

Abstract

Recent advances in studies of biofilm systems have generated a wealth of novel information on multicellular prokaryotic biology and have established models for the formation of biofilms and the biology of their lifecycles. As a prelude to the subsequent chapters in this volume, this introductory article is aimed at identifying the contextual scientific and experimental framework for contemporary biofilm research programs, and addresses the strengths and weaknesses of some of the current key biofilm models. We will discuss whether or not a unique biofilm specific gene expression underpins our observations on biofilm structure and biology. Further, we will highlight the limitations inherent to current genetic and physiological analyses of bacterial biofilms, including the strengths and weaknesses of the molecular toolbox and the biofilm assays commonly employed. Moreover, the extent by which multiple parallel pathways of biofilm formation exist will be addressed, with reference also to applications for novel control strategies based on contemporary advances in studies of bacterial biofilms. The chapter will conclude by discussing the relevance of a consensus view of bacterial biofilm formation and biology.

Biofilm organization and differentiation

Bacterial biofilms are multicellular consortia in which cells are embedded in an extracellular matrix at close proximity to one another. Such consortia are generally studied to assess particular properties of biofilms attached to solid surfaces, but they occur also as multicellular aggregates, flocs and granules suspended in the aqueous phase in many habitats. Biofilms of these kinds can be accommodated by single species, but in most cases in natural as well as artificial systems, they are mixed species consortia, ranging from phylogenetically highly diverse communities (Juretschko *et al.*, 2002; Lyautey *et al.*, 2005; Taylor *et al.*, 2004) to those in which one or a restricted number of species will be numerically and functionally dominant (Tujula *et al.*, 2006).

Intriguingly, whether biofilms consist of one or a mixture of a range of species, they display similarities with respect to architectural features and behavioral responses. For example, the microcolony structure observed in established mature biofilms is strikingly similar across mono- and multispecies biofilms, across different habitats, as well as for different organismal levels (Lawrence *et al.*, 1991; O'Toole *et al.*, 2000; Sauer *et al.*, 2002). The latter applies to the similarities found between the multicellular consortia of well studied

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model bacterial systems, those of highly differentiated multicellular prokaryotes such as streptomycetes and myxobacteria, and colonial or sessile marine invertebrates.

Moreover, strong similarities in behavioral responses and functional features have been reported for mono- vs. multispecies biofilms, as well as across organismal levels on the evolutionary scale. For example, it is now appreciated that biofilms have unique differentiation events that generate subpopulations of cells with apparently defined tasks and hence sharing of labor in the biofilm population (Allesen-Holm *et al.*, 2006; Webb *et al.*, 2003). These events include different cells that build the different parts of the mushroom-like, or equivalent structures, of biofilms, and the generation of specialized dispersal cells or propagules for colonization of new surfaces (Caldwell *et al.*, 2000; Labbate *et al.*, 2004; Mai-Prochnow *et al.*, 2006).

The advances recently made in our understanding of biofilms systems whether well defined or of more complexity, derive in the main from a series of seminal studies on a restricted set of monospecies biofilm (Ghannoum and O'Toole G, 2004). The experimental approaches have preferentially been focusing on high resolution genetic and microscopic techniques. These studies have laid the foundation for our current models of bacterial biofilms, their formation and life cycle, and they have subsequently been extrapolated and assessed for more complex systems (e.g. Auschill *et al.*, 2001; Hope *et al.*, 2002). Specifically, it is now appreciated that biofilm formation can be defined as a process that consists of defined stages. Commonly, we recognize that these stages include: reversible and irreversible attachment; surface motility and initiation of microcolony (or alternative structural arrangements) formation; maturation, ageing and differentiation of microcolonies; and, finally, biofilm dissolution and generation of specialized dispersal cells. It is further appreciated from the research reported on our currently best defined model systems that these stages and structural rearrangements include physiological characteristics and phenotypic responses apparently supportive of suggestions for the presence of a unique biofilm biology.

Intuitively, the appearance of morphological arrangements uniquely observed for biofilm consortia, and the development of traits allowing for remarkable resilience against antimicrobials (Fux *et al.*, 2004; Hall-Stoodley *et al.*, 2004) or removal of biofilm cells by the predatory actions of protozoans and phagocytes (Bjarnsholt *et al.*, 2005; Matz *et al.*, 2005; Queck *et al.*, 2006), to name a few characteristics commonly observed for biofilms, would support the notion of a biology different from that of planktonically living bacteria. In further support of this, recent reports on transcriptomic and proteomic analysis of biofilm vs. free-living cells of different monospecies systems appear to lay a strong foundation for the existence of genes and proteins uniquely expressed by biofilm cells (Hume *et al.*, 2004; Sauer, 2003). For example, swarming, or coordinated population migration on surfaces, involves a defined differentiation event with distinct temporal stages in certain species (Eberl *et al.*, 1999; Hume *et al.*, 2004; Shapiro, 1988; Shapiro, 1998). Following the observation of apparent program-like responses in this and other specific multicellular surface dependent responses, it is logical to anticipate the involvement of a development program of which biofilm specific genes are expressed in a defined order reflective of a biofilm life cycle.

