



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  
Montana State University  
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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.

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

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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.

## 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 characteristics 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, Reimann 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 parahemolyticus* 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 or 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. parahemolyticus* 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, Harshey 1994, 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 involves 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 prototrophy and motility. Two findings from their work were of particular interest. First, the O-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 site-specific recombinase as a gene required for competitive root colonization (Dekkers 1998b). A mutant of the putative site-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 site-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 site-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 *aacCI* (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, *aacCI*, 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 *aacCI* 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 *aacCI* 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.

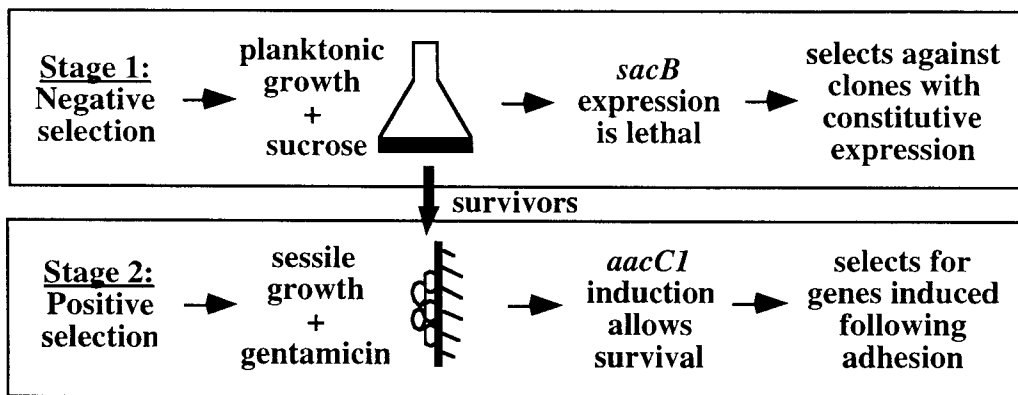


Figure 1: The selection system. Bacterial cells of the genetic library were screened to isolate those clones with the synthetic reporter operon downstream of a gene promoter that is surface growth-induced.

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](http://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 performed 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

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#### 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*, *aacCl* and *gfp* were cloned into the multiple cloning site of pKK232-8. This initial work in constructing the pKK232-derived plasmid, pMF208, was performed by Franklin (pers. comm.). The native promoters of *sacB*, *aacCl*, 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).

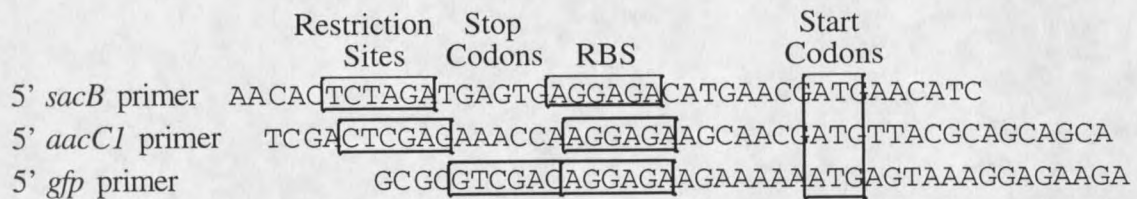


Figure 2: Primers used to PCR amplify genes of the synthetic reporter operon. (3' primers not shown)

The *sacB* gene was amplified from the plasmid vector pEX100T (Schweizer 1992). The *aacC1* gene was cloned from plasmid pUC $\Omega$ Gm (Schweizer 1993). The original *gfp* gene in pMF208 was replaced by a mutant, enhanced *gfp* amplified from pBCgfp (Matthyse 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

stopped by heat inactivation before all sites were acted upon. Inactivating the enzyme before complete digestion ensures that restriction of GATC sites is random. Random restriction thus generates random DNA fragments.

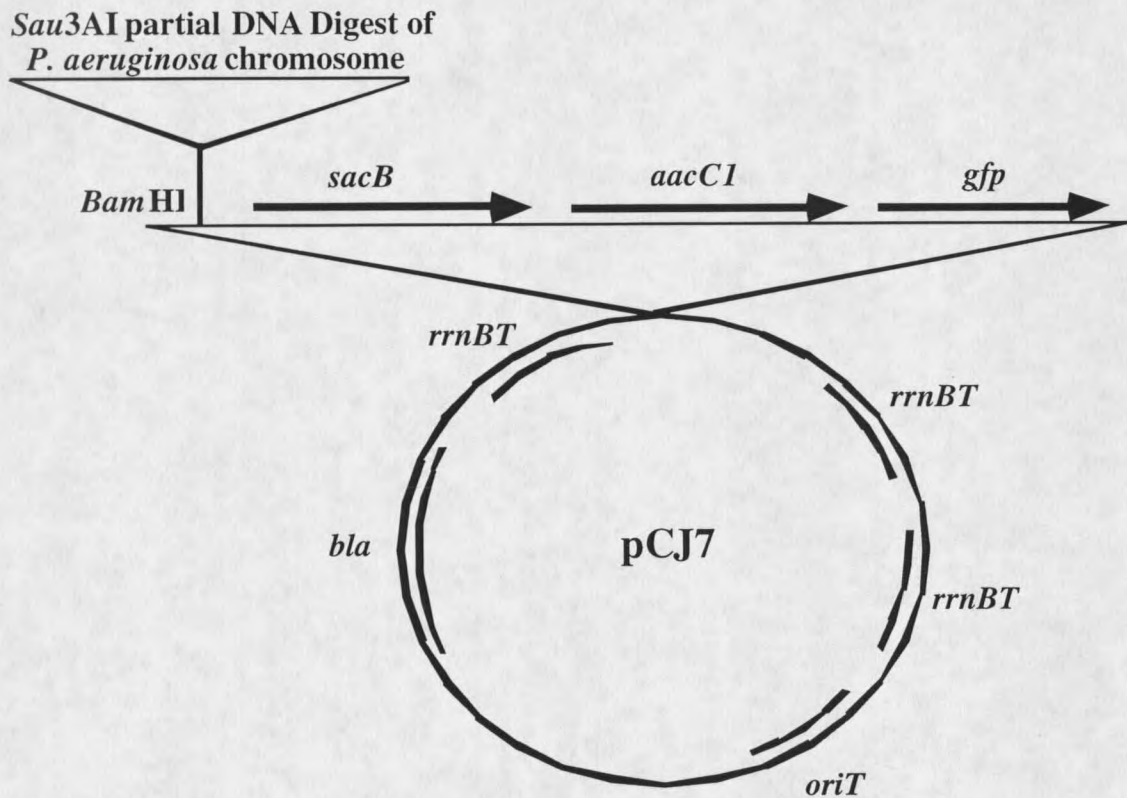


Figure 3: Plasmid map of pCJ7. *SacB* - levansucrase gene; *aacC1* - gentamicin resistance; *gfp* - green fluorescent protein gene; *bla* - beta-lactam resistance; *rrnBT* - transcriptional and translational terminators; *oriT* - origin of transfer.

Next, the DNA was run on a 0.7% agarose gel to separate the fragments by size. A section of the gel containing fragments of one to three kilobase (kb) pairs was excised. The agarose was dissolved and the DNA fragments were isolated and purified. The fragments were then ligated into the *Bam*HI site immediately upstream of the synthetic operon.

The pool of plasmids, each containing a different chromosomal DNA fragment was moved into *E. coli* by electroporation. These *E. coli* cells were plated on agar media with ampicillin. About ten thousand colonies were pooled and collected in liquid media. In order

to analyze promoters throughout the entire genome, the genetic library must contain a proportionately large number of clones. The equation:  $N = \ln(1-P) / \ln[1-(I/G)]$ , was used to calculate the number of clones required to represent the entire genome. In this equation N is the number of clones, P is the probability of obtaining a representative genome, I is the average size of cloned fragments, and G is the size of the target genome. Using this equation, a pool of ten thousand clones has a 95% probability of containing the entire genome of *P. aeruginosa* (Ausubel 1989).

#### Integration into the *P. aeruginosa* Genome

The plasmid gene library was moved from *E. coli* into *P. aeruginosa* by conjugation through the process of triparental mating. The protocol for this procedure is outlined in Table 1. The process of triparental mating involved three bacterial strains. An *E. coli* strain contained the helper plasmid, pRK2013 (Figurski 1979). This plasmid included *tra* genes required for the transfer of plasmid DNA between host and recipient. The host strain was the pooled *E. coli* with the genetic library plasmids. The recipient strain was the wildtype *P. aeruginosa* PAO1. When all three strains were filtered together onto a solid surface the bacteria could transfer plasmids between cells through conjugation. First, the helper plasmid was transferred to the *E. coli* cells that contained the genetic library through a mating with the helper strain. Then the plasmids of the gene library were transferred from *E. coli* into the recipient *P. aeruginosa*.

Once in the *P. aeruginosa* cells the plasmids entered the chromosome by recombination at the sites of homology between the chromosome and the random fragments of chromosomal DNA in the plasmids (Figure 4). The plasmids with the DNA fragments could replicate in *E. coli* because they have the ColEI origin of replication. Once introduced into *P. aeruginosa* however, the plasmids will not replicate autonomously because this plasmid type lacks the origin of replication for the species. In this way a pool of *P.*

*aeruginosa* cells was generated with each cell containing a synthetic reporter operon inserted somewhere in the chromosome. Those cells containing the synthetic operon were selected on carbenicillin media by constitutive expression of the *bla* gene.

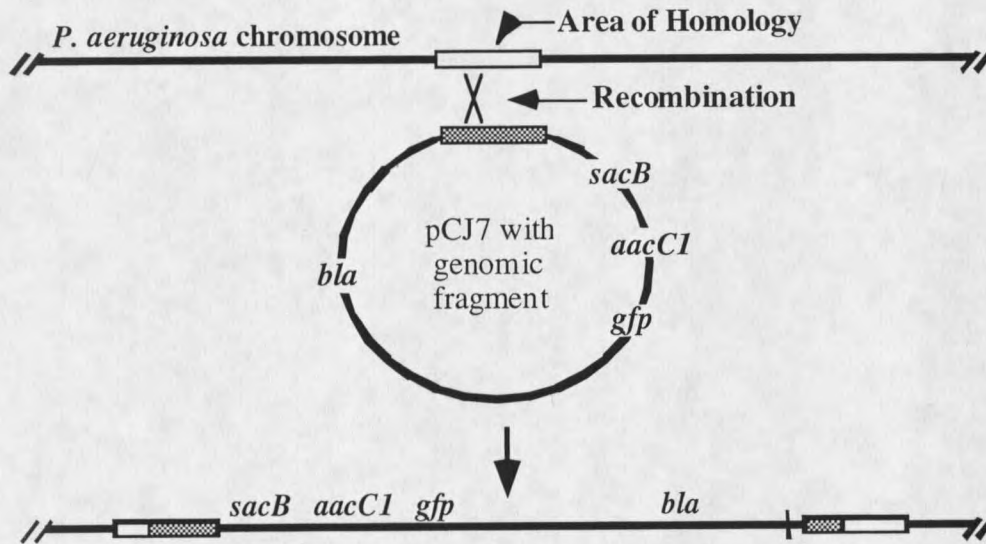


Figure 4: Plasmid integration into the bacterial chromosome. The plasmid moves into the bacterial genome by homologous recombination. Single recombination within the area of homology results in a duplication of the fragment DNA on either side of the plasmid DNA.

The process of integrating the synthetic operon into the *P. aeruginosa* genome serves several purposes. First, the pool of cells can be considered to have the operon inserted randomly throughout the genome because of the random nature of the chromosomal DNA fragments cloned into pCJ7. The later selection process should therefore, be an unbiased selection of bacterial promoters. Second, because a single recombination event will generate a duplication of the area of homology (Figure 4) the function of the gene should not be disrupted. This advantage is in contrast to transposon mutagenesis studies. These IVET studies could be done using plasmids without the need for incorporation into the genome. However, integration into the chromosome provides a stable system with a single copy of the synthetic operon, avoiding the potential bias of multiple copy plasmids. Lastly, a chromosomal fragment on the plasmid may contain only a portion of a promoter region, or

no promoter at all. Using the recombination approach, once the DNA is integrated into the chromosome the native promoter would be restored by the upstream sequence.

