Do Not Fear Commitment: The Initial Transition to a Surface Lifestyle by Pseudomonads

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Abstract

This chapter aims to describe the early stages of biofilm formation, particularly on abiotic surfaces, by focusing on *Pseudomonas aeruginosa*. Specifically, we will dissect the early steps in the establishment of a multicellular community: (i) translocation to the surface from a free-swimming planktonic lifestyle, (ii) initial or reversible attachment, and finally (iii) irreversible attachment. We will also compare the mechanisms used by *P. aeruginosa* to its related fluorescent pseudomonad cousins, *Pseudomonas fluorescens* and *Pseudomonas putida*. We argue that, for pseudomonads, irreversible attachment is the first committed step in the transition to a biofilm lifestyle.

Introduction

It has been understood for some time that the majority of bacteria exist in nature attached to a substratum (see Eberl *et al.* and Hogan, this volume), and more recently it has been suggested that a subset of bacterial infections are the result of surface attached multicellular bacterial communities (Costerton *et al.*, 1999; Vinh and Embil, 2005). One can readily envision the myriad of advantages afforded to a bacterium attached to surface. For example, nutrients are typically most abundant within relative proximity to a substratum (Paul and Clark, 1989). Furthermore, bacteria growing in a biofilm gain protection from protozoan and bacterial predator grazing, phage infection and a variety of other environmental insults (Matz *et al.*, 2004; Patel, 2005; Picioreanu *et al.*, 2000; Sutherland *et al.*, 2004; Matz, this volume).

Relatively recently, molecular and genetic techniques have been applied to the study of this phenomenon. Several model organisms for the study of biofilm formation have emerged and among the most studied microbes is the ubiquitous Gram-negative bacilli *Pseudomonas aeruginosa*. *P. aeruginosa* has been previously noted for its ability to thrive in a vast array of environments ranging from the rhizosphere to medical facilities (Bloemberg and Lugtenberg, 2001; Rahme *et al.*, 1995; Stoodley *et al.*, 2005). This ubiquitous nature is partially due to the relatively large coding capacity of the *P. aeruginosa* genome allowing for both metabolic plasticity as well as the ability to rapidly sense the environment and alter gene expression accordingly, which is attributed to this microbe's myriad of two-component regulatory systems (Stover *et al.*, 2000). There is growing evidence that the ability to attach to surfaces and form complex multicellular communities is also a major factor contributing

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to the metabolically diverse nature of *P. aeruginosa*. It is likely that these features are interrelated, thereby increasing the fitness of this bacterium. The growing attention regarding *P. aeruginosa* and its ability to form biofilms has begun to reveal that the formation of these communities follows a fairly defined series of steps and may be analogous the developmental pathways previously reserved for more complex organisms (O'Toole *et al.*, 2000).

A look back

The phenomenon we currently refer to as biofilm formation has been described in the literature for some time. This should come as little surprise since biofilms are observable to the naked eye. Indeed, when Anthony van Leeuwenhoek first took scrapings from between his teeth and observed them under his crude microscope he observed small "animalcules" embedded in an organic substance (van Leeuwenhoek, 1683). Today we know that these scrapings were oral communities rich in a variety of bacteria, including *Streptococcus* spp., embedded in a polymeric matrix secreted by the microbes (Kolenbrander *et al.*, 1999; Kolenbrander *et al.*, this volume). With the improvement in microscopy over the next several centuries came further study of bacteria and their ability to attach to surfaces. Work carried out by several early pioneers of biofilm formation, including Henrici, Zobell, Anderson, Waksman and many others, demonstrated through a variety of techniques that bacteria from a range of environmental niches were capable of attaching to a surface and proliferating (Henrici, 1933; Waksman and Vartiovaara, 1938; Zobell and Allen, 1935; Zobell and Anderson, 1936; Zobell, 1937, 1943).

Work by Zobell in 1943 demonstrated not only that a large number of bacteria in seawater were capable of attaching to a surface and preferred to do so, but that this attachment seemed to consist of two distinct stages (Zobell, 1943). He noted that when glass slides were incubated with the bacteria for a short period of time the attached bacteria could easily be washed off. However, if the slides were left in the seawater for somewhat more extended periods of time the bacteria were much more difficult to remove through simple washing.