In the following sections we will address whether a unique program, and hence biofilm specific gene expression, underpins the structural and functional features discussed above,

and whether the interpretations made from contemporary molecular analyses of biofilm genetics and physiology accurately reflect the biology of these communities. The implications of our analysis of currently available data sets on biofilm gene expression and seemingly similar behavioral responses of biofilms or multicellular consortia of different organisms will also be explored. In addition, we will discuss whether multiple, parallel, pathways of biofilm formation and development of specific traits exists not only for different taxa, but also within a species. The implications of such parallel pathways for biofilm research and biofilm control measures will also be assessed. Finally, we will address strengths and limitations with current biofilm assays and conclude by discussing whether or not there should be a consensus view on how bacterial biofilms form and function.

Biofilm program and specific gene expression

Recent years have witnessed detailed mechanistic studies of biofilm formation in a range of species, including *E. coli*, *Vibrio cholera*, *Staphylococcus aureus*, and *Serratia marcescens* (Davey and O'Toole, 2000; Ghannoum and O'Toole G, 2004; Labbate *et al.*, 2004). For in-depth studies, one of the preferred model organisms is *Pseudomonas aeruginosa*. There are several good reasons for the many reports on biofilm biology in this organism. First, a number of molecular tools are available for this organism including commercial DNA arrays for the study of genome wide expression. Second, the genus *Pseudomonas* has simple growth requirements and tolerance to a wide range of temperatures (4°C to 42°C), significantly facilitating experimental efforts. Third, the organism represents a highly diverse and ecologically significant group of bacteria. Fourth, *P. aeruginosa* is also the most common bacterium found in nosocomial and life-threatening infections of immune compromised patients for which the development of effective treatments are desirable. The bacterium causes a variety of acute and chronic infections (as listed by Van Delden and Iglewski, 1998) and it is particularly known for its involvement in chronic infections of the respiratory pathways including, diffuse panbronchiolitis, bronchiectasia and cystic fibrosis (CF) (Frederiksen *et al.*, 1997; Hoiby, 1974; Kobayashi, 2005; Koch and Hoiby, 1993).

Much of what we know about *P. aeruginosa* biofilm formation derives from studies of monospecies biofilms established in flow cells. A number of specific features are required for the type of biofilm development we see in these flow cells: (1) Attachment to the surface involving specific adhesive proteins, (2) cell to cell binding involving proteins, extra-cellular DNA and polysaccharides in order for the cells to resist the hydrodynamic forces, and (3) cell motility to enable the cells to crawl on the surface (Ghannoum and O'Toole G, 2004). When the biofilm is fully developed, the bacteria are less likely to be displaced by physical processes including shear forces. Moreover, they are better able to withstand competition by invading organisms and they are more resilient to predation challenges (in nature or by host immune responses). In fact, biofilm cells show a remarkable tolerance to a variety of antimicrobial measures as compared to their, planktonic, growing, counterparts (Bjarnsholt *et al.*, 2005; Drenkard and Ausubel, 2002; Fux *et al.*, 2004; Hentzer *et al.*, 2003; Lewis, 2001). Most standard antibiotics will kill only minor parts of the population (Hentzer *et al.*, 2003b), and similar difficulties in eliminating biofilm bacteria have been documented for the host defense systems (Anderson *et al.*, 2003).

The characteristics detailed above have prompted researchers to suggest the existence of a specific, developmental, program which would operate to ensure proper biofilm formation and differentiation, and account for the tolerance phenotype characteristic for all biofilms. Such proposals are also born out of the formation of characteristic biofilm architectures, including the mushroom-like structures frequently observed in glucose irrigated flow cell biofilms. Recent analysis based on transcriptomics revealed that the bulk of *P. aeruginosa* biofilm cells even at the early stages express genes in a pattern that is reminiscent of gene expression seen in early stationary phase of planktonic cells (Hentzer *et al.*, 2005). This would in part explain the elevated tolerance to antibiotics since many drugs are relatively ineffective against slow or non-growing stationary cells. In addition, quorum sensing (QS) regulated gene expression also contributes to biofilm tolerance. Davies *et al.* (1998) demonstrated that a QS deficient *lasI* mutant of *P. aeruginosa* formed biofilms that were more susceptible to biocides. Likewise, biofilms formed by a *lasR*, *rhlR* double mutant of *P. aeruginosa* is much more prone to killing by tobramycin and hydrogen peroxide (Bjarnsholt *et al.*, 2005), as well as ciprofloxacin and ceftizidime (our unpublished results) and kanamycin treatment, shear forces, protozoan grazing and neutrophil phagocytosis, than biofilms formed by a wild-type counter part (Allesen-Holm *et al.*, 2006; Barraud *et al.*, 2006; Bjarnsholt *et al.*, 2005; Davies *et al.*, 1998; Hentzer *et al.*, 2002; Hentzer *et al.*, 2003; Matz *et al.*, 2004; Matz *et al.*, 2005; Queck *et al.*, 2006; Rasmussen *et al.*, 2005; Shih and Huang, 2002) suggestive of biofilm specific QS controlled genes. Remarkably, we have observed that the majority of QS regulated genes are expressed even at the early stages of biofilm formation.

