Wisconsin Hospital (MMWR 1991). Even though the instruments were routinely treated with a 2% glutaraldehyde solution the infections continued. In this case the automated disinfection system used to decontaminate the endoscopes was found to be colonized by the bacterium.

With all the above information in mind it is not surprising that *P. aeruginosa* is believed to be responsible for 10-11% of all nosocomial infections; third only to *Escherichia coli* and *Staphylococcus aureus* (Botzenhart 1993). In most cases *P. aeruginosa* is only able to sustain an infection in individuals with some sort of pre-existing compromise in immune function (Botzenhart 1993), so the hospital provides a favorable environment for this opportunist. However, the fact that *P. aeruginosa* is an opportunistic pathogen that infects only immunocompromised individuals is somewhat overshadowed by the organism's ability to infect a wide variety of tissue types, generating an array of different malignancies. Among the many infections that *P. aeruginosa* may cause are ear infections, urinary tract infections, and central nervous system infections (Artenstein 1993). This bacterium is noted to infect such diverse sites as the kidneys, heart, bone and lung (Artenstein 1993). *Pseudomonas aeruginosa* is even cited as the leading cause of nosocomial pneumonia in some hospitals (Jarvis 1992). Lung infection, or pneumonia, is of particular concern since 50-90% of patients with cystic fibrosis (CF) are colonized with *P. aeruginosa* (Luraya-Cussay 1976). In these cases the bacterium causes a large degree of the morbidity and mortality associated with CF (Sferra 1993).

From the above information it should be evident that *P. aeruginosa* can be successful in many environments due to some distinguishing phenotypic characteristics. Other phenotypic characteristics may vary with the environment. For example, one of the determining characteristic listed in *Bergey's Manual of Systematic Bacteriology* for differentiating *P. aeruginosa* from other species in the genus is the production of the blue pigment, pyocyanin (Palleroni 1983). However, the level of pyocyanin production may vary with the level of phosphate and nutrients in the culture medium (Sorenson 1993). Further, pyocyanin is an antibiotic active against many gram-positive bacteria (Baron 1981), and a virulence determinant (Sorenson 1993), that is regulated by the cell signaling *rhl* system in a cell density-dependent manner (Ohman 1995, Reimmann 1997).

Importance of the Biofilm Mode of Growth

In the outbreak mentioned in the Wisconsin hospital study a thick film of bacteria attached to the surfaces of the automated disinfection system was the source of contamination. *P. aeruginosa* survived exposure to sterilization solution that was lethal to most other bacteria. When the endoscopes were supposed to be getting cleaned they were actually getting reinoculated with *Pseudomonas* that had detached from the thick layer of cells in the cleaning device (MMWR 1991). Several investigators have found that bacteria, and *P. aeruginosa* in particular, are more resistant to antimicrobials when growing as a layer of cells surrounded by extracellular polymeric substance (LeChevallier 1988, Herson 1987, Anwar 1989), what has become known as a biofilm.

The study of biofilm bacteria in addition to planktonic bacteria has become increasingly important. In the past few years it has been formally recognized that biofilm bacteria may be involved in as many as 65% of bacterial infections (Potera 1999). Some researchers, have also estimated that 99% of all bacteria growing in natural environments exist within biofilms, or associated with surfaces (Costerton 1987). Thus, the greatest proportion of problems caused by bacterial growth involve organisms in an adhered state (Williams 1999).

As mentioned, bacteria growing in layers of cells on a surface are known to be more resistant to antibiotics and other antimicrobials than are planktonic or free-swimming bacteria of the same strain (LeChevallier 1988, Herson 1987, Anwar 1989). Other phenotypic changes occur after bacteria adhere to a surface. Following adhesion to surfaces *Vibrio parahemoliticus* change from polar flagella production for swimming motility, to lateral flagella production for swarming motility (Belas 1986, McCarter 1990). *E. coli* and *S. typhimurium* cells become elongated and hyperflagellated following adhesion (Harshey 1994). After contacting a surface extracellular polysaccharide production is upregulated in

P. mirabilis (Gygi 1995). In a recent review, Goodman and Marshall listed motility, cell wall thickness, exoproduct production, and growth rate as examples of characteristics that change after bacteria leave the liquid phase and grow on a solid or semi-solid surface (Goodman 1995).

The process of bacterial attachment to surfaces is best described in the medical field where bacterial attachment to host cells is often a prerequisite to successful infection. Studies of adhesion to animal cells have shown that bacteria often bind through a receptor-ligand interaction (Irvin 1989, Prince 1992, Pier 1997). Bacterial attachment to inert surfaces, which lack specific receptors, is less well characterized. However, the importance of biofilm formation on the surface of implant devices is emphasized by several recent papers (Costerton 1999, Habash 1999, Reid 1999). Reid (1999) described the extent of the problem posed by biofilm formation on implanted medical devices. Habash and Reid (1999) provided an extensive review outlining the process of biofilm formation on such surfaces and the hurdle that biofilms present to successful treatment strategies. Costerton and colleagues (1999) also reviewed the topic and described many of the characteristics of biofilm infection, as well as some of the advances made in identifying possible targets for treatment options.