The concept that bacterial attachment to a surface consists of two stages, one in which the bacterium is weakly attached to the surface followed by a more secure form of attachment, is further supported in work by Marshall and colleagues in 1971 (Marshall *et al.*, 1971). Their work using both *Achromobacter* and *Pseudomonas* demonstrated that both bacteria undergo an immediate reversible attachment followed by a time-dependent irreversible attachment. Furthermore, their studies showed that the *Pseudomonas* species used in these studies attached via the pole of the cell and could rotate freely about their pole during the reversible stage of attachment (Figure 3.1). This rotation ceased once the bacterium had made the switch to irreversible attachment.

Work by Lawrence and colleagues demonstrated that there were two distinct stages of early adhesion in *Pseudomonas fluorescens* (Lawrence, 1987). Utilizing several different microscopic methods they observed that the bacteria attached initially by one of their poles wherein the bacteria were capable of rotating freely around this pole. This polar attachment was observed to be reversible as some bacteria were seen to re-enter the planktonic phase. Following reversible attachment, the bacteria switched to a more secure interaction with the surface by attaching along their longitudinal axis, followed by microcolony formation

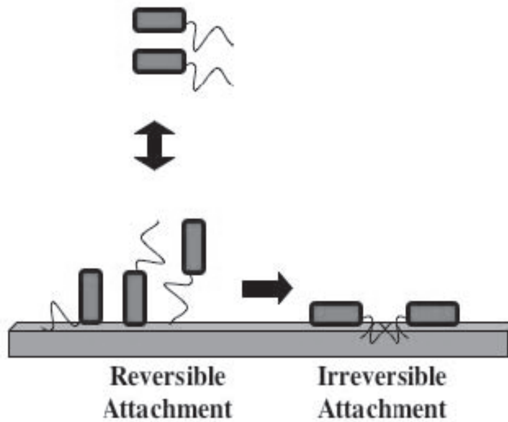

Figure 3.1 Initial attachment to a substratum. Pseudomonads translocate to the surface and adhere via a pole. Which pole, and which part of the cell directly contacts the surface, is unknown. Several possibilities are illustrated here. Bacteria subsequently adhere via the long axis of the cell in a process known as irreversible attachment. Irreversible attachment is the first committed step in the transition to a biofilm lifestyle.

and subsequent biofilm maturation. From these early findings the models of adhesion and biofilm formation arose: bacteria swim to a surface whereupon they initiate attachment through polar, reversible attachment, followed by irreversible attachment, which we will argue below, is the first committed stage of biofilm formation.

Translocation to the surface

One can readily imagine that before a bacterium can attach to a substratum, it must first locate the surface and be capable of translocating to that surface. Approaching the surface may be more difficult than it appears on its face because in order to attach, a bacterium must not only move towards the substratum, but also be capable of overcoming the repulsive forces found at this liquid-surface interface. Both the substratum and bacterial surfaces are often of a similar charge, thereby resulting in electrostatic repulsion between the microbe and the surface to which it may attach. Hydrophobic interactions can also have an impact on initial attachment as these forces can act either as a repulsive or attractive force thereby altering rates of attachment (An and Friedman, 1998; Bos *et al.*, 1999).

Bacteria can overcome repulsive forces as they approach the surface in several ways. The bacterium may move passively towards the surface, subject to the will of gravity, Brownian motion or the simple flow of the liquid environment within which it finds itself. Alternatively, the bacterium can actively translocate to the surface using a variety of swimming appendages. As discussed in greater detail below, there are a large number of flagellated bacteria that form robust biofilms, including *P. aeruginosa*. However, many bacteria lacking any form of swimming organelle are quite capable of forming robust biofilms, as is the case for *Staphylococcus aureus* (Caiazza and O'Toole, 2003; Cramton *et al.*, 1999;

Marrie and Costerton, 1984), suggesting that for these organisms, active translocation to the surface during biofilm formation is dispensable.