### The Selection Strategy

The pool of bacteria can be exposed to selective agents of sucrose or gentamicin under various environmental conditions to select genes that are differentially expressed under specific growth conditions. The growth conditions that were examined in these experiments was growth on a solid surface and growth in liquid culture. Two solid surfaces were examined in this study, the surface of an agar media plate and a glass surface.

The first step in the selection process was to grow the pool of bacterial cells in liquid medium. The bacteria were grown in liquid media without any selective agent added until the culture reached logarithmic growth phase. A sample of this culture was then inoculated into liquid medium containing 10% sucrose. In the presence of sucrose any cell that expressed the synthetic operon was removed from the pool because *sacB* expression was lethal to the cells. In this way constitutive promoters were eliminated from the pool of promoters to be screened. Cells with the synthetic operon downstream of promoters that were only induced during liquid growth were also eliminated from the pool. The pool of cells were incubated in sucrose medium for approximately 24 h so that the cells were exposed to sucrose during all growth phases. The expression of some promoters are growth phase dependent so care was taken to eliminate these promoters before the next step of selection.

A sample of the pool was then grown for 12 h in fresh medium that did not contain sucrose. The cells likely contained intracellular sucrose that could interfere with the gentamicin selection. Any intracellular sucrose was eliminated after several generations of cell division.

The pool was then ready for selection on the solid surface. The pool should contain only bacteria with the synthetic operon downstream of genes that were not induced during

liquid growth. The possible exception were cells with the reporter operon inserted in the wrong orientation. In this case the genes of the synthetic operon may be downstream of an induced promoter but will not generate a transcript because the synthetic operon is in the opposite orientation of the promoter.

The pool was then plated on 1.5% agar plates containing gentamicin at 100 $\mu$ g/ml. All plates were incubated for 18 h. During this growth on the agar plate only those bacteria that expressed the synthetic operon to a sufficient level would survive exposure to gentamicin. All cells that did not express the synthetic operon, did not grow and replicate, and so were removed from the pool. In this way all cells with the synthetic operon in intergenic sequences, or in the opposite orientation from a promoter sequence, were eliminated. The remaining cells were those that contained a synthetic operon downstream of a promoter that was not induced during growth in liquid but was induced during growth on the solid surface.

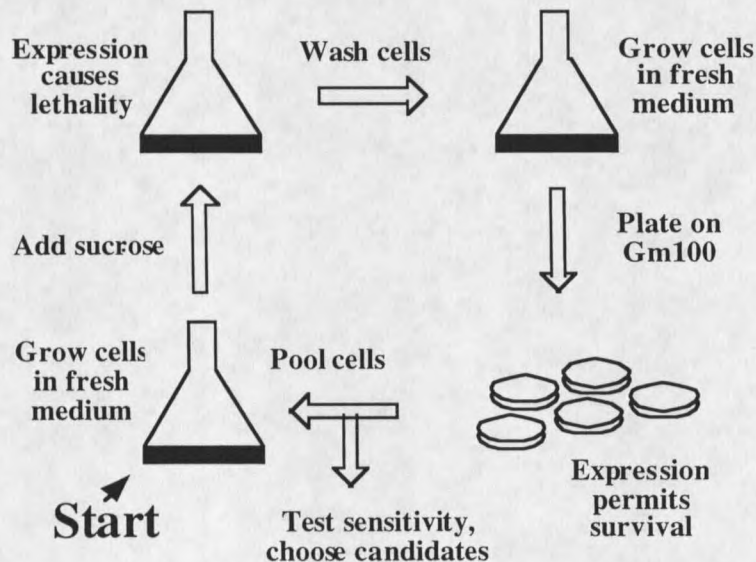


Figure 5: Selection procedure schematic. The genetic library pool of cells are subjected to negative selection by *sacB* expression in liquid culture containing sucrose and positive selection by *aacC1* expression as attached cells on the surface of an agar plate.

The colonies from the gentamicin plates were pooled into liquid medium. This was done by placing 3ml of medium onto the agar surface and using a sterilized glass rod to scrape the surface and get the bacteria into suspension. Cell suspensions from each plate were then pooled and grown in liquid medium until the culture reached stationary phase. At that point, the bacterial pool had gone through one round of selection. Since the efficiency of the positive and negative selection processes was unknown, the pool was put through two additional rounds of selection as described above. An increase in the number of colony forming units (CFU) on gentamicin plates following the second round of selection indicated that additional rounds of selection helped increase the percentage of cells having the selected phenotype.

#### Testing the Level of Expression

Following three rounds of selection, fifty colonies were transferred to agar plates. These fifty clones were then inoculated into test tubes containing 2.5 ml of 10% sucrose media. The same fifty clones were also inoculated into tubes with 2.5 ml media containing 50 µg/ml gentamicin. These cultures were incubated for 18 hrs on a test tube roller. Eight of the fifty clones grew well in sucrose liquid media but not in the gentamicin liquid media.

These eight clones were then tested further in both liquid media, and on agar plates, that contained varying strengths of gentamicin. This testing will be referred to as method #1 for analysis of gene induction. The eight isolates were grown in liquid medium for 12 h, at which point the cells were in logarithmic growth phase. A sterile transfer loop was used to inoculate 2.5 ml liquid cultures containing gentamicin at 10, 25, and 50 µg/ml. For the solid culture conditions a loop inoculum of bacteria was streaked, in one continuous streak, onto plates containing gentamicin at 100, 200, 300, 400, 500 and 1000 µg/ml. The level of resistance to gentamicin, used as an indicator of gene induction, was determined as the highest concentration of gentamicin that allowed growth of the bacterial isolate.

### Microscopy

Bacterial cells were examined using a Nikon LABOPHOT epi-fluorescence microscope at 400-1000X magnification. Fluorescent light was generated by a mercury bulb in a 100 W power supply. Green wavelengths of light were examined using a B-2A filter cube containing an excitation filter of 520-560 nm and a 510 nm dichroic mirror. Bacterial cells from colonies on agar plates were suspended in liquid medium for microscopic analysis. The production of green fluorescence by various isolates was compared to that of the wildtype strain by observing live cells in suspension between a glass slide and coverslip. The level of fluorescence of individual isolates were also compared for potential differences between sessile and planktonic cells of the same clone.

#### Testing of the Level of *sig* Expression - Method #2

The prior measure of gene expression by testing gentamicin resistance in method #1 lacked a quantitative aspect necessary to provide confidence in the level of gene induction. In addition, attempts to assess increased *gfp* expression by analysis of fluorescence of bacterial isolates growing on surfaces provided inconsistent results. Therefore, another measure of the level of *sig* (surface-growth induced gene) expression was determined necessary to improve the degree of confidence in previous data.

The eight *sig* isolates were grown for approximately 24 h in liquid culture. The cultures were then serially diluted. Inocula of 100  $\mu$ l were transferred from a  $10^{-7}$  dilution to a series of test tubes containing gentamicin at 10, 15, 20, 25 and 30  $\mu$ g/ml. Similarly, 100  $\mu$ l samples of the same dilution were spread on a series of plates containing 50, 100, 150, 200, and 250  $\mu$ g/ml gentamicin. After 24 h of incubation the bacterial density of the liquid cultures was determined by absorbance readings at a wavelength of 600 nm. For the surface-associated plate cultures, an attempt was made to generate quantitative data

comparable to that of the liquid cultures. The bacterial colonies on the surface of the agar were suspended into an equal volume of liquid (5 ml) by scraping the plate surface with a sterile glass rod. The absorbance values of this cell suspension was then determined. The two types of cultures were then compared with respect to the highest concentrations of gentamicin that permitted bacterial growth.

#### Sequencing of the Putative Surface-growth Induced Genes

In order to identify the promoter upstream of the synthetic operon, the upstream DNA was sequenced. To facilitate isolation and concentration of the target DNA, a plasmid reconstruction strategy was used. Total genomic DNA from each of the eight clones was isolated. The DNA was digested with restriction enzyme *AatII*. After evaluating several genomic sequences, *P. aeruginosa* DNA was noted to contain *AatII* sites approximately every one to two kilobases. The vector DNA only contained one *AatII* site at the end of the plasmid sequence, so a complete plasmid could be recovered.

DNA ligase was then added to the solution of *AatII* digested genomic DNA so that the two ends of each fragment could ligate and generate circular DNA. Only the fragment containing the synthetic operon could generate a plasmid able to replicate in *E. coli* cells, since this fragment would have the ColEI origin of replication. In addition, this fragment would have the *bla* gene that could be used for selection. The ligation mix was moved into *E. coli* cells by electroporation and then the cells were plated on ampicillin plates. Since the pCJ7 plasmid DNA contained the *bla* gene for beta-lactamase, the *E. coli* clone with the reconstructed plasmid was selected by ampicillin resistance.

The plasmid DNA was then purified and the DNA adjacent to *sacB* was sequenced. A sequencing primer was made of the reverse complement of a portion of the 5' end of the *sacB* gene (5'-GTTGCGCCTCCTGCCAGCAGTG-3'). The resulting sequence thus consisted of the reverse complement of the chromosomal DNA upstream of the *sacB* gene.



### Sequence Identification

The *sigs* were initially assumed to be genes of unknown function, or as of yet unidentified genes of *P. aeruginosa*, so homology searches were performed to identify sequences with similarity in other species. Using this strategy a putative identity can be assigned to a gene that has not been previously characterized in *P. aeruginosa*. The Pseudomonas Genome Project ([www.pseudomonas.com](http://www.pseudomonas.com)) was used to identify genomic DNA surrounding the 200 to 400 bases of nucleotide sequence generated from the sequencing reaction. A ten to twenty kilobase pair section of the genome sequence adjacent to the sequence of the putative surface growth-induced gene (*sig*) was loaded into the computer program MapDraw (DNASTAR Inc.). This program identified where putative open reading frames (ORFs) were within the section of DNA sequence. The sequence of the ORF that contained the *sig* sequence was determined. The DNA sequence of this ORF was then translated into protein sequence using the EditSeq program (DNASTAR Inc.). Protein sequence was entered into BLAST searches of the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov/blast/blast.cgi](http://www.ncbi.nlm.nih.gov/blast/blast.cgi)) protein database. Protein sequences from the database that had similarity to the protein sequence of each *sig* were identified and evaluated as potential homologs. The BLAST search program itself evaluated the degree of similarity or homology between sequences and provided a statistical analysis of the comparisons (Altschul 1997).

### The Flowcell System

A flow-through culture system (Figure 7) was developed that could be used to evaluate bacterial attachment, *gfp* induction and biofilm development in a flowing system using light, and epifluorescence, microscopy. The base of the flowcell was constructed of a transparent polycarbonate material. A glass coverslip was fixed in place to form the top of the flowcell

chamber. Bacterial suspensions were inoculated into the system directly upstream of the flowcell. After a short period for bacterial attachment, 0.5 to 15 min, sterile medium was pumped through the system at 1.0 to 1.3 ml/min so that only attached cells, not planktonic cells, remained within the flowcell. Bacterial attachment and biofilm development were observed by periodically mounting the flowcell on the microscope stage to view the bacteria *in situ*.