Bacterial Attachment and Gene Expression

Even though more and more about the process of bacterial adhesion and biofilm development on inert surfaces is being elucidated, much less is known of the genetic regulation and gene expression necessary for this progression of events (Dalton 1998). There is a great deal of complexity inherent in answering the question of genetic regulation in biofilms. A recent study of the differences in gene expression between planktonic and sessile cells of *E. coli* was performed using a *lacZ* reporter system (Prigent-Combaret 1999). The study indicated that up to 38% of the bacterium's genes are differentially

expressed depending on whether the bacterial cells were recovered from the side of a well in a microtiter dish or from the liquid phase within the well. The authors stated that gene expression patterns within biofilms appear to be controlled by many changing environmental physiochemical conditions which interact with complex regulatory pathways (Prigent-Combaret 1999). An analysis of the *P. aeruginosa* genome has indicated that the regulatory capacity of this species far exceeds that of *E. coli*. Approximately 4.7% of the genome of *P. aeruginosa* is characteristic of sequence involved in regulatory functions, whereas that of *E. coli* is only about 1% of the genome (Stover 1999). In addition, some studies have indicated that gene expression patterns may even vary within the biofilm itself, so that depending on a bacterium's location in an established biofilm, different genes will be expressed at different levels (Davies 1993, Xu 1998).

One can see that the answer to how gene expression varies after bacterial attachment will not be a simple one. Therefore, it is important to consider what is known about the bacterial attachment process and subsequent growth of a biofilm. A great deal is known about initial adhesion and attachment of bacteria to surfaces and this will be described in the following several paragraphs. This information is a demonstration of the utility, but also the limitations, of studies of biofilms to date.

Inert surfaces, when exposed to a liquid, will accumulate a conditioning film (Habash 1999). Various components of the surrounding media or liquid diffuse to the surface and form a coating. Which components are deposited depends upon the particular surface chemistry, charge and hydrophobicity (Habash 1999). Many natural surfaces are negatively charged (Neihof 1972). Since most gram-negative bacteria also have a net negative surface charge, some way of counteracting the repulsion of like charges must exist in order for the bacteria to contact the surface. Positively charged ions such as calcium and magnesium ions have been proposed to function in bridging between the two negatively charged surfaces (Habash 1999). Bacterial appendages such as fimbriae and flagella may also facilitate initial

attachment (Reid 1999). First, appendages may extend through the distance that separates the similarly charged surfaces, making contact with the inert surface to form reversible bonds. Second, some appendages function as adhesins (Irvin 1989) or have adhesins on their ends (Prince 1992) that act in specific receptor-ligand interactions to bind the bacteria to the surface. Mutation studies have shown that bacteria may have reduced ability to bind to a surface if the mutation prevents the formation or proper function of appendages such as pili (Chiang 1998, Pratt 1998) or flagella (Arora 1996, O'Toole 1998a). However, these findings do not necessarily prove that a specific adhesin is the essential component lacking in the mutants. Defects in the particular appendages may cause the bacteria to become non-motile, which may influence a bacterium's ability to contact and bind to a surface. In addition, some mutants of *P. fluorescens* that were non-motile and defective in biofilm formation, were found to attach and form biofilms if grown in the presence of citrate of glutamate (O'Toole 1998b). These results point to the fact that other cell surface components or other unknown biochemical factors may be involved in initial attachment. Adhesion of *P. aeruginosa* is even known to be influenced by the production of the toxin molecule exoenzyme S (Baker 1991). Alginate, an exopolysaccharide of *P. aeruginosa,* may also function in adhesion, but its role as a specific adhesin is questionable (Prince 1992). Thus, adhesion and attachment of bacterial cells to a surface is a complex process, influenced by many cell wall components and environmental factors. More is known about this primary or initial step in biofilm formation than about the subsequent steps in biofilm development.

Surface-associated, or biofilm, growth occurs in an environment where many conditions are different from the planktonic environment. Therefore, many factors may act as signals to induce genetic alteration within the attached bacteria. For instance, bacteria covered by an exopolymeric substance will experience different oxygen and carbon dioxide concentrations than that of the bulk fluid. *LacZ* mutants of *P. aeruginosa* were identified by

Goodman in which increased expression of specific genes was induced by increased carbon dioxide levels (Goodman 1995). Viscosity of the surrounding medium may also be a signal. There is evidence that the switch from polar to lateral flagella in *V. parahemoliticus* is controlled by the difference in viscosity within a biofilm, or at a surface-liquid interface (McCarter 1990). Sheehan and coworkers (1992) found that toxin A of *S. aureus* was regulated by the osmolarity of the surrounding media. Goodman and Marshall (1995) predict that osmolarity and water activity should be higher at a surface, or in a biofilm, than in the bulk fluid, and proposed that this difference may trigger changes in gene expression in *S. aureus.*