P. aeruginosa expresses a single peritrichous flagellum and exhibits robust swimming motility under most conditions. Several studies have implicated a role for swimming motility in biofilm formation. One of the earliest studies of the genetics of biofilm formation found that *P. aeruginosa* mutants lacking a functional flagellum were incapable of forming a biofilm in minimal medium supplemented with glucose and amino acids under static conditions (O'Toole and Kolter, 1998b). In these studies, pseudomonads were inoculated into wells of a 96-well microtiter dish containing minimal glucose plus amino acids medium and incubated under static conditions for up to 24 hours. Following incubation, the individual wells were washed with water and stained with crystal violet, followed by subsequent solubilization of the stain with ethanol to quantify the attachment. Mutant strains lacking a flagellum demonstrated a severe defect in attachment in this system. Microscopic studies confirmed the staining data (O'Toole and Kolter, 1998b).

Work by Ramsey and Whiteley (2004) further supported this notion, only these investigators demonstrated a role for the flagellum in a somewhat more dynamic environment that also incorporated shear stress. In this study, the author's biofilm assay was performed as described above, utilizing a similar minimal medium containing glucose and amino acids, but they also added glass beads to the wells. Rather than incubating the assay plates under static conditions, the microtiter dishes were shaken at high speed, thus the addition of the glass beads allowed for both aeration of the culture and provided a source of shear stress. This model further emphasized the role for flagella in biofilm formation by demonstrating that the cell appendage is not only important in a static environment but also in environments subjected to shear stress. Several genetic studies of soil pseudomonads also demonstrated that a functional flagellum is required for attachment and subsequent biofilm formation on both biotic and abiotic surfaces (De Weger *et al.*, 1987; DeFlaun *et al.*, 1994).

How might the translocation of bacteria to a substratum to initiate reversible attachment be regulated? This precise question has not been directly addressed, however because the regulation of flagellar assembly and function has been studied in detail, and is quite complex (Soutourina and Bertin, 2003), this entire regulatory cascade could be included in a discussion of the regulation of biofilm formation.

One confounding issue regarding interpreting the role of the flagellum in biofilm formation relates to the fact that this structure has two potential roles: as a propeller used to move the cells through a liquid environment and an adhesive appendage (Arora *et al.*, 1998; Landry *et al.*, 2006; Lillehoj *et al.*, 2002). Recent work has allowed us to begin to address each of these roles individually. Using a bioinformatic approach, Toutain *et al.* (2005) and Doyle *et al.* (2004) identified two pairs of genes with sequence similarity to *motAB* of *E. coli* in the *P. aeruginosa* genome. MotAB comprises the stator, the stationary component of the flagellar motor. Both groups showed that in regards to swimming motility, the two sets of motor genes, *motAB* and *motCD*, were redundant—mutations in either stator alone revealed no measurable effect on swimming (Doyle *et al.*, 2004; Toutain *et al.*, 2005). Interestingly, in a more recent study, Toutain and O'Toole (unpublished) showed that mutations affecting either set of stators resulted in a significant reduction in biofilm formation despite the absence of any discernable defect in swimming motility. Disruption of both

sets of stators further exacerbated this biofilm formation defect under static conditions, thus demonstrating that not only is the presence of a flagellum required, but it must also be functional to initiate biofilm formation under these conditions (Toutain and O'Toole, unpublished). Taken together, the lines of evidence above certainly support the notion that for a flagellated Gram-negative bacterium such as *P. aeruginosa*, actively translocating to the surface facilitates attachment to a greater degree than relying on a passive mechanism, and that flagellar-mediated motility plays a key role in early biofilm formation.

Interestingly, there is very little evidence suggesting a role for chemotaxis in biofilm formation. Most studies have focused primarily on the classic *che* mediated chemotactic pathways, leaving room for less or uncharacterized chemotactic mechanisms. Work by Pratt and Kolter (1998) demonstrated that in *E. coli* disruptions in the *che* chemotaxis system did not result in a loss of biofilm formation in a static assay. Similarly, large genetic screens for biofilm mutants in *P. aeruginosa* and *P. fluorescens* did not yield any strains with mutations in the chemotaxis gene cluster that is most similar to *E. coli* (O'Toole and Kolter, 1998a, 1998b).