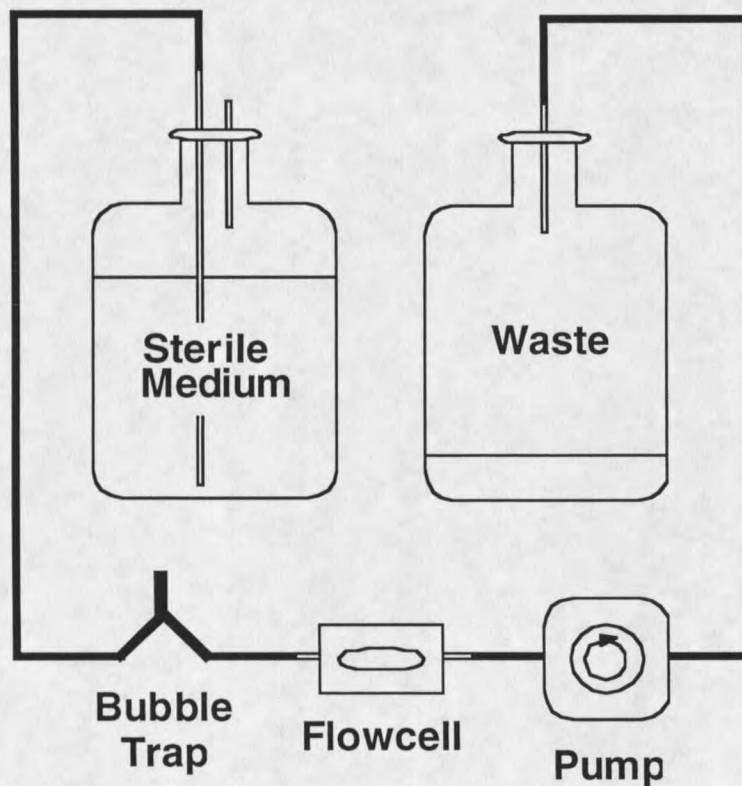


Figure 7: The flowcell system. The flowcell can be placed directly under a microscope while still connected to the system. Bacterial attachment and growth can be observed and selection performed on the attached cells. Induction of a *sig* may be observed *in situ* as fluorescence derived from *gfp*.

The selection strategy described previously was also carried out using the glass surface of the flowcell as the solid selection surface instead of the agar plate surface. Several flowcell selection experiments were conducted. The selection in liquid medium was

performed as described above. Then, instead of plating out aliquots of culture, the cell suspension was inoculated into the flowcell. A period of 4 h was allowed before gentamicin was added to the medium to allow induction of the *aacCI* gene. After 8 to 16 h of incubation in the presence of gentamicin, flow was stopped. The flowcell was opened, the bacteria removed from the surface by swabbing or scraping with a razor blade, and the bacteria were transferred to liquid or solid media.

## RESULTS

Demonstration of the Effect of Genes in the Synthetic Reporter Operon

To examine the effectiveness of the two types of selection produced by the genes of the synthetic operon a plasmid-containing control strain was generated. Plasmid pCJ4 was constructed by cloning the synthetic operon downstream of the inducible *trc* promoter of plasmid pMF54 (Franklin 1994). A plasmid bearing clone of *P. aeruginosa*, designated PAO1(pCJ4), was tested in medium with sucrose and in medium with gentamicin. Expression of the *trc* promoter, and consequently expression of the synthetic operon, was induced by the addition of isopropyl  $\beta$ -D-thiogalactoside (IPTG) to the medium. In the presence of IPTG the clone was able to grow at gentamicin concentrations as high as 500  $\mu$ g/ml and exhibited green fluorescence far greater than the natural autofluorescence of strain PAO1, demonstrating that *aacCI* and *gfp* produced functional gene products. When tested in medium containing 5% sucrose no growth of PAO1(pCJ4) was noted after twenty-four hours of incubation, demonstrating functionality of the *sacB* gene.

To evaluate the effect of expression of the synthetic operon as a single copy inserted into the chromosome, a sample of the recombinant pool of *P. aeruginosa* cells was screened on gentamicin plates and in liquid medium containing gentamicin. An isolate that grew well on both types of media was selected and considered to have the synthetic operon inserted downstream of a constitutive promoter. This isolate, designated PAO1::pCJ13, was tested for growth on sucrose and gentamicin media (Figure 8). The wildtype PAO1 was not affected by sucrose and grew well on sucrose plates. However, PAO1 did not grow in the presence of 50  $\mu$ g/ml gentamicin. In contrast, PAO1::pCJ13 was sensitive to sucrose due to *sacB* expression. PAO1::pCJ13 grew well on gentamicin-containing medium due to constitutive expression of *aacCI*, which made the clone gentamicin resistant.

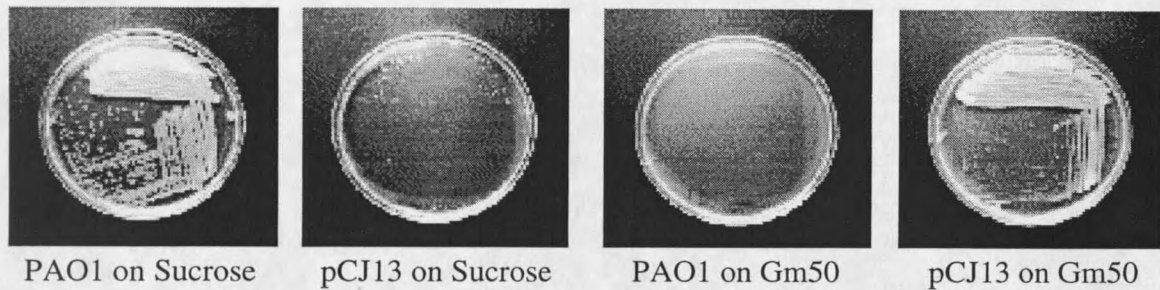


Figure 8: Demonstration of the effect of the genes in the synthetic reporter operon. Wildtype PAO1 was compared to an isolate with constitutive expression of the synthetic operon from a chromosomal insertion (PAO1::pCJ13 is abbreviated pCJ13). Sucrose plates contained 5% sucrose. Gm50 plates contained gentamicin at 50  $\mu\text{g}/\text{ml}$ .

### Growth Curve Analysis

An evaluation of the growth rate of the pool of recombinants was performed for several reasons. First, growth curve data provided an indication of when the pool of cells would be in a specific growth phase. Since the growth phase of a culture may affect gene expression patterns, knowledge of the growth curve was considered in the design of experimental protocols. Second, the effect of sucrose concentrations as high as 5% on *P. aeruginosa* cells was unknown.

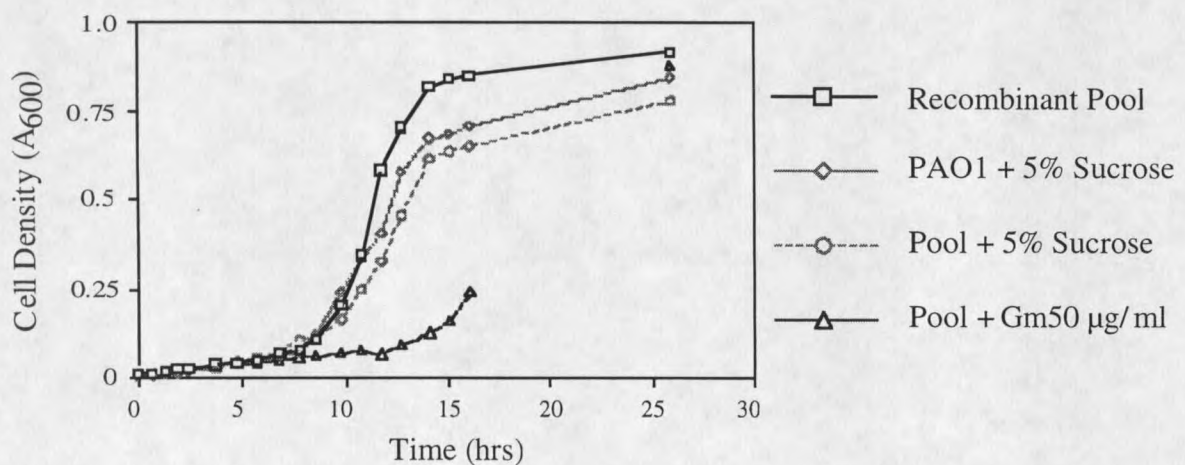


Figure 9: Growth rate comparison. The recombinant pool and the wildtype were grown in the presence of sucrose and gentamicin in liquid culture.

The growth rate of the bacteria containing the genetic library in the presence of sucrose did not differ greatly from wildtype PAO1 in sucrose medium. The maximum cell density of the recombinant pool culture without sucrose was slightly higher than the pool with sucrose suggesting that the combination of *sacB* expression and sucrose inhibited growth of a percentage of the cells. The growth curve of the bacterial pool in medium with gentamicin was substantially different from the recombinant pool without gentamicin in the medium. The culture exposed to gentamicin had a much longer lag phase. However, the final density of this culture was similar to that of the other cultures as indicated by the data point at approximately twenty-six hours. A fraction of the recombinant pool was resistant to gentamicin due to *aacCI* expression driven by upstream promoters.

In addition to the growth curve data, the fraction of cells expressing the synthetic operon was assessed by plating experiments. Several hundred colonies were transferred from media containing only carbenicillin to media with either sucrose or gentamicin. The cells with the synthetic reporter operon downstream of an active promoter should be resistant to gentamicin and sensitive to sucrose. The majority of colonies did not grow on gentamicin media but did grow on sucrose media. These results were as expected from the growth curve data which indicated that only a fraction of the cells contained synthetic operon downstream of an active promoter.

#### Selection and Testing of Surface-Induced Genes (*sigs*)

The pool of recombinants was then put through negative selection in liquid medium with sucrose to screen out clones with the *sacB* gene downstream of promoters that were active during liquid growth. The cells that survived this selection were then plated on solid agar medium with gentamicin. Cells that survived this positive selection should be only those with the *aacCI* gene downstream of a promoter that was induced after the transition from planktonic to sessile growth.

Additional screening identified eight isolates that appeared to contain the synthetic operon downstream of a surface-induced promoter. These isolates grew well in liquid media containing sucrose as well as on agar plates containing gentamicin, suggesting that the synthetic operon was induced during sessile but not planktonic growth. The isolates also exhibited a comparatively high resistance to gentamicin when grown on an agar surface rather than in liquid medium. Testing the level of expression of the operon by this method of analysis of gentamicin resistance is referred to as method #1.

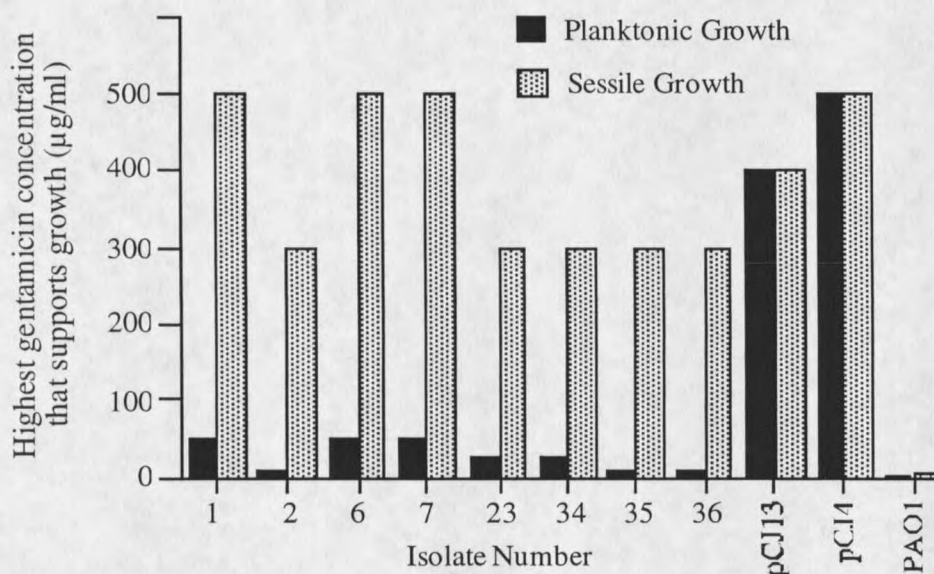


Figure 10: Level of gentamicin resistance of the isolates in liquid and on solid media - Method #1. Strains PAO1(pCJ4) and PAO1::pCJ13 serve as controls with constitutive expression of *aacC1*. Wildtype PAO1 is shown on the far right.