The above mentioned changes, whether involving oxygen, carbon dioxide, ions, or the medium itself, are well established environmental conditions that can undergo changes in concentration within a biofilm. A relatively new area of investigation deals with the concentration of the bacterial cells and their byproducts. Quorum sensing, as it is often referred to, is a method of communication between bacteria that depends upon the density of cells within a bacterial culture or population (Fuqua 1994). As bacteria accumulate on a surface their cell density per unit area increases and so also does the concentration of molecules produced and secreted by the bacteria. Some secreted molecules can function as signals to regulate gene expression. In *P. aeruginosa* two signaling systems, each having a specific signaling molecule, exist that influence the expression of various genes (De Kievit 1999). One of the two systems, the *las* system, has been shown to function in the regulation of several virulence factors including alkaline protease, LasA and LasB proteases, and exotoxin A (Gambello 1993, Latifi 1995, Toder 1991). Van Delden and Iglewski (1998) describe cell-to-cell signaling in *P. aeruginosa* infections and speculate about how these signaling systems may function to help the bacteria overcome the host defenses and produce a successful infection. Parsek and Greenberg (1999) recently provided a review of quorum sensing in *P. aeruginosa* biofilms describing the signaling systems and their

importance in the development of biofilms. Thus, it is expected that these signaling molecules will influence the genetic regulation behind the phenotypic characteristics noted of biofilm cells.

Biofilm Formation and Genetic Regulation

Biofilm formation proceeds through several subsequent steps which involve many changes in bacterial phenotype, allowing the formation of a bacterial community that is very complex in intercellular interaction and in macrocolony structure (Costerton 1995). In the first step after attachment, the bacteria may spread over the surface. Since such a process is fundamentally different from any involved in planktonic growth, many changes in the bacterial cell can be expected. Such changes were previously mentioned with regard to the morphology of *V. parahemoliticus*, *E. coli*, and *S. typhimurium* after they attach to a surface (Belas 1986, Harshey1994, McCarter 1990). These changes enable a type of bacterial locomotion specific to that necessary for spread across a solid surface, and so are expected to require changes in the genetic regulation and the production of new protein. Thus, many of the changes in gene expression that the current work seeks to investigate may occur quite soon after attachment.

Several lines of evidence indicate that there may indeed be many changes which occur early during surface-associated growth. A study of *S. aureus* found that a peak in respiratory activity occurred at only one hour after the start of attached growth (Williams 1999). This sharp peak suggested that regulatory events occurred quite soon after the start of attached growth. Other investigators have found that *P. aeruginosa* transiently increased production of mucoid exopolysaccharide, mainly composed of alginate, following adherence of the bacteria to a silicon surface (Hoyle 1993). Later study of a particular gene in the alginate biosynthesis operon, *algC*, showed increased expression within fifteen minutes after attachment (Davies 1995). This gene expression was determined by fluorescent

intensity of a substrate for the *lacZ* gene fusion product. There is also limited evidence that new protein production is required transiently for a biofilm to develop in the first hour after attachment (O'Toole 1998b). This requirement is indicated by the inhibition of biofilm formation in cells treated with a low level of tetracycline. The level of tetracycline did not reduce bacterial viability but may have inhibited protein production (O'Toole 1998b). All these indications of early gene expression are indirect measurements, and so are considered as merely suggestive evidence. Taken together though, the idea that changes in gene expression occur soon after bacterial attachment seems reasonable.

After these early stages of attachment and spread across the surface, a mature differentiated biofilm with complex architecture develops (Lawrence 1991). Typical mature biofilms have been noted to contain structures of dense growth forming columns, or streamers, interspersed with open channels or areas where only a thin film of cells is present (Costerton 1995). Cell-to-cell signaling may be important in biofilm architecture. A study of *P. aeruginosa* biofilm development indicated that a significant concentration of one of the signaling molecules was necessary for the initial monolayer of cells to differentiate into the mature biofilm with the typical wildtype architecture (Davies 1998). However, a subsequent study of *P. aeruginosa* mutants defective in signal production provided an alternative hypothesis (Stoodley 1999). This study showed that although mutant and wildtype cultures were not identical in biofilm formation it was the flow conditions of the culture vessel, rather than the presence of signaling molecules, that determined the structure of the mature biofilm (Stoodley 1999). Clearly, the involvement of quorum sensing signals, and other factors controlling the later stages of biofilm development, deserves further investigation.