However, the results generated by different research groups have failed to consistently identify a specific biofilm regulon. More generally, the genes classified by the different research groups as differentially expressed by biofilm cells (as compared to planktonic cultures present at different stages of the growth cycle) do not reveal the presence of a specific program operating in *P. aeruginosa* during conditions of sessile growth and biofilm formation. This failure to detect common themes in gene expression profiles in different reports on *P. aeruginosa* biofilms leaves us with the possibility that biofilm development in this organism is mainly governed by adaptive responses. Such a conclusion is strengthened by the finding that biofilm development is associated with the expression of genes required for various physiological life styles (Hentzer *et al.*, 2005). For example, mature *P. aeruginosa* biofilms express a large number of genes for anaerobic respiration and iron metabolism (Hentzer *et al.*, 2005). We submit that although the experimental conditions would differ in different experiments, the existence of a specific biofilm program would always require a core set of key genes to be expressed, regardless of the experimental conditions. To date, we conclude that transcriptomic studies of *P. aeruginosa* biofilms have not delivered such an outcome.

Clearly, the field of biofilm transcriptomics is at an early stage. Hence, the lack of expression of a consistent set of biofilm genes in *P. aeruginosa* does not necessarily translate to other biofilm forming bacteria. In fact, development of fruiting bodies in *Myxococcus* spp. biofilms (which are reminiscent of the mushroom structures and the stationary phase physiological response in *P. aeruginosa* biofilms) is controlled by a genetically programmed series of events regulated by signal molecules (Sager and Kaiser, 1994). Intriguingly, many of the features assigned to *Myxococcus xanthus* development, such as the structure of fruiting bodies, role of signal molecules, and type IV pili dependency appear to have strong

similarities with analogous features and events in *P. aeruginosa*, and it is possible that further more in depth studies will reveal the presence of a program also in *P. aeruginosa* (Lux *et al.*, 2004; O'Toole, 2003). While our understanding of gene expression by biofilm cells of other bacterial species has also increased significantly in recent years, the studies of differential gene expression in these organisms do not yet allow for comparative measures of the existence of genes specific for the biofilm mode of life. Hence, the generality of the findings for *P. aeruginosa* remains to be addressed.

With respect to the expression of the entire set of QS genes by early biofilms, it is still likely that the QS system in *P. aeruginosa* regulates gene expression in a similar fashion in both biofilm and planktonic cells. It may be proposed that QS is not required for biofilm formation *per se*, and that the QS mediated communication between the cells is a consequence of increased cell density and growth physiology. Again, comparative studies in other bacteria species for which the QS system plays a key role for biofilm development and function, such as *S. marcescens* and *V. cholerae*, will provide much needed information on this issue.

The failure, in analyses of *P. aeruginosa* biofilm and planktonic cells, to detect common themes in biofilm gene expression profiles, may also be caused by the severe limitations of current transcriptomic research. These refer to the poor sensitivity (10^8 – 10^9 cells are required) of transcriptomic analysis and the fact that it only provides us with an average view of the phenomenon of how cells behave in compartmentalized three dimensional biofilms. Application of confocal laser scanning microscopy has shown that *in vitro* biofilms of several bacterial species often display elaborate multicellular structures separated by channels and void spaces (Allesen-Holm *et al.*, 2006; Lawrence *et al.*, 1991). For example, *P. aeruginosa* differentiates into two distinct subpopulations, a process that has implications for the antibiotic resistance of biofilms. One of these subpopulations is fully susceptible to the drug (the antimicrobial peptide colistin) whereas the motile, migrating, subpopulation found on the top of the microcolonies in mature biofilms is orders of magnitude more resistant to the peptide (Janus *et al.*, 2006).