The level of resistance for the wildtype was an important consideration since others have found an inherent increased resistance to antibiotics of surface grown cells (Anwar 1989, Stewart 1999, Vransky 1997). However, when wildtype culture was streaked onto gentamicin medium plates and inoculated into liquid it demonstrated less than a two fold increase in resistance. The wildtype strain PAO1 grew on agar plates that contained up to, but not greater than, 5 µg/ml gentamicin. In liquid medium the wildtype grew in medium with gentamicin concentrations of 3 µg/ml, but no higher.

Under the conditions of this analysis of gene induction the control strains PAO1::pCJ13 and PAO1(pCJ4) were able to grow on either solid or in liquid media with concentrations of gentamicin of 400 to 500  $\mu\text{g/ml}$ . In these two strains, constitutive expression of the *aacC1* gene produced equivalent levels of resistance in either planktonic or sessile cells. On solid medium the *sig* isolates exhibited resistance to gentamicin concentrations of 300 to 500  $\mu\text{g/ml}$ . These levels of resistance for the isolates on solid medium were comparable to those of the constitutive controls. In contrast, the highest concentration of gentamicin that supported growth of the eight isolates in liquid medium was only 10 to 50  $\mu\text{g/ml}$  gentamicin. Thus, the *sig* isolates appeared to be 10 to 30 fold more resistant to gentamicin when grown on a surface than when growing in liquid medium. The data for the *sig* isolates compared to the controls is consistent with the hypothesis that the putative *sigs* are induced by the switch from planktonic to sessile growth.

#### Analysis of *aacC1* Expression - Method #2

In an attempt to generate more quantitative and statistically significant data on the level of induction of the synthetic operon, additional tests of the level of gentamicin resistance of these isolates were conducted. To avoid the effect that a large inoculum may have had on previous tests, medium containing gentamicin was inoculated with only a small number of individual cells. Liquid overnight cultures were diluted and inoculated into new liquid medium containing 15, 20, 25, 30 and 35  $\mu\text{g/ml}$  gentamicin. The same volume of inoculum was spread onto agar plates containing 0, 50, 100, 150, 200 and 250  $\mu\text{g/ml}$  gentamicin. After incubation, colonies from the agar surfaces were suspended into 5ml of liquid medium. Cell density of these suspensions were then compared to that of the 5 ml liquid cultures. Absorbance readings were taken to determine the level of gentamicin that prevented growth. These readings provided a more quantitative determination of the highest concentration of gentamicin that could support growth of a particular isolate.

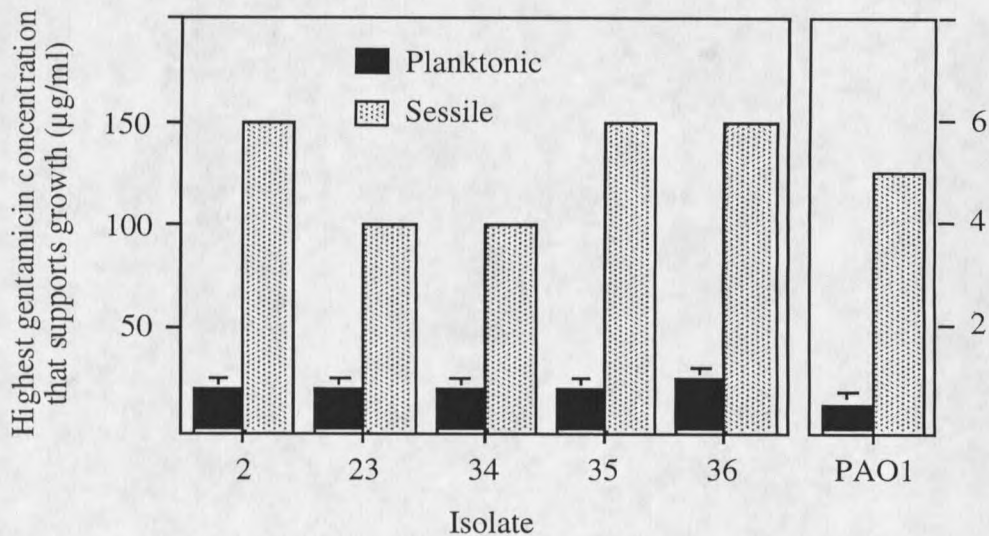


Figure 11: Level of gentamicin resistance of planktonic versus sessile cultures - Method #2. The level of resistance was defined as the highest incremental concentration of gentamicin in the media that produced liquid turbidity above background (as determined by absorbance values at 600nm). Mean and standard deviation of three separate experiments were calculated and represented by error bars. Values for sessile cultures did not vary. Values represented above were rounded to the nearest increment of gentamicin concentration.

The level of gentamicin resistance in the wildtype PAO1 strain was measured in liquid medium at 0.1 µg/ml increments from 0.3 to 0.9 µg/ml gentamicin. The mean of three experiments indicated that the highest concentration of gentamicin which supported growth of the wildtype in liquid medium was approximately 0.5 µg/ml. On the agar plates, wildtype PAO1 produced several CFU per plate up to 5 µg/ml gentamicin. No CFU were noted on plates with 6 µg/ml gentamicin or higher. The data indicated that the wildtype may be 10 fold more resistant to gentamicin when growing on a surface than when growing in liquid, while the other analysis described in Figure 10 indicated that the increase in resistance for the wildtype was less than 2 fold.

On average the *sig* isolates were able to grow in liquid culture with up to 20 µg/ml gentamicin. On solid medium the highest concentration of gentamicin that supported growth was either 100 or 150 µg/ml, depending on the particular isolate. Thus, the increase in gentamicin resistance for the isolates tested using this methodology was between 5 and 7.5

fold. The increase in resistance for the *sig* isolates appeared to be lower than that of the wildtype. However, if the statistical error of the data is considered, the magnitude of the increase in resistance to gentamicin for the *sig* isolates is not statistically different from that of the wildtype.

A clone that was resistant to high levels of gentamicin in liquid and on solid culture was also tested as a control assumed to have constitutive expression of the synthetic reporter operon. This strain was different from PAO1::pCJ13 shown in Figure 10, but was isolated in the same manner. The level of resistance for this isolate was equivalent in either environment. The clone grew on agar plates and in broth that contained up to 500 µg/ml gentamicin. Thus, the positive control again exhibited no difference in the levels of resistance for the two different environments, while the *sig* isolates showed an increased resistance to gentamicin on the agar surface.

In evaluating plates with varying concentrations of gentamicin, it was noticed that the CFU count for each isolate decreased consistently as the gentamicin concentration increased. Apparently the sensitivity of each cell in the culture to gentamicin was not identical. This discrepancy was considered with respect to possible dynamic changes that might be occurring in the regulation and expression of the promoters upstream of the *aacCI* genes. However, a similar trend of decreasing CFU counts was noted for the wildtype control.

#### Analysis of Gene Induction by *gfp* Fluorescence

If the synthetic operon is inserted downstream of a gene that is induced after the bacteria attaches to a surface, then all the genes of the operon should have a concomitant increase in expression. Therefore, *sig* induction should cause sessile cells to express *gfp* more strongly and be more fluorescent than the same cells growing planktonically. All of the eight isolates were observed by epifluorescence microscopy to compare their level of

fluorescence when grown in liquid medium or on an agar plate. The relative fluorescent intensity of these isolates varied and was not consistent. For instance, during one analysis cells of isolate number 2 that were taken from a colony on an agar plate were observed to be noticeably more fluorescent than those from a liquid overnight culture. However, when the experiment was repeated weeks later none of the samples tested appeared to be more fluorescent than any other, whether sessile or planktonically grown. Other of the isolates also demonstrated increased fluorescence of sessile cells during some experiments but not during others.

The control strain PAO1(pCJ4) contained a plasmid with the synthetic operon downstream of an inducible promoter. This strain always displayed fluorescence far greater than the wildtype, whether grown on a surface or in liquid suspension. Control strain PAO1::pCJ13 had the synthetic operon inserted into the chromosome in a location that was assumed to be downstream of a constitutive promoter. If the culture was allowed to grow for more than 24 h strain PAO1::pCJ13 was generally more fluorescent than wildtype PAO1 cells, but this observation was not always consistent. This difference in the control strains' fluorescence may be due to the fact that PAO1(pCJ4) contained the operon on a multicopy plasmid while PAO1::pCJ13 contained the operon as a single copy inserted into the chromosome. Results of *gfp* analysis were therefore inconsistent and not considered as a dependable indicator of *sig* induction.

#### Recovery of Plasmids from the Chromosomal Insertions

The eight isolates identified in the screening procedure were chosen for sequencing of the DNA upstream of the synthetic operon. To facilitate sequencing, plasmids containing a portion of the putative *sigs* were isolated from the chromosome of each isolate and reconstructed as described in Figure 6. The plasmids were then examined to identify any replicate isolates as well as to be sure that the plasmids were composed of the vector, pCJ7,

and some upstream chromosomal DNA. These plasmids were digested with restriction enzymes *ClaI*, *NdeI* and *PstI* to generate multiple fragments that could be distinguished as either chromosomal DNA or vector DNA. The fragments of each plasmid were separated by agarose gel electrophoresis and compared to the fragment banding pattern of the control plasmid pCJ7.

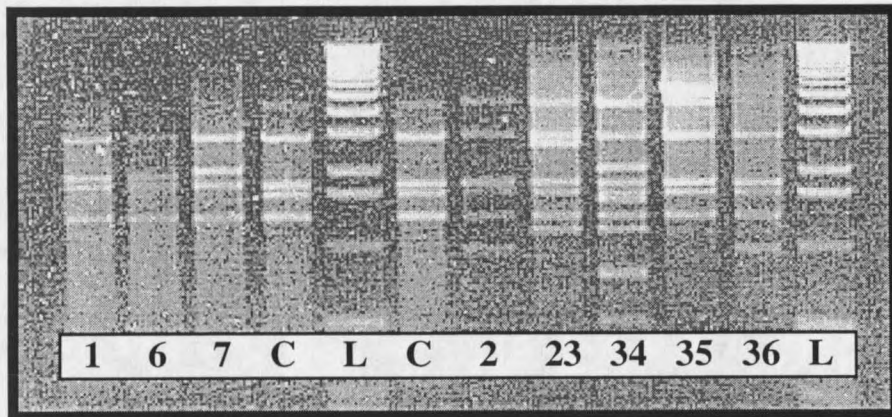


Figure 12: Restriction patterns of plasmids isolated from the chromosome of each recombinant (indicated by number). Plasmids were digested with endonucleases *ClaI*, *NdeI* and *PstI*. L indicates the lane containing 1kb ladder DNA for size reference. C indicates the pCJ7 control plasmid.

The DNA fragment banding pattern for the restriction digest of plasmid pCJ7 produced four distinguishable fragment bands. The restriction fragment patterns of isolates 2, 23, 34, 35 and 36 each included the control bands as well as additional bands. These additional bands represented the chromosomal DNA that was upstream of the synthetic operon in the *P. aeruginosa* genome and was recovered with the original vector DNA. Isolates 2 and 36 both produced identical restriction patterns as would be seen for sibling isolates of one clone. There were obvious differences in the DNA fragment sizes of digests for isolates 2, 23, 34 and 35. These different bands represent different DNA sequences, an indication that each of these four *sigs* were likely different genes. Restriction patterns of isolates 1, 6 and 7 were similar to one another, but different from that of pCJ7, in that these

three patterns lacked one of the bands that each of the other isolates and the control possessed but contained another band of a larger fragment size.