Molecular Genetic Technology

Some of the earliest investigation into bacterial genetics was performed using mutational analysis. Much has been learned from mutagenesis studies but there are many

problems inherent in this approach (Botstein 1985). The general methodology for mutagenesis involoves generating a pool of mutants by exposing the bacteria to a mutagenic agent that causes random mutations throughout the genome. Then a mutant that is defective in the specific phenotype is selected. The gene involved in producing this phenotypic characteristic is then found by introduction of a plasmid containing the wildtype DNA that can complement the mutation. This mutagenesis approach was unfavorable for several reasons. Random mutagenesis may create defects in the cell that can not be predicted or even detected under normal circumstances. A phenotypic characteristic noted of a mutant may be the direct result of an identified mutation or may be an indirect result of regulatory functions affected by some downstream process. Care must also be taken in mutagenesis studies to be sure that more than one mutation does not occur in a single cell.

Using transposons as the mutagen overcame some of the undesirable aspects. Depending upon the transposon used, mutagenesis is basically random and usually generates only one mutation per genome or bacterium. Further, gene identification no longer required complementation since the inserted transposon provided known DNA sequence for PCR amplification of adjacent sequence (Botstein 1985).

Some of the gene products necessary for the first stages in biofilm development have been identified by transposon mutagenesis. Some of this work has been done using the Tn5 transposon in *Pseudomonas fluorescens* (Dekkers 1998, O'Toole 1998b). From their work with strain WCS365, O'Toole and Kolter (1998b) concluded that multiple signaling pathways were involved in biofilm formation on the side of polyvinylchloride microtitre dish wells. They examined mutants that could not attach to the side of the plate wells. Some of the mutants defective in biofilm formation could be rescued, or made to attach to a degree similar to that of the wildtype, if the growth medium was supplemented with specific components. A mutant in flagella biosynthesis was defective in attachment in the standard media used, but could attach and form biofilms if the medium was supplemented with citrate

or glutamate. Another mutant which was motile but had a mutation in a gene with unknown function, could only attach if supplemented with citrate. Still other motile, as well as non-motile, mutants could be rescued with the addition of exogenous iron to the media. The authors proposed that the substances, citrate, glutamate and iron, function as signals that affect gene expression so that new protein production may overcome the defect and allow attachment to a surface. However, the mechanisms by which these putative signals function in attachment were not described.

Other mutagenesis studies on the same strain of *P. fluorescens* were performed by Dekkers and co-workers (1998a). Most of the adhesion mutants were defective in known colonization traits such as amino acid prototropy and motility. Two findings from their work were of particular interest. First, the 0-antigen of lipopolysaccharide (LPS) was found to be essential to the bacterium's ability to bind to the root surface. This mutant was also significantly less competitive when grown in liquid media in the presence of the wildtype. Interestingly, another mutant which could make a shortened , or truncated, version of the O-antigenic side chain was defective in its ability to colonize the root surface but was not defective in its ability to compete with wildtype cells in liquid media. These findings suggests that the O-antigenic side chain of LPS is involved in attachment to the root surface but perhaps not other aspects of competitive growth.

The second finding from their work that is of particular interest is the identification of a putative sight-specific recombinase as a gene required for competitive root colonization (Dekkers 1998b). A mutant of the putative sight-specific recombinase did not show a significant defect in competitive growth in liquid media or in root colonization when tested in mono-culture. However, the mutant showed a significant defect in its ability to compete and colonize a root surface when tested in mixed culture with the wildtype strain. This indicated that the sight-specific recombinase was probably not important in initial attachment to the surface but was important in later stages of competitive growth.

An idea that links these two findings is also quite interesting and important in other attempts to understand genetic regulation involved in surface-associated growth. The idea is that the sight-specific recombinase may be involved in the regulation or modification of the O-antigenic side chain of LPS itself. The authors suggested that this putative recombinase may function in generating DNA rearrangements that would cause phase variation in a cell surface molecule such as LPS, surface lipoprotein, or a flagellar protein (Dekkers 1998b). This idea points to the fact that the genes that are important in producing a specific phenotypic characteristic may be difficult to identify unambiguously. If recombination events can be directed to a locus to cause changes in what might be a surface-associated phenotype, and this may occur over time irrespective of environmental cues, then identification of corresponding genetic sequences may be quite difficult.

This complexity is well illustrated by a recent paper on bacteriophage FIZ15 which causes lysogenic conversion of *P. aeruginosa* PAO1 (Vaca-Pacheo 1999). The phage uses the O-antigen of LPS as a receptor to infect the bacterium. The phage DNA then incorporates into the chromosome and causes changes in several phenotypic characteristics. The site of integration was not identified, but experiments demonstrated that the lysogen had increased adhesion properties and increased resistance to phagocytosis. These virulence associated phenotypes were proposed to be due to some type of cell surface modification of the LPS (Vaca-Pacheo 1999).