There are several lines of apparently contradictory data concerning the role of flagellar motility in biofilm formation. Microscopic studies by Klausen *et al.* (2003) demonstrated that under flowing conditions, in medium containing citrate as the sole carbon source, *P. aeruginosa* flagellar mutants were quite capable of forming biofilms albeit with gross morphological differences from those formed by the wild-type bacterium. Similar results were observed for strains lacking either or both flagellar stators (Toutain and O'Toole, unpublished data). These results seem at odds with those outlined above, however several differences should be noted. First, the biofilm formed by the wild-type bacterium when grown on citrate is significantly different morphologically from that formed in medium supplemented with glucose, and furthermore, biofilms formed under static conditions are morphologically distinct from those formed under flowing conditions. Furthermore, there is a significant difference in the time frame of these two assays—typically the static biofilm assays are incubated for 8 hours while those experiments using the flow conditions were incubated for 24 hours or more. Finally, it is also important to consider that the flagellum also plays a role in surface-associated swarming motility. How flagellar-mediated swarming contributes to biofilm formation is still an open question. Taken together, we believe that the data presented here can be interpreted to mean that there is a more active role for flagellar-mediated motility in the formation of a biofilm than simply getting the bacterial cell to the surface.

Reversible attachment

Once the bacterium has overcome the various repulsive forces found at the surface/liquid interface, it must attach to the substratum. As is quite evident from several early studies, this attachment consists of two distinct steps: an initial and reversible attachment defined by a relatively unstable, polar adhesion event and the ability of the bacterium to rapidly return to the liquid environment, followed by a more stable irreversible attachment via the long axis of the cell (Lawrence *et al.*, 1987; Marshall *et al.*, 1971; Zobell, 1943). While this phenomenon has been noted in the literature for several decades, in depth investigation of the molecular mechanism of reversible and irreversible attachment has been lacking.

Recent microscopic analysis of reversible attachment has suggested that the bacterium is indeed attached in a polar manner and may be attached via its flagellum (Caiazza and O'Toole, 2004; Sauer *et al.*, 2002) as reversibly attached bacteria appear to rotate about their polar axis while attached to the substratum. Direct proof that the flagellum is indeed responsible for this initial attachment has been elusive thus far as strains lacking these structures mutants are also impacted in their ability to translocate to the surface as previously mentioned.

It has been postulated by a variety of groups that during the initial attachment stage the bacterium may be sensing the local environment of the surface prior to committing to the surface (Caiazza and O'Toole, 2004; Hinsa *et al.*, 2003; Lawrence *et al.*, 1987; Marshall *et al.*, 1971; Zobell, 1943). This is a fairly sensical assumption as this affords the bacterium the ability to either return to the liquid environment, in the case of an unsuitable environment, or further commit if the surface and the local environs are indeed suitable to promote initial biofilm formation and maturation. If the transition from reversible to irreversible attachment is actively controlled, there must presumably be set cues leading to commitment, however, few if any of these cues have been identified but several candidates have been proposed (Marshall *et al.*, 1971). These attachment signals may include sensing carbon availability, carbon nitrogen ratios, presence of other bacteria or the surface itself.

Work by Otto and Silhavey (2002) demonstrated that the *cpx* signaling pathway was not only required for surface adhesion but also played a key role in signaling upon adhesion of *E. coli* to a surface under certain conditions. The *cpx* system had previously been shown to play a role in sensing perturbations and stresses within the cell envelope (Raivio and Silhavy, 1997). These experiments utilized a *lacZ* reporter system that served as a marker for induction of the *cpx* system. Upon adhesion to the surface a *cpx-lacZ* reporter system was induced and reached maximal expression 1 hour following attachment, while planktonic bacteria did not show this same level of induction. The model put forth would suggest that upon contact with the surface there is some form of perturbation of the cell envelope due to contact with the surface, the *cpx* system interprets this contact and relays the information resulting in increased expression of adhesins and subsequent biofilm formation (Raivio, 2005). Little is still known concerning the signals that mediate the decision to return to the planktonic stage or further commit to the surface. It seems likely that the bacterium is capable of receiving and acting upon various environmental cues, whether they be metabolic as would be the case for nutrient conditions, or mechanical such as sensing the surface.