### Sequence Analysis

After a portion of the chromosomal fragment from each plasmid was sequenced, the sequences were compared to one another and were searched in the database of the *Pseudomonas* Genome Project database ([www.pseudomonas.com](http://www.pseudomonas.com)). Sequences from isolates 2 and 36 were nearly identical, having less than five nucleotide base differences over 200 bases of sequence. These differences were attributed to error inherent in the sequencing process. The match of sequences of isolates 2 and 36 may have resulted because the two were progeny of a single clone that were isolated independently. Similarly, isolates 23 and 34 were found to be identical, despite the different restriction patterns of their plasmids (Figure 12). The plasmid of isolate 34 was assumed to have had additional DNA, not in that of 23, because of incomplete digestion by the *Aat II* restriction enzyme reaction. No sequence could be generated from plasmids of isolates 1, 6 and 7 though several sequencing reactions were attempted for each.

The sequence from isolate 2 and 36, hereafter referred to only as *sig 2*, was identified within the *P. aeruginosa* genome database. When the DNA sequence on either side of the location of the *sig 2* sequence was analyzed by the program MapDraw (DNASTAR Inc.) several open reading frames (ORFs) were identified as shown in Figure 13. The direction of transcription for the reading frames identified immediately adjacent to the *sig 2* sequence was opposite that of the reading frame of the synthetic operon, so analysis of the surrounding DNA region was also performed. The amino acid sequence of each ORF was determined and used in an Advanced BLAST search for proteins with homology (NCBI, NIH, [www.ncbi.nlm.nih.gov/blast/blast.cgi](http://www.ncbi.nlm.nih.gov/blast/blast.cgi)). This database search identified proteins with amino acid sequence similarity and performed comparisons of the regions of sequence that

were most similar. Proteins with the highest homology, homologs, are listed below in Table 2. The protein database search program defined various criteria for comparing the similarity of protein sequences and generated data on how statistically similar two target sequences were to one another. Some data on the degree of homology is provided below.

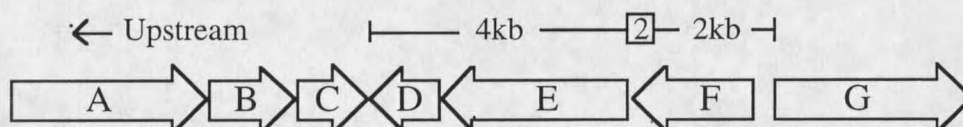


Figure 13: Diagram of the ORFs surrounding the *sig 2* sequence. The location of the *sig 2* sequence is indicated by the boxed-in number 2. The position of the synthetic operon would be directly downstream of this site. Distances to ORFs in the same orientation as the *sig* sequence are shown in kilobase pairs (kb).

Table 2: Putative identity of the genes surrounding the *sig 2* sequence.

Gene	Species	%ID <sub>a</sub>	Sim <sub>b</sub>	Length <sub>c</sub>	Prob <sub>d</sub>	Function	
A	<i>hmwA</i>	<i>H. influenzae</i>	33%	50%	298	$2 \times 10^{-31}$	adhesin
B	<i>lpfE</i>	<i>S. typhimurium</i>	25%	43%	139	$9 \times 10^{-4}$	fimbrial protein precursor
C	<i>rcsB</i>	<i>E. coli</i>	38%	63%	205	$6 \times 10^{-36}$	capsular synth. regulator
D	<i>csgA</i>	<i>M. xanthus</i>	32%	45%	227	$1 \times 10^{-22}$	signal transduction
E	<i>safA</i>	<i>M. xanthus</i>	31%	46%	995	$10^{-111}$	saframycin synthetase
F	<i>pbsX</i>	<i>D. radiodurans</i>	70%	86%	62	$4 \times 10^{-18}$	transcriptional regulator
G	<i>styD</i>	<i>P. fluorescens</i>	48%	63%	499	$10^{-131}$	dehydrogenase

(a) indicates the percent of amino acids that were identical to those in the homolog sequence. (b) indicates the percent of residues that had similar properties. (c) indicates the length of the amino acid sequence that was compared. (d) score given to each comparison that describes the probability of such a pattern arising simply by chance (Altschul 1997).

Each of the genes described in Table 2 were considered for their potential as surface-induced genes. The *sig 2* sequence is 2 to 4 kb downstream from either ORF that would be transcribed in the same orientation as the synthetic operon. The *sig 2* sequence is immediately upstream of the *safA* homolog, but this putative gene would be read in the opposite direction. In cases such as this, where sequences are found in areas with opposite polarity, others have suggested the possibility that the promoter driving expression of the

synthetic operon might be from an antisense RNA (Camilli 1995, Valdivia 1997, Valdivia 1998). An RNA polynucleotide could be transcribed from the opposite strand of DNA which would be complementary to the leader sequence of the *safA* homolog's mRNA. Thus, the antisense RNA could act as a negative regulator of the *safA* homolog.

The sequences of *sigs* 23 and 35 were found to be only about one kilobase apart from one another in the *P. aeruginosa* genome. Analysis of the DNA surrounding these sequences did not identify any ORFs immediately around either sequence. However, an ORF that started approximately two kilobases upstream was found to be highly homologous to a tyrosine-tRNA synthetase (60% identical over 396 residues). This discovery lead to the *sig* 23 and 35 sequences being searched as nucleotide sequences rather than amino acid sequences. The blast(n) searches found these sequences to be greater than 95% identical to *P. aeruginosa* 16S rRNA and tRNA sequences, respectively. Single base differences were attributed to sequencing error.

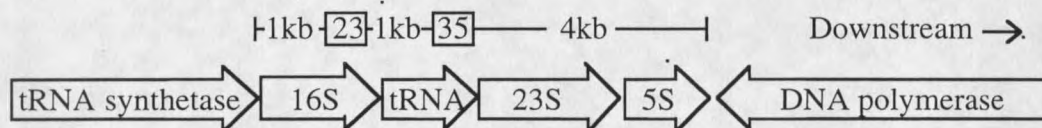


Figure 14: Diagram of the ORFs surrounding the *sig* 23 and 35 sequences. The relative positions of each sequence are indicated by their respective numbers. The DNA polymerase homolog identified downstream was 47% similar to a DNA polymerase III of *P. fluorescens* (Prob.  $5 \times 10^{-87}$ ).

## DISCUSSION

Understanding of bacterial processes such as host colonization or contaminant degradation have been greatly improved by the use of molecular genetic techniques. Genetic analyses of bacterial infection have been undertaken with a number of goals in mind. Knowledge of the process of host invasion and sustained infection in general can be increased by identifying the genes and gene products necessary for the bacteria to survive within a host environment. This knowledge can provide information on aspects of the infection process that may be targeted by control strategies. If the induction of a particular gene or operon is required for a bacterium to survive and mount an infection, then preventing that expression, or interfering with the gene product's function, may prevent infection. Technologies such as the *in vivo* expression technology (IVET) show much promise in furthering these goals.

An appreciation of the role biofilms play in infection, as well as in the natural environment, has spawned increased investigation into surface-associated growth, or the sessile lifestyle of bacteria. In this study an adaptation of IVET systems to study bacterial processes at interfaces was developed and tested for many of the same reasons described above. For example, bacteria are much more difficult to eliminate from a system once they have attached to a surface and are growing as a layer of cells surrounded by extracellular substances (Anwar 1989, Herson 1987, LeChevallier 1988). Thus, if genetic targets can be identified in the process of biofilm formation, then these more resistant infections may be more easily prevented or treated.

On the other hand, biofilm formation may be beneficial in some environments. A healthy biofilm community may increase contaminant degradation rates in aquatic as well as terrestrial environments (Ascon 1999, Ascon-Cabrera 1995, Molin 1985). Many plants are also known to benefit from a film of bacteria that coat the surface of the roots (Geels 1983,

Schippers 1987, Weller 1988). In these instances identifying factors that help generate healthy biofilm growth would be quite valuable.

There is significant interest in the ability to exploit bacterial colonization and growth where they are beneficial and to prevent biofilm accumulation where it is detrimental. Understanding changes in gene expression that occur after bacteria attach to a surface should help further many such goals of biofilm research. With these goals in mind the IVET-type system described in this study was developed and tested as a tool to identify and evaluate changes in gene expression that are induced following bacterial attachment to a surface.

#### Development of The Selection System

Previously developed IVET systems were not applicable to the study of surface associated bacterial growth, so a new system was developed. This new system utilized a gentamicin resistance gene, *aacCI*, for positive selection. The *aacCI* gene was chosen because *P. aeruginosa* is sensitive to relatively low levels of gentamicin. In contrast to previous IVET systems, this new system also included a gene for negative selection, the *sacB* gene. Expression of *sacB* in the presence of sucrose is lethal to *P. aeruginosa*. The *gfp* gene was also added to the system for a reporter gene that could be used to confirm when an upstream promoter was induced. These three genes were cloned together into a synthetic operon that was inserted throughout the chromosome of *P. aeruginosa* cells. The genes of this synthetic operon should not be expressed unless a chromosomal promoter directly upstream is induced.

This combination of positive and negative selection was then used to isolate clones from the pool that have the synthetic operon inserted downstream of a gene that is induced by an experimental condition or environment. Expression of *sacB* in the presence of sucrose was used to eliminate clones that have constitutive expression of the synthetic

operon. Expression of *aacCI* was then used to select clones that had the synthetic operon inserted downstream of genes of interest, those induced by the environmental stimulus.

In this study the genes of interest were those induced following attachment of the bacterium to a surface. However, this technology has potential for successful evaluation of gene expression within many different environments. Negative selection can be performed in a control setting under standard conditions. Positive selection can then be performed in various experimental environments or when the bacteria are exposed to an experimental stimulus. In this work, the efficacy of the selection provided by these genes was tested for use in identifying surface-associated growth induced genes (*sig*).

#### Evaluation of the Efficacy of *aacCI* for Positive Selection

The *aacCI* gene was an important reporter gene in this selection system. Resistance to gentamicin formed the basis of the positive selection required to isolate genes of interest. The efficacy of the *aacCI* reporter gene in this system was tested and evaluated in several separate experiments.

Control strains having constitutive expression of the synthetic operon were generated and used to test the utility of the *aacCI* gene. Strain PAO1(pCJ4) was generated by cloning the synthetic operon directly downstream of a constitutive promoter in a plasmid which replicated autonomously in *P. aeruginosa*. This strain then contained multiple copies of *aacCI* behind a strong promoter. When tested in media containing gentamicin at various concentrations, strain PAO1(pCJ4) was able to grow in liquid and on solid media having gentamicin concentrations as high as 500µg/ml.

Another control strain was isolated from the genomic library pool of PAO1 cells. One hundred isolates from the pool of cells were tested in liquid medium with gentamicin and on solid medium with gentamicin. An isolate that was resistant to 100µg/ml gentamicin in both liquid and solid media was chosen and considered to have constitutive expression of the

synthetic operon. This strain, PAO1::pCJ13, had the synthetic operon inserted into the chromosome as a single copy. Additional testing of this isolate demonstrated that a single copy of the *aacCI* gene on the chromosome could provide resistance to gentamicin concentrations as high as 400µg/ml. This level of resistance was nearly as high as that provided by the multicopy control plasmid.

The results from these control strains demonstrated that expression of the *aacCI* gene provided substantial resistance to gentamicin, up to a 100-fold increase in resistance compared to the wildtype. Even a single chromosomal copy could provide a significant level of resistance to gentamicin. Thus, the *aacCI* gene provided strong positive selection pressure for cells that have induced expression of the synthetic operon.

The growth curve experiment also provided information on the expression of *aacCI* within the pool of recombinants. The recombinant pool grown in the presence of gentamicin had an extended lag phase as compared to the pool culture not exposed to gentamicin. Though a growth curve was not constructed for the control strain PAO1(pCJ4) that had constitutive expression of the *aacCI* gene this strain did not seem to exhibit such an extended lag phase. This extended lag suggested that in most cells the synthetic operon was inserted at sites without promoters, was inserted in the wrong orientation, or was inserted downstream of relatively inactive promoters. Alternatively, the lag may have resulted because it took some time for cells in the inoculum to inactivate a large enough portion of the gentamicin present in the medium to allow a more rapid reproduction rate. If this were the case the control strain may have had less of a lag phase because it generated more AacCI protein than the recombinant pool cells were able to. Nonetheless, a percentage of cells in the pool did grow and replicate, generating a culture density, within twenty-four hours, that was equivalent to the density of the control culture. This finding indicated that some of the recombinants in the pool contained the synthetic operon downstream of a promoter that was induced to a degree that would provide the cells resistance to gentamicin.