IVET Technology

The above information describes what has been learned from mutagenesis studies and illustrates the complexity involved in the investigation of specific phenotypic characteristics. New technologies have been developed in the last decade in order to overcome some of the limitations inherent in mutagenesis studies, and to better understand changes in gene expression. Mutagenesis work can identify genes that are required for a specific phenotype.

However, mutagenesis studies may not be able to elucidate how genetic expression changes over time. The technique to be described is powerful because it can provide information on yet unidentified phenotypic characteristics that change throughout a developmental process such as biofilm formation.

In vivo expression technology (IVET) was initially developed by Dr. John Mekalanos and coworkers at the Harvard Medical School. This strategy was used to identify genes of the pathogen *Salmonella typhimurium* that may be involved in virulence (Mahan 1993). This system identified not only genetic elements that were related to a specific phenotype, but also gene induction necessary for the development of that phenotypic characteristic. Thus, the technology could identify genes that were not expressed under one environmental condition but were then induced when the bacterium entered a new environment, for example in the host environment.

In their work, a synthetic operon containing promoterless *purA* and *lacZY* genes was inserted randomly into the chromosome of a purine auxotroph of *S. typhimurium* (Mahan 1993). Growth of the bacterium could not occur *in vivo* unless the purine mutation was complemented by the expression of the *purA* gene. Expression of *purA* could only occur if the operon was inserted downstream of an induced promoter. Therefore, which bacterial cells survived depended upon which genes were induced *in vivo*. The *lacZY* genes were supplied in order to monitor expression of the genes *in vitro* by observing a color change.

A pool of the bacteria with the synthetic operon inserted randomly into the chromosome was injected into mice. Genes that were induced in the mouse model of infection were selected by the resulting downstream expression of the *purA* gene. Some of these isolates which were collected after a certain period of infection contained insertions downstream of genes which were expressed constitutively. The genes of interest though, were those specifically induced only after entry into the mouse. This desired group of genes could be selected by identifying those bacterial clones which survived in the mouse but

showed low to no expression of the *lacZY* genes on nutrient agar plates. A random sample of such clones was examined to identify genes with *in vivo*-induced expression. Interestingly, of the sequences identified in the first use of the IVET, one was found to be within an operon encoding about twenty genes involved in O-antigen synthesis (Mahan 1993) the importance of which will be revisited.

Mahan and coworkers (1995) later developed a new IVET system that utilized an antibiotic-based selection instead of the selection using purine auxotrophy. This system allowed investigation into the genetic regulation of bacteria where purine auxotrophs, or the genetic tools to generate an auxotroph, were not yet established. The chloramphenicol resistance gene (*cat*) was fused to *lacZY* genes and *in vivo* expression experiments were performed with mice treated with chloramphenicol. Thus, in order for bacterial clones to survive *in vivo* they must have the *cat* gene inserted downstream of an induced promoter sequence. This system was also used to identify genes that were induced when bacteria were phagocytosed by cultured macrophages, illustrating the flexibility of the system (Mahan 1995, Heithoff 1997).

Valdivia and coworkers (1996 and 1997) used a green fluorescent protein (*gfp*) gene fusion system together with automated cell sorting to identify genes induced within the host cell environment, in this case within macrophages. An advantage of this system was that the fluorescence could be determined when a macrophage was passed through the fluorescence-activated cell sorter. In this way the bacteria did not have to be removed from the specific environment being tested.

The IVET has also been applied to *P. aeruginosa* (Wang 1996a, Wang 1996b). The original strategy using a purine auxotroph was used to identify genes induced in a mouse infection model (Wang 1996b). This study identified two general types of *in vivo*-induced genes, one containing genes involved in sensory transduction or genetic regulation, and one containing genes involved in amino acid biosynthesis. One identified locus was found to be

homologous to the *pilG* gene of *P. aeruginosa,* a gene involved in pilus biosynthesis and twitching motility. As previously mentioned, mutagenesis studies of *P. aeruginosa* showed that mutations in *pilB, pilC,* and *pilY* prevented biofilm formation (O'Toole 1998a). This convergence of experimental findings indicated that pili gene expression and pili function are important in colonization of both abiotic and host cell surfaces.

An IVET study using the purine auxotroph system in *P. aeruginosa* was also done to identify genes induced in cystic fibrosis (CF) infections (Wang 1996a). This study involved inoculating bacterial cells into respiratory mucus from CF patients. Three loci of importance were identified. One genetic locus is known to be involved in iron acquisition, a known virulence factor (Mekalanos 1992). Another locus had homology to a transcriptional regulator, and one gene was involved in biosynthesis of lipopolysaccharide or exopolysaccharide (Wang 1996a). Again, the importance of LPS and of transcriptional regulators was emphasized.