How do pseudomonads mediate polar attachment to the surface? While a fundamentally important question, very little is known regarding this point. As described above, flagellar motility is thought to be important in pseudomonads for their translocation to the substratum. It has been postulated that the flagellar filament also serves as an adhesin. Furthermore, several lines of evidence demonstrate that very defined portions of the flagellum, and perhaps not the organelle in its entirety, play a key role in attachment of *P. aeruginosa* to various surfaces. Arora *et al.* (1998) demonstrated in *P. aeruginosa* that disruption of *fliD*, which codes for the flagellar cap protein at the tip of the flagellum, resulted in an inability to attach to mucin, while strains incapable of producing flagellin, the structural subunit of flagella could still attach to mucin. Further work by Landry *et al.* (2006) demonstrated that under flow conditions *fliD* mutants were capable of attaching to a glass surface

at a rate comparable to the wild-type bacteria. However, if the surface was coated with mucin, the *fliD* mutant was significantly attenuated in attachment while the *flgK* mutant, which lacks the flagellar filament, showed a dramatic decrease in attachment under both conditions when compared to the wild-type bacteria (Landry *et al.*, 2006). These data suggest that in addition to its role in motility, the flagellum, through FliD, mediates contact with some types of surfaces. However, the observation that flagellar mutants do indeed eventually attach and form a biofilm suggests that bacteria have other adhesive molecules on their surface to promote cell-surface interactions.

One obvious mechanism for promoting reversible attachment would be an outer membrane protein (OMP) that functions primarily as an adhesin, or alternatively, an OMP that may be a part of, for example, a secretion system with a secondary role in cell-surface attachment. Several OMP have been implicated as adhesins (Hinsa and O'Toole, 2004), including OprF in *P. aeruginosa*, which has been shown to play a role in anaerobic biofilm formation and adhesion to epithelial cells (Azghani *et al.*, 2002; Yoon *et al.*, 2002). Another potential adhesin is the LecB lectin, which is localized to the OM of *P. aeruginosa* (Tielker *et al.*, 2005). Mutating this fucose-binding protein results in a severe biofilm formation defect, however it is not clear where in the biofilm formation pathway this block occurs (Tielker *et al.*, 2005). The lack of any single, clearly defined adhesin required for reversible attachment might indicate multiple, redundant adhesins for attachment to a wide range of surfaces. Alternatively, there may be multiple adhesins whose primary function is to bind to one particular surface but with enough cross-specificity to make it difficult to identify these proteins via typical genetic approaches. The identification of adhesins required for polar interaction of bacteria during biofilm formation is an area that warrants further study.

It is also possible that LPS plays a direct or indirect role in reversible attachment. LPS structure appears to play a role in adhesion of pseudomonads, as mutants lacking O-polysaccharide are decreased in their ability to attach to a variety of surfaces. Work by DeFlaun and colleagues on the soil bacterium *Burkholderia cepacia* G4, a close relative of *P. aeruginosa*, identified a biofilm-defective mutant utilizing a sand column assay (DeFlaun *et al.*, 1999). It was found that this mutant strain, ENV435, demonstrated significant differences in the lipopolysaccharide O-antigen compared to the wild type suggesting that, indeed, lipopolysaccharide composition plays a role in attachment to abiotic surfaces (DeFlaun *et al.*, 1999). A role for LPS O-antigen in attachment was not entirely novel amongst pseudomonads as work by Dekkers and colleagues in 1998 had demonstrated that mutants of *P. fluorescens* WCS365 defective in O-antigen synthesis were defective for colonization of roots (Dekkers *et al.*, 1998). Because of the pleiotropic effects changes in LPS are likely to cause, it is not currently possible to develop any firm mechanistic models to explain why altering the O-antigen results in a biofilm defect.

Irreversible attachment: commitment to the surface

As stated previously there are a number of different studies that have described the phenomenon of reversible and irreversible attachment in a variety of different bacterial systems. Teleologically, one might postulate that bacteria would develop the ability to initially attach to a surface, sense the suitability of the local environment, and either commit to the surface via irreversible attachment or to re-enter the planktonic stage and seek out a more suitable

niche. This choice to "stay or go" is best typified by the observable change in orientation of the bacterium to the surface, from a polarly attached cell capable of rotating freely about its axis to a longitudinally attached cell that is firmly bound to the surface.

Work by Caiazza and O'Toole (2004) provided some of the first insight into the molecular mechanisms underlying irreversible attachment to a surface. These investigators demonstrated that a cytoplasmic protein of unknown function in *P. aeruginosa*, designated SadB, was required for the transition from reversible to irreversible attachment. Furthermore, the *sadB* mutant was severely defective for biofilm formation under static conditions irrespective of the carbon source present. This study further demonstrated that the biofilm formation defect of a *sadB* mutant was also observable under flowing conditions.