### Evaluation of the Efficacy of *sacB* for Negative Selection

The gene *sacB* provided the negative selection in this expression system. This gene encoded levansucrase which, when in the presence of sucrose, generated a homopolymer of fructose that was lethal to the bacterial cells. The *sacB* gene was relied upon to remove those cells from the recombinant pool that had the synthetic operon downstream of promoters that were induced at times other than following attachment of the bacteria to a surface, such as during planktonic growth.

The two control strains PAO1(pCJ4) and PAO1::pCJ13, which had constitutive expression of *sacB*, were tested in liquid and on solid media containing sucrose. In both culture conditions the control strains were sensitive to sucrose. The controls did not grow in sucrose media because expression of *sacB* in the presence of sucrose was lethal to the cells. This result indicated that cells which express the synthetic operon constitutively would not be able to survive the negative selection process. Taken together with the analysis of the *aacC1* gene expression, these findings demonstrated that induction of the synthetic operon to a degree which provided resistance to gentamicin also produced sensitivity to sucrose.

The growth curve of cultures grown in sucrose containing medium was also informative. The growth curve of the recombinant pool in sucrose medium was generally similar to that of the wildtype PAO1 in sucrose medium. The similarity of the two curves indicated that most of the cells in the recombinant pool were not actively expressing the genes of the synthetic operon. Only a portion of the pool of cells were restricted from growing in the presence of sucrose due to *sacB* expression and the action of levansucrase. The limited growth of a portion of the recombinant population may have caused the reduced slope of the growth curve of the culture compared to that of the wildtype. Thus, *sacB* expression did provide effective negative selection against the portion of the pool that had an induced promoter upstream of the synthetic operon.

Information from the growth curves of the recombinant pool in sucrose medium and in gentamicin medium were in agreement with the idea that a bacterium living in a given environment will express only a portion of all genes in the chromosome. Thus, only a portion of the pool of recombinants had the synthetic operon downstream of an induced promoter. This portion was selected against in sucrose media but selected for in gentamicin media, demonstrating the efficacy of both the negative and positive selections.

#### Efficacy of *gfp* as a Reporter of Expression

The control strain PAO1(pCJ4) that had constitutive expression of the synthetic operon from a plasmid exhibited bright green fluorescence. This strain produced brightly fluorescent cells in liquid and on solid media. Control strain PAO1::pCJ13 exhibited fluorescence under epifluorescence microscopy after the culture grew for a period of days on an agar plate. However, when tested in liquid medium, or after only overnight growth on agar medium, cells of this strain were not fluorescent. A single copy of *gfp* on the chromosome may not be sufficient to provide visible emission under epifluorescence microscopy unless the fluorescent protein accumulates within the cell for several days.

Some surface associated growth-induced genes are likely only transiently expressed after the transition to sessile growth and therefore may not produce sufficient quantities of protein to be visualized. Further, if an evaluation of what genes are induced early during sessile growth is desired, then time constraints may not permit significant accumulation of GFP. Confirmation of expression of the synthetic operon, and induction of the upstream promoter, using *gfp* was therefore not practical.

#### Applying the Selection System

After confirming that the positive and negative selection strategies were functional in *P. aeruginosa*, the pool of clones in the genetic library was screened to identify clones with

the synthetic operon inserted downstream of potential *sigs*. The pool was first grown in liquid medium containing sucrose. In the presence of sucrose those cells with the synthetic operon downstream of an induced promoter were selected against by *sacB* expression and the lethal effects of levansucrase. Thus genes with constitutive expression, and genes that were expressed during planktonic growth, were eliminated.

The information provided by the growth curve data suggested that only a small fraction of the cells in the pool were eliminated in this negative selection step. Most of the pool survived and was then transferred to agar plates where positive selection was applied. Once attached to the surface and exposed to gentamicin, clones not expressing *aacCI* were prevented from growing. The only bacterial cells that were selected for were those that did not express the synthetic operon in liquid culture but did induce expression once attached to a surface. All other cells were selected against. Only cells with the synthetic operon downstream of a *sig* should have been allowed to proliferate on the surface. However, it is possible that this selection process actually selected cells with the synthetic operon downstream of genes unrelated to surface-associated growth. Cells with a mutation in the *sacB* gene may have been able to survive sucrose selection and replicate more than nonmutants. This larger pool of mutants could then provide a larger inoculum for gentamicin selection that might bias their chances of survival and recovery. The fact that positive selection was performed on agar plates could also have resulted in the selection of isolates with the synthetic operon inserted downstream of genes that were induced by stimuli other than exposure to a surface. Exposure to agar or exposure to an air interface could provide a stimulus able to induce expression of genes not necessarily related to surface-associated growth. Exposure to gentamicin itself probably also caused the induction of many genes as will be discussed later.

Though the production of gentamicin resistance by *aacCI* expression and sensitivity to sucrose by *sacB* expression were effective, the efficiency of selection by these genes was

questionable. Confirmation that the potential *sigs* were indeed induced in sessile, or biofilm, cells was desired. In addition, some sources of error needed to be reduced.

The difference in osmolarity between media with and without sucrose may have had an effect on gene expression. Differences in osmolarity are known to cause changes in gene expression (Csonka 1991). Changes in the ionic strength of the medium can require that the bacterial membrane and osmotic potential of the cytoplasm be adjusted. Therefore, any genes identified as induced when the bacteria were moved from planktonic to attached growth should be carefully considered for their potential role in regulation during osmotic shock. The possibility exists that putative *sigs* identified by the selection system used in this study could have been repressed by osmoregulation and then de-repressed after the transfer to media without sucrose. This possibility is unlikely however, because the *sigs* identified do not appear to be associated directly with any obvious osmoregulatory function. If the same selection process were used in the future some means to normalize the osmotic strength of all media would be beneficial. Possibly another disaccharide that is not catabolized by *P. aeruginosa* could be used in all media that did not contain sucrose.

#### Evaluation of Induction of the *sigs* by Gentamicin Resistance

After selection was performed on the pool of clones approximately 200 clones were selected for further testing. Since *sigs* should be induced when the bacterial cell is growing on a surface, as opposed to growing in liquid, the *aacCI* gene supplied downstream should make the sessile bacteria more resistant to gentamicin than the planktonic cells. This fact was then used as a measure of the level of induction of the potential *sigs*.

Selected isolates were grown in planktonic culture and then inoculated into liquid medium, and onto agar plates containing increasing concentrations of gentamicin. Eight isolates demonstrated elevated levels of resistance to gentamicin when they were cultured on an agar surface. These eight isolates appeared to be ten to thirty times more resistant to

gentamicin when growing as surface-associated cells than when growing as single cells in liquid medium (Figure 10). Importantly, the wild type PAO1 was only about twice as resistant to gentamicin when grown on a surface. Therefore, the much greater increase in resistance for the *sig* isolates suggested that the promoters driving expression of the synthetic operon were indeed induced upon the bacterium's transition to growth on the agar surface.

The experimental procedure used to generate these data, method #1, contained possible sources of error, however. The increments of gentamicin concentration used for the culture media were large. The use of such incremental measurements cannot accurately identify a specific minimal inhibitory concentration. The process of inoculation of the media could have also biased the results. Both liquid and solid media were inoculated with a loop of stationary phase culture. The inoculation of this large a quantity of cells on the agar surface may have affected the results. A portion of the cells in the inoculum could have consumed most of the gentamicin within a local area, allowing a small portion of cells to grow in the area of the streak depleted of the antibiotic.

With these sources of error in mind, other means of producing more quantitative data were sought. The possible inoculum effect was avoided by diluting stationary phase cultures so that only a small number of individual cells was inoculated. Inoculating 100 $\mu$ l of a  $10^{-7}$  dilution into liquid medium and spread plating the same 100 $\mu$ l inoculum yielded consistent inoculation of 25 to 50 cells. The increments in gentamicin concentration were made smaller and assays were also performed in at least three separate experiments in an effort to generate more statistically significant data.

Method #1 yielded a rather qualitative measure of when the bacterial inoculum stopped growing. Each increase in gentamicin concentration resulted in a less dense culture that appeared less healthy, so determination of the level of resistance to gentamicin was

somewhat ambiguous. To move from this qualitative analysis to a quantitative measurement, culture densities were compared in method #2. For each culture plate all colonies on the agar surface were suspended into 5ml of liquid medium. The optical density of these cell suspensions were then compared to that of the corresponding 5ml liquid cultures. The highest concentration of gentamicin that permitted bacterial growth to produce absorbance readings above background levels was recorded for each isolate on liquid and solid medium. These concentrations of gentamicin were then plotted as quantitative data (Figure 11).

Using method #2 the degree to which gentamicin resistance was increased by the transition to sessile growth appeared smaller than in method #1 (Figure 10). These new data suggested that the intrinsic increased resistance of the wildtype strain was actually larger in magnitude than that of the *sig* isolates tested. The wildtype exhibited growth at 5 $\mu$ g/ml gentamicin on agar medium but could not grow past 0.5 $\mu$ g/ml in liquid medium. These data represented a ten fold increased resistance, while that of the *sig* isolates was four to eight fold increased.

The findings provided by method #2 may then indicate that the genes identified in this work are constitutively expressed rather than induced by surface-associated growth. If the promoter upstream of the synthetic operon were induced as a consequence of surface associated growth, the increase in resistance to gentamicin for the *sig* isolates should have been much greater in magnitude than that of the wildtype. Possibly the reason that the *sig* isolates exhibited an increase in resistance to gentamicin when grown on solid media was that sessile bacteria may possess intrinsic resistance to antibiotics (Anwar 1989, Herson 1987, LeChevallier 1988).

The idea of the genes identified in this study as being constitutively expressed, rather than induced, may be supported by other aspects of this work as well. During the selection process the bacteria were moved from liquid culture directly to agar media containing gentamicin. Since gentamicin is considered bacteriocidal rather than bacteriostatic, the

bacterial cells would need to be resistant to gentamicin immediately upon contact with the surface. This aspect of the selection process may then actually have promoted the isolation of bacterial cells with moderate levels of constitutive expression of the synthetic operon. The identities of the putative *sig*s by homology comparison also bring to question the idea that such genes would be more induced during surface-associated growth.

Evaluating *sig* induction by comparing levels of resistance to gentamicin possessed many potential conceptual flaws that could not be avoided. Exposure to gentamicin was always different for cells that were suspended in liquid rather than attached to a surface. Cells in batch liquid culture were surrounded by gentamicin in the medium. The amount of reactive gentamicin present in batch culture likely decreased gradually over time as the antibiotic molecules were inactivated by AacC1 enzymes and natural degradation. Exposure of cells on an agar plate to gentamicin came from only one direction. Though the gentamicin could diffuse through the agar medium toward areas where colonies were depleting the local supply of the antibiotic, after several hours of culture colonies on the agar surfaces likely experienced a lower concentration of gentamicin than did planktonic cells grown for an equal period of time in batch culture. Within the flowcell system exposure to gentamicin was again quite different. Cells attached to the surface of the flowcell were exposed to a continuous supply of gentamicin that may have surrounded the cells to varying extents as biofilm accumulated. As cells grew and divided in the different types of culture environments their exposure to gentamicin changed. Planktonic cells divided and separated from one another whereas sessile cells accumulated around one another. This accumulation of cells, as well as the possible accumulation of extracellular material, may have provided adjacent cells some degree of protection from exposure to gentamicin. The physiology of cells in different locations within a biofilm or bacterial colony could also be quite different from that of planktonic cells. These differences in physiology can also affect a bacterium's susceptibility to antibiotics (Stewart 1999).