An IVET-type approach to study changes in gene expression of sessile cells has several advantages over previous studies. Some of the advantages over mutagenesis studies have already been described. Mutagenesis of a gene usually inactivates that gene completely, so that the phenotype one is able to examine is fixed, and cannot change with changes in environment. The IVET approach will not, in most cases, inactivate the genes of interest for reasons that will be described in the experimental procedures. Mutagenesis studies can only examine the effect of the presence or absence of a gene product. However, expression technologies using reporter genes like *gfp* (green fluorescent protein) and *lacZY* can examine the level of gene expression associated with a specific environment and at a specific point in time. Also, due to innovations in experimental design to be described later, the different stages in biofilm initiation and formation can be monitored microscopically *in situ,* leaving the bacterial cells within the specific environment. This type of technology shows promise in unraveling gene expression and regulation involved in surface-attached growth.

The Genetic Selection System

The phenotypic characteristics of *P. aeruginosa* that are of concern in this study were those induced after the bacteria attach to a surface and begin to form a biofilm. After contact with a surface bacteria undergo many changes, including, and facilitated by, changes in gene expression. Therefore, differential gene expression after bacterial attachment to a surface was the focus of this investigation.

In vivo expression technology (IVET) systems are powerful tools to identify specific genes because they can select for clones under one environmental condition and against others under another environmental condition. Initial IVET studies involved positive selection of *in vivo* induced clones followed by differentiation of non-induced clones on an agar surface. The current system required that positive selection of induced clones be performed on a surface, so differentiation of non-induced clones by the previous method was not practical. A new technology had to be developed to identify and evaluate bacterial genes that are induced after attachment to a surface. For the system developed in this study, clones must go through negative selection in liquid culture and positive selection when growing attached to a surface. The inclusion of a negative selection permitted the elimination of undesired clones prior to positive selection of sessile growth-induced clones. In this way selection of genes that are induced after attachment of the bacteria to a surface could be performed *in situ*. To perform this selection, a synthetic operon was developed that incorporated the *sacB* (levansucrase) gene for negative selection, the *aacC1* (gentamicin resistance) gene for positive selection, and a *gfp* gene for confirmation of expression.

The gene *sacB* was chosen for negative selection because *sacB* expression has been shown to be lethal to cells in the presence of sucrose. The gene *sacB* encodes levansucrase, which is an enzyme that functions to transfer fructose subunits of sucrose to a growing homopolymer of fructose called levan (Dedonder 1969). If *sacB* is expressed in the

presence of sucrose, levan will build up in the cell, disrupt cellular functions, and may cause cell lysis. However, if there is no sucrose in the growth medium, the cell is unaffected by the expression of *sacB*. Selection by *sacB* expression has been previously carried out in *E. coli* by Gay and coworkers who found that *sacB* expression caused cell lysis or inhibition of growth (Gay 1985). The *sacB* gene has also been utilized as a negative selection in allelic exchange strategies in *P. aeruginosa* (Schweizer 1992). In that work, expression from a single copy of *sacB* on the chromosome made *P. aeruginosa* cells sensitive to the presence of five percent sucrose in culture media.

For positive selection the gentamicin resistance gene, *aacC1*, was chosen because *P. aeruginosa* is sensitive to this antibiotic. In this work wildtype *P. aeruginosa* PAO1 cells were not able to grow in media containing gentamicin at any concentration higher than 5μg/ml. The gene product provides resistance by transferring an acetyl group to the gentamicin molecule (Biddlecome 1976). This modification prevents gentamicin from acting upon the ribosomal subunit of the bacterium. Therefore, cells with the *aacC1* gene downstream of an induced promoter can be selected for by their increased resistance to gentamicin.

Lastly, the green fluorescent protein gene, *gfp*, was included for visual confirmation of gene expression. The *gfp* fluoresces brightly when exposed to ultraviolet light, allowing the visualization of gene expression under epifluorescence microscopy. This green fluorescent protein gene is a mutant of the original *gfp* from the jellyfish, *Aequorea victoria*, which was selected by fluorescence-activated cell sorting for enhanced fluorescence (Cormack 1996). Bacterial colonies on an agar surface may fluoresce under a UV lamp if expressing *gfp*. Thus, fluorescence of attached bacteria can provide confirmation that the operon is fused downstream of an induced promoter.

Selection of surface induced gene (*sig*) promoters was achieved by successive rounds of negative and positive selection to isolate those promoters upstream of the

synthetic operon that were up-regulated after attachment of the bacterium to a surface. First, *P. aeruginosa* cells were grown in liquid culture with sucrose in the media. Cells with promoters that are induced in liquid culture, and constitutive promoters, were eliminated from the pool of cells during liquid selection because *sacB* expression was lethal to those cells. The remaining cells were moved to the second stage of selection where they were plated on gentamicin containing agar. In this environment cells not expressing the synthetic operon were not resistant to gentamicin and were thus eliminated from the pool. Cells with the synthetic operon downstream of promoters that were induced on the surface of the plate were permitted to grow due to their *aacC1* expression. The only bacterial cells that should have survived the two stages of selection were those with the synthetic operon inserted downstream of a promoter that was not induced during growth in liquid culture but was induced following attachment to a surface.