The formation of the mushroom-shaped structures in glucose-grown *P. aeruginosa* biofilms occurs in a sequential process involving the non-motile bacterial subpopulation that forms the stalks and a migrating bacterial subpopulation which subsequently forms the mushroom caps via a process which requires type IV pili (Allesen-Holm *et al.*, 2006). Recent detailed examination of the dispersal cells of *P. aeruginosa* and *S. marcescens* biofilms also provides clear evidence for different subpopulations of cells, as well as significant phenotypic variation among dispersal cells (Boles *et al.*, 2004; Drenkard and Ausubel, 2002; Koh *et al.*, 2006; Mai-Prochnow *et al.*, 2006). The data suggest that motile dispersal cells are different from the cells of the microcolony hollow structure remaining after the biofilm dissolution event and that the dispersal cells in turn are stable genotypes, following an increased frequency of mutational events in the mature microcolony. This leads to a broad variation of phenotypes pertinent for the optimal colonization of new surfaces, during a range of conditions. Hence, transcriptomic analysis of the entire biofilm population clearly will not offer information specific to biofilm subpopulations.

Additional examples of compartmentalized populations of biofilm cells include the recently emerging evidence that the biosurfactant rhamnolipid is preferentially produced

by subpopulations present in the stalks of the mushroom structures (Lequette and Greenberg, 2005). Similarly, the generation of the matrix component of extracellular DNA is primarily confined to the stalks of the mushroom-shaped structures in *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006). Intriguingly, both these QS controlled processes involve distinct subpopulations. By first impression this contradicts the view of QS as a method for pooling the activities of the entire population. However, spatial limitations that will contribute to increased local cell concentration taken together with the observation by van Delden and Iglewski (1998) who reported induction of QS regulated genes independent of cell density but in response to nutrient unavailability and gradients, might contribute to local divergence in QS regulated gene expression.

Biofilm development

In support of the apparent lack of a specific core set of genes characteristic of the biofilm mode of life, it is possible that biofilm formation may proceed through multiple pathways of development. First, different species appear to acquire similar structural and functional endpoints during biofilm formation, including the different stages of microcolony formation, matrix embedded mature biofilms, and tolerance to antimicrobial agent or other stress factors. This has been well documented for *P. aeruginosa*, *E. coli* and *V. cholerae* (Davey and O'Toole, 2000; Ghannoum and O'Toole G, 2004) perhaps the best studied model organisms for elucidating the detailed stages of biofilm formation. However, these species may or may not employ cell surface structures such as pili, flagella, LPS, and exopolymeric substances at specific stages. Second, the use of different pathways for biofilm formation and function occur also within a species. Perhaps the best example is the development of tobramycin resistance in *P. aeruginosa*. Mah *et al.* (2003) recently reported on the presence of a specific gene product which inactivates tobramycin by binding to the drug in *P. aeruginosa* PA14. The locus identified, *ndvB*, is required for the synthesis of periplasmic glucans. These periplasmic glucans interact physically with drugs and therefore might prevent antibiotics such as gentamicin, ciprofloxacin, chloramphenicol and ofloxacin from reaching their sites of action by sequestering the antimicrobial agents in the periplasm. However, this gene is not QS regulated neither does the gene product contribute significantly to tobramycin tolerance in the sequenced *P. aeruginosa* PAO1 strain. Nevertheless, also *P. aeruginosa* PAO1 tolerates the exposure to high levels of tobramycin without loss of viability during biofilm formation (Bjarnsholt *et al.*, 2005). This illustrates the notion of multiple converging pathways for biofilm development. Importantly, this is pertinent to some of the hallmarks, i.e. tolerance to antimicrobials and stress, of the biofilm mode of life biofilm.

Further examples of parallel pathways for development of common biofilm characteristics include different phenotypic characteristics across a range of clinical *P. aeruginosa* isolates during biofilm formation and dispersal. Although exopolysaccharides encoded by *psl*, *pel* and the *alg* genes (Evans and Linker, 1973; Friedman and Kolter, 2004; Vasseur *et al.*, 2005) have been reported to be critical matrix components where a carbohydrate-rich matrix component appears to be sufficient for mature biofilm formation, Nemoto *et al.* (2003) found that mature biofilms formed by four independent clinical *P. aeruginosa* isolates could be dissolved by DNase treatment, suggesting that extracellular DNA is the primary cell-to-cell interconnecting compound in mature biofilms. Murakawa (1973a,