Isolation and Sequencing of the Putative *sigs*

A plasmid reconstruction technique was used to successfully isolate the synthetic operon, together with some of the upstream chromosomal DNA, out of the bacterial chromosome of the eight isolates. The chromosomal DNA segment was then amplified in *E. coli* as part of the recovered plasmid. This amplification helped to generate a higher concentration of template for sequencing.

Repeated attempts to sequence three of the eight isolates (numbers 1, 6 and 7) did not produce sequence of good quality. The plasmid reconstruction technique was tried again for these three isolates, but still the results remained . The restriction digest patterns of the plasmids from these isolates have apparent similarities (Figure 12). These similarities suggest that the three isolates were siblings of one selected clone. The restriction patterns of these plasmids were also distinctly different from those of the other isolates and the control plasmid. This difference, together with the failure of the sequencing attempts, may indicate that the plasmid DNA within the area of homology to the *sacB* primer was altered in some way that prevented proper priming for the sequencing PCR reaction. Mutations in DNA sequence such as deletion, base substitution, or recombination may have caused the loss of a restriction site.

After sequence was generated for each of the other five *sigs*, two sets of identical sequences were noted. This left three unique sequences recovered from the selection system. Though far more sequences were anticipated, the fact that sequences were recovered, and that two sets of replicates were identified, provided evidence that the techniques developed in this study can be used to identify genes with selected expression characteristics. The identification of two genes which were only about one kilobase apart in the *P. aeruginosa* chromosome was also encouraging. The 16S rRNA gene (*sig 23*) may be expressed as part of the same transcript with the tRNA gene identified (*sig 35*). The fact that

two of the three sequences identified appear to be in one operon out of the entire genome of *P. aeruginosa* implies that the selection of those sequences was not simply random.

### Identity and Regulation of *sig 2*

The length of sequence surrounding the *sig 2* sequence is interesting for many reasons, though the identity of the promoter driving expression of the synthetic operon is unclear. Homology searches of the amino acid sequences of the ORFs (open reading frames) upstream of *sig 2* identified proteins involved in the synthesis of an adhesin and of fimbriae. Fimbriae, or pili, are known to be produced by *P. aeruginosa* when it begins growing on a surface (O'toole 1998a). The pili of *P. aeruginosa* are also known to contain an adhesin (Irvin 1989). Thus, the fimbriae and adhesin homologs would be obvious candidates for surface-induced genes. However, these two genes are more than five kilobases upstream of the *sig 2* sequence. A promoter this far upstream is unlikely to be effective in driving the expression of *sig 2*.

The most immediate upstream ORF with the same polarity as the plasmid insert was found to be reasonably homologous to a capsular synthesis regulator protein. The RcsB protein is a positive regulator of genes involved in the synthesis of the capsular polysaccharide colanic acid of *E. coli*. In *P. aeruginosa*, some studies have reported that the extracellular polysaccharide alginate may be up-regulated following attachment of the cells to a surface (Davies 1995, Hoyle 1993). The *rscB* homolog would accordingly also be a good candidate for a surface induced gene. However, the similarity between colanic acid and alginic acid is less important than the overall significance of this ORF as a regulatory protein. Interestingly, most of the homologs identified by the BLAST searches of ORFs around the *sig 2* sequence are known or presumed to function in genetic regulation.

The ORFs that were identified on either side of the *sig 2* sequence are in the opposite orientation to the direction of the synthetic operon when it was inserted at the site.

Consequently a promoter driving their expression could not initiate the production of active SacB or AacC1 proteins. The two genes directly upstream of the *sig* sequence are both homologs of genes in *Myxococcus xanthus*, a bacterium that produces fruiting bodies during surface-associated growth. One of the *M. xanthus* homologs identified was the *csgA* gene. This gene encodes a protein that is found on the surface of cells and is involved in sensing cell to cell contact. The extracellular signaling pathway is referred to as C-signaling and is required for the coordinated developmental process of fruiting body formation (Lee 1995). The existence of a protein in *P. aeruginosa* that is homologous to the CsgA protein would be interesting to investigate. However, the existence of a theoretical ORF of this type does not necessarily indicate that such a protein exists in the species.

The other *M. xanthus* homolog is the *safA* gene, an antimicrobial synthetase gene. This gene is involved in the production of a saframycin antibiotic. The saframycin family of antibiotics is interesting in that these metabolites are not only bacteriocidal to a wide range of species (Lown 1982), but are also known to exhibit antitumor activity by targeting specific DNA sequences (Ishiguro 1978).

One of the aspects of the *P. aeruginosa* DNA sequence that is also particularly interesting is that it aligns quite well with the *safA* sequence over a length of almost one kilobase. Many other sequence comparisons only exhibit homology over a few hundred bases, but this comparison shows a 46% similarity and 31% identity of amino acid residues over a 995 amino acid stretch. This length over which the similarity holds is one reason for the high probability score ( $10^{-111}$ ) generated by the BLAST program. The majority of all other sequences that were identified by the BLAST program were also antimicrobial synthetase genes of various types.

The amino acid sequence of the ORF at the 5' end of the *safA* homolog was found to be 70% identical to a transcriptional negative regulator. This homology was only described

over a sequence of 62 amino acids. Consequently, the probability score this comparison generated was only  $4 \times 10^{-18}$ , considerably less than other scores. This type of sequence is known to generate a helix-turn-helix motif that binds DNA. Due to the high degree of identity for this sequence, the ORF most likely generates a protein with regulatory function.

The possibility of an antisense RNA promoter driving the expression of the synthetic operon has been proposed by other investigators working with IVET systems (Camili 1995, Valdivia 1996, Valdivia 1997). In these cases, as is the situation with the *sig 2* sequence, the polarity of the ORFs surrounding the identified sequences was opposite that of the genes of the synthetic operon. An antisense RNA generated at this location upstream of the *safA* homolog might effect the translational regulation of that ORF by base pairing with the ribosomal binding site of the mRNA.

To examine the possibility of the existence of an upstream promoter with the same orientation as the *sig 2* sequence, a search was performed using the most conserved nucleic acid bases of the -35 and -10 sequences of *E. coli* (TTGNNN...N(16)...NANNNT). This sequence was found within the *sig 2* sequence itself. This sequence motif was also searched for throughout a 28kb region around the *sig 2* sequence. The promoter-like motif was identified only eight times, four of which were at sites directly upstream of identified ORFs. The existence of such a motif does not necessarily mean that the sequence functions as a bacterial promoter. The sequences of bacterial promoters, even within *E. coli*, are quite variable. This variation prevents the conclusive identification of a promoter, yet allows the possibility that the sequence identified upstream of the *sig 2* sequence is a functional promoter.

The last ORF shown in Figure 13 is approximately 2kb downstream of the *sig* sequence and is in the same transcriptional orientation. This ORF has high amino acid homology (probability score of  $10^{-131}$ ) to the StyD protein of *P. fluorescens*. The StyD

protein is an phenylacetaldehyde dehydrogenase that functions in the degradation of styrene. A styrene catabolism gene initially seemed an unlikely candidate for a surface induced gene compared to the other putative genes in the area. Interestingly though, the petri plates used in all phases of this study were made of polystyrene. The question therefore arose considering whether small amounts of styrene could dissolve in the agar medium used in the selection process and induce the expression of the synthetic operon. At least two sources of information tend to dispute the above idea. First, the *styD* homolog lies approximately two kilobases downstream from the *sig* sequence. The only ORF in the intervening sequence is oriented in the opposite direction. Since no ORF with correct orientation could be identified for the intervening space, a section of DNA sequence in that gap was entered into a BLAST search. This search did not yield any identifications of sequences with probability scores more significant than 0.5. Thus, if a promoter within , or upstream of, the *sig 2* sequence was driving the expression of the *styD* homolog, the mRNA transcript of the intervening space would seem to be an unusually long sequence of unproductive message. Second, research on the genes of the styrene catabolism pathway in *P. fluorescens* indicated that *styD* exists in an operon downstream of *styA*, *B* and *C*. These three genes are proposed to code for a hydrolase, an oxygenase and an isomerase, respectively, all of which are required for the degradation of styrene (Beltrametti 1997). The absence of significant homology between the DNA sequence downstream of the *sig 2* sequence and that of *sty A*, *B* or *C* indicates that the native promoter of this operon is probably also absent.

#### Identity and Regulation of *sigs 23* and *35*

The identity of the genes immediately upstream of the synthetic operon in isolates 23 and 35 were unambiguous. Two hundred nucleotide bases of sequence generated by the sequencing reactions were used in searching the NCBI database. In both cases the

sequences had greater than 95% identity to sequences within what appeared to be an rRNA operon of *P. aeruginosa*. The putative *sig* of isolate 23 was found to be a 16S rRNA gene. The putative *sig* of isolate 35 was determined to encode tRNA for isoleucine or alanine. This tRNA sequence lies between the 16S and 23S sequences of the putative rRNA operon.

The regulation of these genes is much less clear than their identities. In *E. coli*, rRNA synthesis is known to be subject to at least two different types of regulation. Stringent control is dependent upon the intracellular availability of amino acids. If the cell is starved for an amino acid ribosomal activity ceases and the transcription of rRNA operons is inhibited. The level of rRNA within a cell also varies with the growth rate of the cell. Fast growing cells require more ribosomes for increased protein synthesis and consequently require increased production of ribosomal RNA (Gourse 1996).

The regulation of rRNA operons also involves levels of complexity still under investigation. The mechanism behind growth rate regulation remains unclear. However, available information suggests that most bacterial species have the ability to induce or repress rRNA synthesis in response to various environmental and physiological signals (Gourse 1996).

In support of rRNA as a surface growth induced gene are experiments involving protein synthesis in the initial stages of biofilm formation. O'toole and Kolter (1998b) demonstrated that the inhibition of protein synthesis by tetracycline exposure has a dramatic effect on biofilm initiation. Cells exposed to tetracycline during the first thirty minutes of colonization of a surface formed significantly less dense accumulations than cultures without tetracycline. When the cultures were allowed to attach for thirty minutes before tetracycline treatment the level of accumulation of cells on the surface was not significantly different from controls. These results were taken to suggest that newly attached cells may have increased protein synthesis requirements. However, the authors did not mention the possible effect of increased resistance to the antibiotic after bacterial attachment. If

planktonic cells are more sensitive to antibiotics than are sessile cells, then accumulation of cells from the planktonic state would be expected to be more dramatically inhibited by this antibiotic. In addition, their data clearly indicates that the extent of biofilm formation did not increase after thirty minutes. There was no difference in the level of biofilm formation in the controls that were allowed to grow for either thirty minutes or for sixty minutes. Thus, it is difficult to say how, or even if, biofilm formation is affected by the inhibition of protein synthesis in the second thirty minutes of incubation, since the level of biofilm formation itself was unchanged during this time period.

A study of *S. aureus* indicated that the amount of protein within the cell increased by roughly 35% in the first five hours of surface-associated growth (Williams 1999). However, these data were not a measurement of the concentration of protein, but of total protein in the cell. Since cell size also increased during this time period, an increase in total protein values is not surprising.

Differences in the regulation of protein expression could be expected during a transition from different growth states such as planktonic and sessile growth. However, regulatory changes in protein expression due specifically to this fundamental difference are more difficult to prove. Further complicating the question of whether rRNA genes could actually be surface induced genes is the possible effect of the experimental design itself. Since the *P. aeruginosa* cells were exposed to gentamicin as a consequence of being on the surface of the agar, the effects of gentamicin exposure in these experiments cannot be separated from the influence of surface-associated growth itself. Gentamicin acts by binding to and inactivating the 70S ribosome complex (Tangy 1985). If the cells that were put through the selection process did indeed increase expression of rRNA genes after the switch to surface-associated growth, the increase may have resulted from one or more different causes. The bacteria may have increased rRNA expression due to a concomitant increase in growth rate when they arrived in a new environment with increased availability of

nutrients. Alternatively, the bacteria may have had to increase rRNA expression in order to counteract the effect of gentamicin inactivating the existing ribosomes.