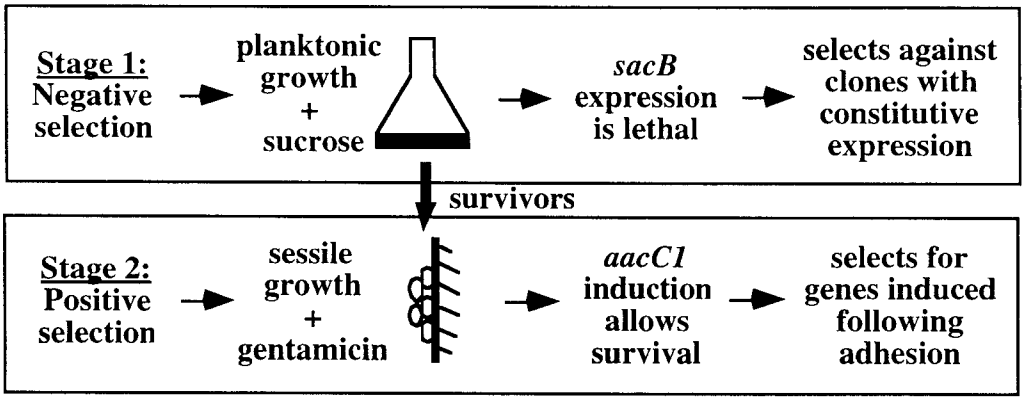

Figure 1: The selection system. Bacterial cells of the genetic library were screened to isolate those clones with the synthetic reporter operon downstream of a gene promoter that is surface growth-induced.

The success of the selection system described above depended on the proper and efficient functioning of the constructed synthetic reporter operon. The efficacy of each of the genes in the operon, and the selection system in general, was carefully tested and evaluated in this work. The synthetic operon generated in this work permitted the

development and application of a novel selection system to study changes in gene expression previously impossible by conventional methods.

MATERIALS AND METHODS

Bacterial Strain

P. aeruginosa strain PAO1 was used in these studies. This strain is the type strain for the majority of medical, as well as some environmental, studies of the species. A joint project by the University of Washington Genome Center, PathoGenesis Corporation and the Cystic Fibrosis Foundation has made available the nearly complete genome sequence of this strain as part of the Pseudomonas Genome Project, which is available via the worldwide web (www.pseudomonas.com). Thus, the genome can be searched to identify specific genes using various computer software.

Genetic Manipulations

General cloning procedures such as restriction enzyme digestion, ligation, and alkaline phosphatase treatment were performed according to the manufacturers instructions (Promega Corp.). Transformation of competent cells, electroporation, partial digestion of genomic DNA, and sequence amplification by the polymerase chain reaction (PCR) were all preformed according to procedures outlined in *Short Protocols in Molecular Biology* (Ausubel 1989). Plasmid purification was performed with the QIAprep Spin Miniprep Kit, (QIAGEN Inc.). DNA fragment purification from agarose gels was accomplished with the QIAEX II Gel Extraction System (QIAGEN Inc.). Purification of genomic DNA was achieved using the Wizard Genomic DNA Purification Kit (Promega Corp.).

All cloning was first done in *E. coli* strain HB101. Single plasmids were moved into *E. coli* via transformation of competent cells. Plasmids of the genetic library were introduced into *E. coli* by electroporation (Ausubel 1989). Typical protocols for shuttling DNA into *E. coli* by triparental mating or electroporation did not provide efficient transfer

rates for *P. aeruginosa.* Modifications were made to existing protocols for triparental mating. The main adjustment to previous protocols involved growing the recipient *P. aeruginosa* cells at 42°C rather than 37°C. The growth phase, or period of incubation for the recipient and donor were also optimized for transfer efficiency. Specific steps in the protocol are described in Table 1.

Table 1: Triparental Mating Protocol for Introduction of DNA into *P. aeruginosa.*

1. Grew recipient (PAO1) in Luria Broth at 37° C for 18 to 24 hrs.

2. Transferred 2 ml of the recipient culture to 25 ml LB and incubated at 41-42°C for 12 hrs
   - 27 ml culture was placed in a flat bottom flask
   - shaker was set at approximately 125 rev/min

3. Donor strain, as well as *E. coli* with helper plasmid pRK2013, was taken from frozen stock, thawed, and 100 µl added to 5 ml LB in a test tube
   - frozen stock was made of stationary phase culture mixed 1:1 with 10% skim milk

4. Incubated the two test tubes on a roller at 37°C for approximately 6 hrs

5. Transferred 200 µl of each culture (donor, recipient and helper) to one test tube containing 2.5 ml LB and mixed by swirling

6. Filtered cell suspension onto 0.2 µm polyethersulfone filter

7. Placed filter, with bacteria on upper surface, onto LB agar plate

8. Incubated the agar plate at 42°C for 10 hrs

9. Placed filter in a test tube containing 5 ml saline and vortexed until cells were in suspension

10. Spread plated 100 µl of cell suspension onto Pseudomonas Isolation Agar (Difco Laboratories) plate containing 150 µg/ml carbenicillin

11. Incubated spread plate at 37°C for 18 to 24 hrs

Media and Culture Conditions

Luria broth (LB) was used at approximately twenty percent of the standard strength (1/5 LB) so that each liter of media contained 1 g of yeast extract and 2 g of tryptone.