Upon close inspection of micrographs and time-lapse images of *sadB* mutants in a flow cell, no obvious defect in reversible attachment was observed, a majority of the *sadB* mutant bacteria were bound by their poles and rotated freely about the attached axis of the cell. In contrast, most wild-type bacteria under similar conditions were irreversibly attached via their long axis (Caiazza and O'Toole, 2004). How SadB mediates irreversible attachment is still not known. Interestingly, the gene coding for SadB is conserved amongst the pseudomonads begging the question of whether this system is integral for biofilm formation in all members of this genus.

Levels of SadB protein, as assayed by Western blot, are upregulated in strains carrying mutations in *rpoN*, which codes for an alternative sigma factor that regulates a large number of genes including those required for flagellar-mediated motility, and *fleR*, which is involved in regulation of flagellar biosynthesis (Caiazza and O'Toole, 2004). These data suggest that the regulation of irreversible attachment is co-regulated with flagellar-mediated motility and thus reversible attachment events.

Studies in *P. fluorescens* have identified another series of components required for the switch from reversible to irreversible attachment that utilizes a large adhesin, termed LapA, which weakly associates with the bacterial cell envelope, and a predicted ABC transporter encoded by the *lapEBC* genes (Hinsa *et al.*, 2003). Mutations in *lapBCE* are still capable of making LapA, but this protein is localized only to the cytoplasm and is not found outside of the cell. These data suggest that the LapBCE system is required for the secretion of the LapA adhesion. Hinsa (2003) identified the Lap system through a genetic screen to identify genes required for biofilm formation in a static assay. Mutations in the Lap system resulted in bacteria, that when observed microscopically, were incapable of progressing past the reversible stage of adhesion.

Adjacent to the *lapABCE* cluster is *lapD*, which is shown to also be required for irreversible attachment in *P. fluorescens* WCS363 and *P. putida* (Gjermansen *et al.*, 2005; Hinsa and O'Toole, 2006). LapD is an inner membrane protein, and strains lacking LapD have a significant decrease in the amount of LapA found associated with the cell surface (Hinsa and O'Toole, 2006). Therefore, it is possible that LapD participates in the secretion of LapA or its association with the outer membrane. Interestingly, LapA is not found in *P. aeruginosa* and a mutation in the ABC transporter of this microbe with the greatest sequence similarity to LapBCE has no biofilm defect on abiotic surfaces (Hinsa and

O'Toole, unpublished data), suggesting that these related organisms, while both capable of irreversible attachment, commit to the surface via distinct mechanisms.

Espinosa-Urgel and colleagues identified several genes critical for attachment of *P. putida* to corn seeds, and furthermore several of the mutants from this screen were deficient in attaching to abiotic surfaces as well. Of the eight genes identified, four of them encode proteins predicted to localize to the cell envelope suggesting that one or more of them may act as an adhesin during attachment (Espinosa-Urgel *et al.*, 2000). Furthermore, one of the proteins identified has sequence similarity to LapA, suggesting that this adhesin is also involved in irreversible attachment (Hinsa *et al.*, 2003).

Recent work by Overhage and colleagues demonstrated that the *pslA* gene, a member of the exopolysaccharide synthesis *psl* regulon in *P. aeruginosa*, played a role in initial attachment—approximately a third as many *pslA* mutants were attached to PVC as the wild type in minimal media supplemented with glucose (Overhage *et al.*, 2005). Furthermore, they demonstrated, using a P_{pslA}-*gfp* transcriptional fusion, that the *pslA* gene was induced upon attachment to the surface (Overhage *et al.*, 2005). These data are quite interesting, because in addition to the often-mentioned role of exopolysaccharide in the stabilization of the mature biofilm structure (Friedman and Kolter, 2004a, 2004b; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Vasseur *et al.*, 2005; Pamp *et al.*, this volume), this works suggests that exopolysaccharide may also play a role in early events in biofilm formation, such as irreversible attachment.