1973b) reported on the chemical composition of “slimes” from 20 clinical *P. aeruginosa* isolates and found that slimes from 18 strains consisted primarily of DNA, while two strains with a mucoid phenotype produced slimes composed primarily of polyuronic acid, an important component of alginate. Also Wozniak *et al.*, (2003) demonstrated that alginate was not the important matrix component of non-mucoid PAO1 and P14. Additionally, a recent study in our laboratories assessed the role of filamentous phage in inducing differentiation, autolysis of a subpopulation of cells in mature microcolonies, and subsequent biofilm dissolution through the release of specialized dispersal cells. This study followed the initial demonstration that filamentous phage Pf4 in *P. aeruginosa* PAO1, as well as some clinical isolates, is involved in mediating active dispersal from biofilms (Webb *et al.*, 2003). Our recent study showed no phage induction in some isolates and variation in the timing of phage induction, and hence lack of correlation between phage appearance and autolysis and dispersal in other isolates (unpublished results). It is also clear that different filamentous phage, and hence different genes, are induced during biofilm formation in the different clinical *P. aeruginosa* strains (Mooij *et al.*, 2005). A final example of parallel pathways for development of biofilm traits is the use of cell surface associated adhesins for the same outcome. For example, *Staphylococcus epidermidis* produces a polysaccharide intercellular adhesin (PIA) which is involved in biofilm accumulation. However, infections of PIA-negative strains are not uncommon, suggesting the existence of PIA-independent biofilm accumulation mechanisms (Rohde *et al.*, 2005).

The examples above are in favor of a model in which various environmental conditions select for different parallel pathways by which biofilm formation and dispersal proceed across different species, as well as across strains within a given species. In these cases, the structural and functional endpoints of the biofilms are similar at the level of characteristics used to describe the biofilm life cycle. Furthermore, we suggest that the capacity of forming a multicellular biofilm consortium from planktonic cells developed through parallel evolutionary processes and at different times. While several observations appear to support this view, there are also examples of the use of very similar systems and genes for biofilm formation in different bacteria. For example, within the genus *Serratia*, different species employ the QS system for biofilm formation, however, the organization and function of these systems vary (Givskov *et al.*, 1996; Givskov *et al.*, 1998; Horng *et al.*, 2002) possibly suggestive of a common ancestral QS system which has been acquired by different species through lateral gene transfer.

Implications of different biofilm processes

The notion that multiple parallel processes for biofilm formation and dissolution are employed by not only different species of bacteria but also a different strain within a species, has implications for our understanding of a range of bacterial colonization events. For example, an immediate outcome of our ability to detail the various stages in biofilm formation and hence colonization of host surfaces by bacterial pathogens, is the development of novel biofilm control strategies. Novel and effective biofilm control require an in depth understanding of biofilm processes. One biofilm control measure has been suggested to be based on interference of bacterial QS systems (Givskov *et al.*, 1996; Hentzer *et al.*, 2002; Ren *et al.*, 2001). For example, a number of pathogens appear to control much of their

virulence arsenal by means of QS that relay on extracellular signal molecules (Dunny and Winans, 1999). Most diseases in which QS regulation is expected to play an important role originate from infections caused by opportunistic pathogens. The sessile biofilm mode of growth is recognized in all implant related infections and with every foreign medical equipment inserted into a patient there is a potential risk that a bacterial biofilm will form over time on that artificial surface and that the infection develops into a chronic state (Hentzer *et al.*, 2003).

Combined with the involvement of QS in biofilm tolerance to various antimicrobial measures and modulation of both the cellular and adaptive immune systems, it is feasible to propose that QS signaling and interference with important host defense mechanisms afford the bacteria with a mechanism to strategically cause disease. A drug capable of blocking signaling will be expected to attenuate bacterial pathogenicity and cause the biofilm cells of the infectious organism to become susceptible to the host defense and consequently be cleared from the host. Further, an important and appealing aspect is that antibiofilm measures directed against QS systems interfere with non-essential phenotypes and therefore are not expected to create a similar harsh selection pressure for development of resistance as seen with conventional antibiotics. Furthermore, being non-toxic they are not expected to eliminate communities of helpful and beneficial bacteria present in the host. Obviously, QS interference with the aim to remove or prevent biofilms constitutes a powerful tool for biofilm control on all surfaces at which bacteria form and maintain biofilms in a QS dependent fashion. Hence, the application of QS blockers is expected to have wide utility.

In the case of *P. aeruginosa*, the majority of animal model experiments strongly suggests that QS plays an important role in the ability of this bacterium to cause disease but the direct involvement in for example the CF pulmonary infection is still largely based on circumstantial evidence (Hardman *et al.*, 1999; Middleton *et al.*, 2002). Given the possibility that biofilms may be formed by multiple pathways (see above), the lack of clear evidence for a role of QS systems is not surprising. However, it is likely that the environment in the lung tissue constitutes a rather defined selection pressure and hence allows for one or a limited number only of parallel pathways for biofilm formation by the infecting organisms. While such a scenario clearly calls for further studies, the results obtained from the animal models referred to above nevertheless justifies the development of pharmacological relevant QS blockers as an important constituent of future chemotherapies for treatments of a variety of *P. aeruginosa* based infections. Finally, an increasing number of clinical isolates show multiple resistances to conventional antibiotics which only speak in favor of speeding up the pace in this particular field (Oliver *et al.*, 2000).