This hypothesis could also explain why the *sig* isolates exhibited less of an increase in their level of gentamicin resistance than did the wildtype. Data in Figure 11 suggested that the wildtype PAO1 was naturally about ten-fold more resistant to gentamicin when growing on the agar surface. In contrast, the *sig* isolates demonstrated only a four to eight-fold increased resistance to gentamicin when growing on a surface. If the isolates increased expression of the rRNA operon once they contacted the surface, they would also have increased expression of the gentamicin inactivating system of the *aacCI* gene. This increased expression level would not only result in more ribosomes but also less reactive gentamicin. Whether exposed to gentamicin on the agar surface or in liquid medium the isolates could have increased expression of the rRNA genes simply due to gentamicin exposure. The subsequent expression of the *aacCI* gene may then have offset the effects of this bacterium's intrinsic increased resistance.

The fact that the *sig* isolates did not display a level of gentamicin resistance that was more than ten-fold increased suggested that the genes upstream of the synthetic operon of these isolates may not actually have been induced by the transition to surface-associated growth. However, the control strains that exhibited constitutive expression of the synthetic operon provided support for the idea that the *sigs* were indeed induced by surface-associated growth. Every analysis of the constitutive expressing clone's level of resistance to gentamicin provided similar results. The constitutive expressing clones either exhibited no increased resistance to gentamicin when grown on solid medium compared to liquid medium, or exhibited only a two-fold increased resistance. The *sig* isolates always displayed a greater relative difference in resistance to gentamicin when grown on a surface versus in liquid than the constitutive controls displayed. These constitutive controls may be considered more appropriate for comparison to the *sig* isolates than the wildtype control.

The constitutive expressing controls all contained the synthetic operon while the wildtype did not. Thus, more evidence indicated that the *sigs* were indeed surface-induced genes.

### Improvements to the Experimental Design

If this work is to be validated some more accurate way to test the level of expression of the synthetic operon may be needed. The test of planktonic versus sessile expression should be as direct as possible in assaying gene induction and less time consuming and labor intensive than hybridization blotting.

Why the single copy *gfp* reporter did not function reliably during experiments of this study is unknown. However, further work by other individuals provided a likely explanation. Their experimentation with the *gfp* indicated that the gene may have been altered in some way that prevented proper fluorescence from the protein product (pers. comm.). The *gfp* gene was cloned from plasmid pCJ7 into a pUC18 vector with a constitutive promoter. When introduced into *E. coli* cells, the plasmid promoter should have initiated the production of sufficient amounts of GFP to make the cells fluorescent, as was seen with strain PAO1(pCJ4). However, the *E. coli* did not appear fluorescent. In contrast, when the original *gfp* was cloned from a previous source into the high copy pUC18 vector, cells containing this plasmid were noticeably fluorescent. These results indicated that the *gfp* of pCJ7, and therefore in all the recombinant clones of the pool, was either nonfunctional itself or produced a nonfunctional protein. These findings provided hope that the *gfp* reporter could be useful in future investigation.

Use of the *gfp* also holds much promise because other investigators have found it an effective reporter of gene expression. Valdivia, Falkow and Cormack developed the mutant *gfp* (Cormack 1996) used in this work for their work with an IVET system in *Salmonella typhimurium*. They sought to develop a system which utilized fluorescence-activated cell sorting (FACS) to identify acid-inducible genes and so developed a GFP that was optimized

for FACS analysis. They tested the mutant *gfp* as a promoterless gene fusion to library DNA of a plasmid-based system. After identifying specific clones with acid-inducible expression of the *gfp* fusions they made several chromosomal insertions of the *gfp* vector at the corresponding sites on the *S. typhimurium* chromosome. These chromosomal fusion strains demonstrated similar acid-inducible fluorescence induction, but lower fluorescence intensity than the plasmid vector fusions. The difference in the absolute fluorescence induction was assumed to be copy number dependent. Still, the increase in fluorescence, which was below the four to twenty-fold increase of the plasmid containing clones, was of a magnitude detectable by the FACS (Valdivia 1996). These results suggest that the sensitivity of FACS analysis may permit efficient screening of the *P. aeruginosa* chromosomal recombinants already generated.

The use of GFP in conjunction with FACS would correct or improve at least two major deficiencies of this study. First, analysis of fluorescence would obviate any dependence upon potentially confusing gentamicin resistance characteristics. Though the absolute fluorescent intensity of a particular clone can be affected by several characteristics of the GFP itself, the measure of fluorescence-induction is much more direct an indicator of gene induction than is gentamicin resistance or sucrose sensitivity. Testing the fluorescent intensity of a clone could also be done without the need to expose a clone to different selective factors like gentamicin or sucrose. The cells would be exposed to UV light for a short time rather than being continuously exposed to an agent like sucrose or gentamicin that so dramatically affects cell physiology.

The use of FACS would also make analysis of gene expression much more unbiased. The selection process itself could be performed by the FACS. The recombinant pool already constructed could be grown in planktonic culture and then analyzed by FACS. Only those cells with no, or low, fluorescence would be collected. These cells would then be grown in the flowcell for a period of time and later removed by scraping the surface. These

resuspended cells could then be run through the FACS and cells with the highest level of fluorescence selected. Later, individual clones could be tested in monoculture. The level of induction of the synthetic operon would be determined as a value of fluorescent intensity. In this way, the data would be generated by the FACS; avoiding the potential bias of manual screening, and providing a specific, nonincremental, indicator of promoter activity.

In order to identify genes with low or transient expression of the synthetic operon a plasmid-based system rather than a chromosomal insertion scheme may be required. Lee and Camilli (1999) have found that the important *in vivo*-induced virulence genes of *Vibrio cholerae* are temporally expressed within an infant mouse model. They utilized an elegant IVET system based on the expression of a recombinase gene fusion. A strain of *V. cholerae* was developed with a chromosomal insertion containing a tetracycline resistance cassette flanked on both sides by resolvase substrate sequences. The recombinase reporter gene was then randomly inserted into the chromosome of this strain. When the recombinase gene fusion was expressed, the recombinase enzyme acted upon the tetracycline insert and removed it from the chromosome (Camilli 1995). Thus, strains which expressed the synthetic operon even transiently could be identified by their newly developed sensitivity to tetracycline.

This system is now referred to as RIVET, for recombinase-based *in vivo* expression technology. The technology should yield new insight into *V. cholerae* genetics, but is not expected to function in *P. aeruginosa*. The use of a plasmid-based *gfp* vector in *P. aeruginosa* may function to help identify transiently expressed genes.

Since GFP is relatively stable (Tombilini 1997) it may be possible to isolate clones that only expressed the synthetic operon at a high level for a short time. Several plasmid copies of the *gfp* gene would help ensure that sufficient message was produced to be detected later. In order to identify genes which are transiently expressed from those that would be continually expressed by sessile cells, unstable GFP could also prove useful. GFP

constructs have been made that are rapidly degraded in the cell (Andersen 1998). This type of unstable protein could be used to investigate what genes are expressed at different time periods after surface colonization.

If selection were based upon the expression of the *gfp*, then *sacB* and *aacCI* might be used to confirm the induction of particular selected clones. The sucrose or gentamicin could be added to the flowcell system after a certain period of attached growth. Viable cell counts could then be determined as previously proposed.

#### Use of the Flowcell System

Though selection in the flowcell system did not prove successful this was probably because of a lack of efficient negative selection by *sacB*. In the allelic exchange work mentioned previously a single copy of *sacB* was cloned behind a strong constitutive promoter. The sensitivity of *P. aeruginosa* to *sacB* expression from a less strongly induced promoter may not be as dramatic. Tests performed on subsequently isolated clones showed that some isolates which grew at gentamicin concentrations of 150 to 200µg/ml on an agar plate were also able to grow on sucrose agar. This surprising result indicated that while a particular level of *aacCI* expression generated a relatively high resistance to gentamicin, the same level of expression of *sacB* did not lead to sucrose sensitivity.

In general, the flowcell system developed in this study worked well and promises to be quite useful in examining gene expression *in situ* on various material surfaces. The flowcell system is considered preferable to the agar surface for several reasons. The flowcell can be adapted to more closely model medical biofilms on implant devices. A flowing system, as compared to an agar plate, more closely resembles systems of industrial and medical importance such as a pipe or waterline, or even a mammalian circulatory vessel. In addition, the fact that components can be added to the flowcell system at various time points makes the system more convenient and desirable than the rather static agar plates. If bacteria are

cultured in the flowcell the developing biofilm can also be examined microscopically at various time points.

If a plasmid-based system were used, and *gfp* expression were induced at high enough levels, spatial and temporal patterns of expression might be investigated. Valdivia and Falkow describe the detection of fluorescence within eight minutes of induction of their mutant *gfp* in *E. coli* (Cormack 1996). If the *gfp* performs similarly in *P. aeruginosa* it may be possible to view cells growing within the flowcell over time and see when and where gene induction first occurs in the newly forming biofilm.

### Conclusions

A genetic selection strategy to identify genes that are induced during surface-associated growth of *P. aeruginosa* was developed and tested. The utility of the *sacB* and *aacCI* genes in the investigation of gene expression during biofilm formation was evaluated. If these two genes are expressed strongly enough the negative and positive selection they provided did function as required. However, the selection process may not be as effective as was anticipated in identifying genetic expression changes that are less dramatic. For assessing the level of expression of the synthetic operon, neither the effects of *sacB* nor *aacCI* expression produced results that were irrefutable. Using the methods tested in this work, the utility of the *gfp* gene was also unconvincing. However, the use of a different *gfp*, or the *gfp* in a plasmid-based system, may correct some of the deficiencies identified during this study.

The flowcell system created, and the techniques developed for flowcell experiments, should prove useful for future work. Though the flowcell system did not function to improve the outcome of the selection process itself, the apparatus did prove quite useful. The flowcell system should be amenable to many different applications and types of selection.

Conclusive proof that the putative *sigs* were induced by growth on a surface was not produced though many trials using several different procedures were undertaken. Two of the three sequences recovered appeared to be part of an rRNA operon. The function of the third sequence was unclear. Still, there was evidence to support the idea that the genes identified in this work were induced by surface-associated growth. Experiments to examine *sig* induction by evaluating gentamicin resistance using method #1 demonstrated that the *sig* isolates were more resistant to gentamicin when growing on an agar plate than when growing in liquid medium. The control strains displaying constitutive expression of the synthetic operon never differed in their sensitivity to gentamicin whether grown as planktonic or sessile cells. The *sig* isolates were not consistently more fluorescent when attached to a surface but were never more fluorescent when growing planktonically.

The results of *aacCI* induction testing by method #2 suggested that the *sig* isolates may not have been more resistant to gentamicin when growing on a surface than was the wildtype PAO1, but these results did not prove that the synthetic operon was not induced in the isolates. The effects of the induction of the synthetic operon as determined by gentamicin resistance may have been masked by the biofilm bacterium's intrinsic increased resistance to antibiotics. Therefore, though this experiment did not indicate that the putative *sigs* were more induced during surface-associated growth, it did not provide conclusive evidence to the contrary.

Although assessment of *sig* induction was not definitive, the power of the selection system itself was indicated by commonalities between the *sig* sequences. Each of the eight isolates had something in common with at least one other isolate. Numbers 2 and 36 were found to be replicates, as were numbers 23 and 34. Numbers 1, 6 and 7 all had similarities in their plasmid restriction patterns. The sequences of the *sigs* of numbers 23 and 35 appeared to be part of the same operon. These findings indicated that the selection system was effective in identifying genes that were similarly induced or regulated.

Application of the selection strategy did not yield the results anticipated, but much was accomplished as a foundation for future work. The genes identified in this study cannot be described definitively as being induced by the transition to sessile growth, but the selection process certainly did select for genes with a certain type of expression. Consequently, sequence was successfully recovered and analyzed to determine the identity of the genetic promoters that drove the expression of the synthetic operons.

The selection system developed in this study might be modified and improved to provide a powerful technology for investigating genetic regulation during biofilm development by *P. aeruginosa*.

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