As mentioned above, the process of reversible attachment allows an individual bacterial cell to sample the surface, integrate the appropriate local environmental signals and either release from the surface or trigger the transition to irreversible attachment. Because irreversible attachment appears to be the first stable interaction with the substratum, we proposed that this step is the "first committed" step in biofilm formation. In part, the choice of the phrase "committed step," typically used within the context of metabolic pathways, was chosen to convey the idea that the initial step in surface commitment is part of a larger set of behaviors of which *P. aeruginosa* is capable. It is possible that *P. aeruginosa*, subsequent to reversible attachment to a surface, is able to initiate other surface behaviors, such as twitching motility or swarming in addition to biofilm formation. A greater understanding of the early steps in biofilm formation is needed to understand this surface behavior in the greater context of the lifestyle of *P. aeruginosa*.

Genomics and proteomics

While the switch from reversible to irreversible attachment is evident when observed microscopically, is this transition accompanied by changes in gene or protein expression in the cell? Recently, several studies have sought to determine the extent of gene expression changes during early stages of biofilm formation. Generally, the results of genomic or proteomic studies have demonstrated a profound difference in gene expression in cells attached to a surface compared with those grown in the liquid environment, and in one study, approximately 25% of all proteins were upregulated in 1 day old biofilms as compared to planktonic cultures (Sauer *et al.*, 2002; Sauer, 2003; Southey-Pillig *et al.*, 2005). Sauer and colleagues also tracked the changes in protein expression over several stages of biofilm formation, breaking the pathway into three distinct stages: maturation 1 and maturation 2 and

dispersion (Southey-Pillig *et al.*, 2005). This work compared the proteome of *P. aeruginosa* during these three stages as well as during planktonic growth and was able to demonstrate that a large number of proteins were differentially regulated during these stages and that a distinct set of proteins is expressed at each stage in biofilm maturation (Southey-Pillig *et al.*, 2005).

Work by Waite and colleagues explored the transcriptional profile of *P. aeruginosa* during logarithmic and stationary growth, and four different points in biofilm formation: 8, 14, 24, and 48 hours post inoculation, by growing *P. aeruginosa* on nitrocellulose membranes placed on LB agar (Waite *et al.*, 2005). The percentage of the genome differentially regulated depended upon the comparison made. For example, by 8 hours 0.8% of the genome was differentially regulated in the model biofilm used in this study compared to a log-phase planktonic culture, but close to 10% of the genes were differentially regulated when an 8 hour biofilm was compared to a stationary phase culture grown in the same medium (Waite *et al.*, 2005). However, the significance of these changes and whether they are a direct or indirect consequence of growing in a biofilm-like environment is unclear.

Few transcriptome- or proteome-based studies have been conducted that have focused on the switch from reversible to irreversible attachment. One study that has tackled this issue head-on comes from work in *Vibrio cholerae*. Moorthy and Watnick (2004) used genetic studies to indicate that there were differences between planktonic bacteria and bacteria which had formed a monolayer. We believe that this monolayer stage may be thought of as the functional equivalent of irreversibly attached bacteria. Furthermore, these investigators showed that *V. cholerae* could be locked in a monolayer stage by growing them in a medium that lacked monosaccharides (Moorthy and Watnick, 2004), thus providing an excellent tool for specifically accessing differences between planktonic and irreversibly attached bacteria. Moorthy and Watnick (2005) used microarrays to analyze differences between planktonic- and monolayer-grown bacteria and found 150 genes were differentially regulated (91 upregulated and 59 downregulated). These data suggest that even the early transition to irreversible attachment results in changes in the biology of the microbe compared to their planktonic counterparts.

Summary

While the majority of prokaryotic research has focused on studying planktonic bacteria grown in liquid media, it is evident that the majority of bacteria exist in nature attached to a substratum in what we now refer to as biofilms. This field has gained a fair amount of attention in recent years leaving one with the impression that it is a relatively new area of research, and while there have been many recent advances in our understanding of this process, history has proven that much of the early groundwork in the field performed in the 1930s and 40s is on the mark. Zobell's early recognition that there were two types of attachment, an immediate yet unstable attachment and a more secure, time-dependent attachment has been repeatedly confirmed in a variety of different organisms. With the advent of modern molecular genetic and biochemical techniques, this evidence has been augmented further with the identification of adhesins and regulatory proteins that regulate the switch from reversible to irreversible attachment. The large number of genes involved in the switch from reversible to irreversible attachment suggests that this is an important stage

in biofilm formation. In fact, we argue that this is the first committed step in the transition from a planktonic to biofilm lifestyle.

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