The use of QS inhibitors to inhibit biofilm formation by Gram positive staphylococci and streptococci, has also been argued as an attractive novel therapeutic tool. However, the use of peptide blockers or inhibitors requires careful investigation in every specific pathogen and type of infection, as a consequence of alternate biofilm processes. Moreover, according to Yarwood (this volume) the signal used can be varied, and the responses to the different signals are complex in staphylococci. For example, peptide signaling via the Agr system may facilitate early biofilm and prevent late biofilm formation. Hence, signal inhibitors may prevent the acute phase of infection but facilitate chronic biofilm based infections.

Methodologies for biofilm analyses

While the last few years have witnessed much progress in our understanding of the means by which bacteria form biofilms on different surfaces and in different habitats, it is also clear that the methodologies employed to study biofilm populations and the rather intricate differentiation processes that appear to underpin biofilm development are unsatisfactory. In fact it has been argued that the progress has been limited in several aspects of biofilm research. This concern speaks to the range of approaches used to study biofilms, i.e. including biofilm assays, such as current batch and flow cell systems, and transcriptomic and proteomic analyses yielding information on average activities rather than assessments of discrete activities reflective of the compartmentalized nature of three dimensional biofilms.

Activity of specific regulatory systems

There is clearly a need for the development of several assays for detection of specific traits in biofilms, such as improved assays for the detection of specific regulatory elements. For example, the typical screen for QS blocker activity involves a gene fusion of a QS regulated promoter to a reporter gene. In the presence of exogenously added signal molecules, the reporter gene is activated to express its corresponding phenotype. If in addition to AHL an exogenous blocker is also present, expression of the reporters is reduced or switched-off. Rasmussen *et al.* (2005) developed an alternative system in which growth of the screening-bacterium indicates the presence of QS blocker activity. Briefly, by genetic manipulation QS regulates expression of a detrimental gene product, however presence of a QS blocker compound blocks expression of the “killer” gene, rescues the host cells and enables growth. Performed as an agar based diffusion assay it enables a quick verification of the inhibitory as well as toxic properties of a test compound or extract. This novel paradigm for bioassays can be applied not only to QS systems and their inhibition, but extends into a multitude of key regulatory systems of direct value for biofilm research.

Detection of differential gene expression in biofilms

In order to fully capitalize on novel high resolution and specific assays, we require a much enhanced understanding of the differential gene expression that occurs in biofilm communities. However, this is not easily achieved at present. The current “omic” technologies are limited in regard to resolution. While mechanical tools, such as micromanipulators exist to sample individual cells or biofilm compartments, the sample size is not sufficient to support current analyses by transcriptomic or proteomic tools. Ideally, the sensitivity of such tools needs to be improved by at least six orders of magnitude, allowing for analysis of a population of ideally less than 1000 cells. While studies of biofilms have improved significantly using methods for detection of individual cells, by means of for example micromanipulator assisted analysis, GFP tagging, CARD FISH, confocal scanning laser microscopy, and the separation of cells by flow cytometry and cell sorting, current “omic” technologies require a return to sample preparation which includes entire biofilms. Ideally, our experimental toolbox should include the analysis of genome-wide expression by single cells.

If the latter scenario can be applied, a range of experiments addressing central questions on gene expression in bacterial biofilms and their individual cells upon interactions

with hosts as well as other organisms in the environment, including protozoan grazers, may be conducted. Such high resolution methods might enable the detection of key responses in individual cells, reflective of the true interaction between cells and their hosts or predators. Currently, our measurements only depict the average signal or response from all cells in the population which may reflect the majority of cells that do not respond to the presence of the recognition events mediated by the eukaryotic opponent. If analysis of genome-wide expression by single cells can be accomplished we will be in the position to identify relevant gene responders which in turn can be used in the construction of appropriate bioscreens. Such screens would be ideal for the identification and design of interfering chemical compounds.

Biofilm assays

There are several examples of *in vitro* biofilm devices, including Robbin's device (Kharazmi *et al.*, 1999), spinning disk biofilms (Charlton *et al.*, 2001), run through flow cells (Moller *et al.*, 1998) and microtiter dish based batch systems (often referred to as static biofilms). The former three systems produce biofilms irrigated with fresh medium and therefore constitutes biofilms that experience a continuous flow supplemented with fresh nutrients. The latter system generates biofilms that have exhausted important nutrient components at the end of an over night incubation. The key features of this system are that numerous biofilms can be handled at any given time, and does not require time consuming sterilization and setup procedures allowing it to be used as a high through put system for biofilm analysis. Importantly, it provides a basis for a rapid screening procedure of biofilm mutants. For example, a library of random mutants generated by transposon mutagenesis can and has been readily screened for the expression of adhesive properties (Kulasekara *et al.*, 2005), biomass development and biofilm forming capacity (O'Toole and Kolter, 1998; Watnick and Kolter, 1999), as well as extracellular matrix composition (Friedman and Kolter 2004a, 2004b). Essentially, detection of any phenotype that can be processed by a microtiter reader can be handled by this system and mutants can be isolated in pure cultures. Hence, to a limited extent the batch based microtiter analysis can be used to study functional aspects of the biofilm linked to a certain genotype. On the other hand, the system is incompatible with confocal scanning laser microscopy based investigations, the preferred methodology to reveal structural aspects of biofilms. Structural biofilm analyses require the use of irrigated biofilm systems.