Sodium chloride was added at 7.8 g/L to keep the ionic strength approximately that of saline. When LB agar plates were used, the medium was prepared as above and 15 g of agar per liter was added. All media used for genetic selection experiments contained carbenicillin at 150 μg/ml. Other components of the medium were added as indicated. Sucrose medium was prepared as above with sucrose added at 10% by weight, unless otherwise indicated. Gentamicin medium was prepared as above with 100 μg/ml gentamicin added, unless otherwise indicated. All cultures were maintained at 37 °C. Broth cultures were grown in aeration flasks within an incubator shaker set at 250 rpm, unless otherwise specified.

Construction of the Synthetic Operon

For use in a reporter system, the synthetic operon must first be placed in a plasmid and then moved into the bacterial chromosome. The plasmid vector chosen for this purpose was the ColEI-based plasmid pKK232-8 (Pharmacia Biotech). This plasmid replicates in *E. coli* but will not replicate autonomously in *P. aeruginosa*. The vector contains three transcriptional termination sequences (*rrnBT*). Since other genes on the plasmid such as the beta-lactam resistance gene (*bla)* have constitutive promoters, the *rrnBT* sequences help reduce aberrant expression of the synthetic operon that might be caused by transcriptional readthrough. A multiple cloning site of pKK232-8 contains many restriction sites for ligating in the reporter genes.

The genes *sacB, aacC1* and *gfp* were cloned into the multiple cloning site of pKK232-8. This intial work in constructing the pKK232-derived plasmid, pMF208, was performed by Franklin (pers. comm.). The native promoters of *sacB, aacC1,* and *gfp* were removed so that expression of the synthetic operon would only occur if a promoter was supplied upstream of the synthetic operon. Exclusion of the native promoters was accomplished by PCR amplification of each gene exclusive of its native promoter sequence. The upstream, or 5', primer for the *sacB* gene included several important sequences that

were added to the synthetic operon. An *Xba*I restriction site was included to facilitate ligation of the PCR product into the multiple cloning site. Three stop codons, each in a different reading frame, prevent translational fusions to *sacB* that might otherwise disrupt levansucrase function. A ribosomal binding site (RBS) was included in this primer. The 5' primer also contained the ATG start codon for initiation of translation (Figure 2).

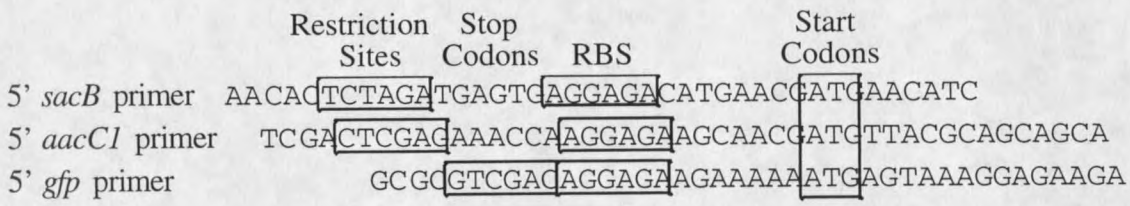

Figure 2: Primers used to PCR amplify genes of the synthetic reporter operon. (3' primers not shown)

The *sacB* gene was amplified from the plasmid vector pEX100T (Schweizer 1992). The *aacC*1 gene was cloned from plasmid pUCΩGm (Schweizer 1993). The original *gfp* gene in pMF208 was replaced by a mutant, enhanced *gfp* amplified from pBCgfp (Matthysse 1996) during this work to create pCJ7.

Construction of the Genetic Library

In order to screen the entire genome for genes which are increased in expression after bacterial attachment, the synthetic operon must be inserted randomly throughout the *Pseudomonas aeruginosa* genome. Individual strains with random insertions were collected into a pool called a genetic library. The genetic library was first constructed in a plasmid vector and then moved into the *P. aeruginosa* chromosome.

A gene library was constructed in the plasmid pCJ7 (Figure 3). Chromosomal DNA was isolated from *P. aeruginosa*. The DNA was then partially digested with restriction enzyme *Sau*3AI. This endonuclease cuts double stranded DNA at GATC nucleotide sequences which are frequent in the genome of *P. aeruginosa*. The digestion reaction was