The irrigated biofilm flow systems are counterparts of chemostats normally employed for studying physiological steady state conditions in planktonic prokaryotes. An important distinction, however, is that the irrigated and flow through cell systems allow for studying the development over time and space of the biofilm. This includes non-destructive studies of temporal and spatial expression of selected genes—as limited by the range of fluorescent reporters available—biofilm structure, and the complete life cycle of biofilm formation and dispersal. Importantly, several of the findings on unique behavioral responses by biofilm cells and subpopulations derive from observations made using irrigated biofilm flow systems e.g. (Davey and O'Toole, 2000). Such information can not be generated using static microtiter batch systems. Moreover, other studies of biofilm characteristics such as toler-

ance to antimicrobial measures require the use of one of the irrigated biofilm systems for reliable data acquisition (unpublished observations).

Genomics

The field of genomics is currently experiencing enormous progress in sequencing technology and strategies (Margulies *et al.*, 2005; Shendure *et al.*, 2005; Goldberg *et al.*, 2006) that will open exciting opportunities for biofilm research. In fact, we expect that sequencing of individual cells, populations or natural communities will become an indispensable tool in biofilm research.

An important, recent observation in biofilm research has been that a large number of stable, genetic variants are generated during the process of biofilm formation and dispersal; a process that ensures a next generation of cells with better fitness for environmental adaptation (Boles *et al.*, 2004; Mai-Prochnow *et al.*, 2006; see also section “Biofilm program and specific gene expression” above). Applying whole-genome sequencing on entire dispersal populations would help to quickly and effectively identify the mutational processes (such as reduced mismatch repair or increased transposition) that cause the generation of variants. Furthermore, information about the positional bias of mutations in certain genes or genomic regions will point towards selection of specific genotypes in the process from initial attachment to final dispersal. This in turn can enhance our understanding of the evolutionary forces of the biofilm cycle. Recent studies of the genomes of *P. aeruginosa* CF strains isolated from patients at early and late stages of the disease have provided a strong case for the power of such genomic-based approaches (Smith *et al.*, 2006). Comparing the “early” and the “late” stage derived genomes showed that the strains undergo numerous genetic adaptations during the disease progression. Interestingly, virulence factors such as the QS regulator LasR and the multi-drug efflux pump MexZ are selected against during the chronic phase of the disease. This highlights the notion that advanced CF strains are quite different from “wild-type” *P. aeruginosa* and might offer new strategies for treatment. The study by Smith *et al.* (2006) also shows that genome analysis can generate results that are initially counterintuitive to our current understanding of a biological system, while also challenging us to define new hypotheses for the observations made. With the dramatic reduction of sequencing cost expected in the near future, we will see genomics becoming increasingly integrated into other defined, experimental systems of biofilm research.

High-throughput DNA sequencing is now well established as a powerful tool to explore and describe the diversity of natural, microbial communities in a field termed environmental genomics or metagenomics (Handelsman, 2004; Riesenfeld *et al.*, 2004; Venter *et al.*, 2004; Tyson *et al.*, 2004; DeLong *et al.*, 2006). In fact the first large-scale environmental sequencing effort was applied to a biofilm community from an acid mine drainage system (Tyson *et al.*, 2004). The relatively low complexity of this microbial system (less than 10 species) allowed for the reconstruction of nearly complete genomes for the populations of the two most-abundant species, *Leptospirillum* group II and *Ferroplasma* type II. While the *Leptospirillum* group II showed very little nucleotide polymorphism, the *Ferroplasma* type II exhibited extensive homologous recombination resulting in a complex, mosaic genome for its populations. This study illustrates that natural biofilm populations can undergo significant genetic diversification yet the degree of variation might depend on

the organism itself or the specific evolutionary pressure experienced. It is also tempting to speculate that these observations reflect cell differentiation required for the adaptation to complex, environmental conditions or the biofilm lifestyle itself. Clearly, there is a need to expand these studies to other naturally occurring biofilm systems in order to obtain a better and more refined picture of the genetic diversification and its underlying mechanisms.

Should we expect there to be a consensus view on biofilms?

Contemporary biofilm research has the potential to reveal novel prokaryotic biology. While there may not be one specific biofilm program, as discussed above, there are certainly a range of observations that suggest the elucidation of prokaryotic biology not previously assigned to planktonic cells or cell cultures. Moreover, we expect that the field of biofilm research will continue to deliver novel information on prokaryotic biology and hence greatly improve our understanding of how biofilm consortia in the environment drive biogeochemical cycles and other key ecological process, as well as unravel disease mechanisms used by bacterial pathogens. Given these predictions, and the fact that several reports on biofilm diversity and heterogeneity have been published in recent years, it is surprising that an almost consensus view on bacterial biofilm biology appear to prevail today.

This rather narrow model possibly derives from the focus on mushroom-like structures. It would appear that many bacterial species have the capacity to form such structures *in vitro*, should appropriate nutrient conditions be provided, and such architectures have become a focal point in biofilm research. In fact, this has lead to a widely held view that biofilm research is the science of mushroom-like structures as studied by CLSM. However, it is not clear that such structures are common in natural habitats. For example, biofilms on living surfaces studied in the authors' laboratories, e.g. those of the surface of lung tissue, chronically infected wounds and marine sessile organisms, do not display mushroom-like structures. Rather, these biofilms exist as microcolonies (Rao *et al.*, 2006) and in some instances filamentous biofilms (Labbate *et al.*, 2004). Also, other naturally occurring biofilms such as those in the phyllosphere (Jacques *et al.*, 2005; Morris *et al.*, 1998), the oral cavity (Auschill *et al.*, 2001; Hope *et al.*, 2002) and the rhizosphere (Timmusk *et al.*, 2005; Walker *et al.*, 2004), form aggregates or microcolonies rather than mushrooms. Hence, many current studies appear to be directed at a phenotype that may be of limited relevance in natural habitats. It may be argued that this prevailing consensus view of biofilms has directed the field into a much too narrow research path. Contributing to this situation is the lack of technologies which allow for more detailed analysis of the activity and interactions of single cells, or subpopulations, beyond the current microscopy based descriptions of biofilm structures, as discussed in sections above.

What should biofilm research also address and what do we expect will be the focal points in this research area in the near future? Several issues may be considered in this regard. We assume that the driving force for cells to form multicellular arrangements relates to bacterial survival strategies in hostile environments. Hence, studies of the mechanisms by which cells form a multicellular sanctuary or a fortress, as have been the descriptions used for biofilms in which cells are protected from environmental conditions or engage in competitive interactions with other organisms, will be crucial to explore differences between planktonic and sessile prokaryotic lifestyles and identify elements key to the ecological suc-

cess of biofilms. Further, a much improved understanding of the genes and pathways the cells need to call on for achieving the shift from a planktonic to a true multicellular life style will not only disclose targets for biofilm control but likely also reveal evolutionary traits common to prokaryotes and eukaryotes.

With respect to both structural stability and tolerance of biofilms, the exact composition and role of the extracellular matrix in which biofilm cells are embedded should be explored in considerably more detail than is hitherto the case. It is likely that such studies will reveal structural arrangements, akin to the lattice recently reported for *Staphylococcus aureus*, following the dissolution of mature biofilms (Costerton, J.W. personal communication), or the proposed biofilm backbone characteristic of filamentous phage fibrils embedding *P. aeruginosa* cells (Webb *et al.*, 2004). These arrangements are likely to represent novel “nanostructures,” potentially contributing also to rapid advances in the nanosciences. Related to the observation of a network of phage filaments around these cells are current reports on predominantly DNA containing, rather than other macromolecules, extracellular biofilm matrices (Allesen-Holm *et al.*, 2006). Obviously, the implications of DNA as the biofilm matrix are profound as discussed by Pamp *et al.*, this volume.

Finally, perhaps the most rapid and ecologically relevant contribution to our understanding of biofilms will derive from precise mechanistic based studies of increasingly complex multispecies biofilms. It is possible that only the inclusion of high resolution genetic based analyses of defined mixed species biofilms, representing a broad range of habitats, will deliver truly meaningful information of prokaryotic multicellular communities. This speaks to their intrinsic biology as well as their ecosystem functions. It may be argued that in depth analyses of relevant mixed species biofilm communities are also required to fully understand a range of bacterially mediated diseases. As discussed in the previous section, current rapid progress in whole bacterial community sequencing and functional metagenomics may make such studies a reality within a very short period of time.

We suggest that the apparently prevailing consensus view on bacterial biofilms may not reflect the organization and function of most biofilms, neither in *in vitro* situations nor in their natural habitats. We also submit that the current consensus view is not helpful in allowing us to embrace the taxonomically and functionally diverse biofilms that constitute the predominant prokaryotic biomass in host and environmental settings.

Acknowledgments

We wish to express our sincere thanks to our colleagues at the Centre for Marine Biofouling and Bio-Innovation at UNSW and BioCentrum at DTU for their contributions to the topics discussed in this chapter, and Anne Mai-Prochnow and Torsten Thomas for their help in preparing the chapter.

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