Developmental regulation of microbial biofilms

Luanne Hall-Stoodley and Paul Stoodley*

Sophisticated molecular and microscopic methods used to study biofilm formation are rapidly broadening our understanding of surface-attached microbial communities in a wide variety of organisms. Regulatory mechanisms involved in the attachment and subsequent development of mature biofilms are being elucidated. Common themes are beginning to emerge, providing promise for the development of sophisticated control strategies.

Addresses
Center for Biofilm Engineering, Department of Microbiology and Civil Engineering, 366 EPS Building, Montana State University, Bozeman, MT 59717, USA
*e-mail: paul_s@erc.montana.edu

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Abbreviations
EPS extracellular polysaccharides or extracellular polymeric substances
QS quorum sensing
WT wild type

Introduction

Biofilms are complex communities of microorganisms that develop on surfaces in diverse environments. They contaminate industrial pipelines, dental unit water lines, catheters, ventilators and medical implants and can cause disease in humans, animals and plants. Biofilms are now being studied in a wide variety of organisms, across the phylogenetic spectrum. The tendency is toward an ever-widening appreciation that microorganisms have the ability to occupy and modify ecological niches within biofilms. There is a notable trend toward the identification of pathogenic surface-attached organisms in such diverse genera as *Pseudomonas*, *Vibrio*, *Escherichia*, *Salmonella*, *Listeria*, *Streptococcus*, *Staphylococcus* and *Mycobacteria*. A variety of sophisticated molecular and microscopic approaches are now used to interrogate biofilm development. These methods are generating an explosion of data on the developmental processes and regulatory mechanisms involved in biofilm formation, some of which will be discussed in this review. This pervasive microbial response may be profitably examined and exploited to reduce biofouling or the contamination of medical devices and pharmaceuticals. Knowledge gained in this area could also be exploited to enhance bioremediation systems or symbiotic plant–microbe interactions.

Structure and biofilm formation

A central tenet of biofilm formation is that it is dynamic. Current models, based largely on *Pseudomonas* spp., depict biofilm formation as a linear process that commences when free-floating bacterial cells attach to a surface. This attachment is followed by growth into a mature, structurally complex biofilm and culminates in the dispersal of detached bacterial cells into the bulk fluid [1,2•]. These various phases of microbial interactions with the surface appear to require the production of extracellular microbial structures that assist in initial adhesion, maintenance of biofilm structure, and detachment from matrix-enclosed aggregates. This is a crucial aspect of biofilm investigation, as microbial phenotypic behavior may be quite distinct during the different phases of biofilm formation [2•].

Initial attachment and development of biofilm structures

Structurally, biofilm development occurs by several mechanisms. One is by the redistribution of attached cells by surface motility. In *Pseudomonas aeruginosa*, flagella and type IV pili-mediated twitching motility both play important roles in surface aggregation [3]. In *Escherichia coli*, flagella, type I pili and curli fimbriae have been implicated in biofilm formation [4••]. Sauer *et al.* [2•] noted that a flagellar mutant of *P. aeruginosa* was unable to colonize a glass flow cell as efficiently as the parental strain. In *Vibrio cholerae*, motility also appeared to facilitate surface colonization as flagellar mutants had larger voids between the biofilm microcolonies than wild-type (WT) O139 [5]. Although motility appears to assist colonization of surfaces by Gram-negative organisms, it is not a prerequisite for biofilm formation as several non-motile bacteria such as streptococcus, staphylococcus, and mycobacteria readily form biofilms. In *Staphylococcus epidermidis*, protein and polysaccharide adhesins have been linked to adherence. When the polysaccharide intercellular adhesin/hemagglutinin (PIA/HA) was mutated, surface colonization was reduced in two animal models of biomaterial-based infection [6]. In *Staphylococcus aureus* a novel biofilm-associated protein (bap) was similarly found to be involved in initial attachment and in intercellular adhesion [7].

Another mechanism of biofilm formation results from the binary division of attached cells [8,9]. As cells divide, daughter cells spread outward and upward from the attachment point to form cell clusters, in a similar manner to colony formation on agar plates. This type of growth has been monitored microscopically by measuring the radial expansion of *Mycobacterium fortuitum* microcolonies on a silicon surface over time [10]. A time-lapse video of microcolony formation in a mixed species biofilm can be found at the American Society for Microbiology (ASM) MicrobeLibrary (‘Growth and detachment of biofilm cell cluster in turbulent flow’: www.microbelibrary.org/Visual/page1.htm).

Surface-associated aggregation can also occur by recruitment of single cells [9] or cell flocs [10] from the bulk fluid

to the developing biofilm; however, in both of these studies recruitment played a minor role in colonization compared with replication. Lastly, the shear induced flow of large patches of biofilms or 'rolling' of entire micro-colonies over surfaces may allow mature biofilms to colonize new surfaces downstream and to form secondary structures such as 'nets' or organized ripples [11]. The relative contribution of each of these mechanisms to biofilm formation will depend on the organism(s), the properties of the surface being colonized [12••], and the physical and chemical conditions of the aqueous environment.

Biofilm formation is a dynamic process

Biofilms take variable periods to reach structural maturity on the basis of microscopically measured physical dimensions and visual comparison [8,11,13•]. In oligotrophic environments, mature biofilms may consist of little more than a sparse covering of cells with little structural complexity. Given that biofilms can take long periods to develop, care must be taken not to misinterpret retardation in the initial events of attachment and biofilm development as the total suppression of biofilm formation. Baty and colleagues [13•,14] investigated bacterial subpopulations on an artificial chitin surface using the marine chitin-degrading bacterium *Pseudoalteromonas* S9. Surface-associated growth rates were examined over 200 h and the number of attached cells decreased nearly three logs between initial surface proliferation and a steady-state of the attached population. Interestingly, these studies suggested a 'division of labor' in the adherent cell community. One subpopulation of adherent cells that degraded chitin was associated with high levels of chitinase expression, while another subpopulation consisted of actively replicating chitinase-inactive cells that released daughter cells into the bulk fluid. The authors speculate that the chitinase-inactive cells used chitin degradation products that were secreted by their chitinase-active neighbors, sequestered in the extracellular matrix and transported via water channels in the biofilm. This study suggests a holistic integration of both planktonic and biofilm phenotypes in this organism.

Biofilm phenotypes

Sauer *et al.* [2•] used microscopy and molecular methods to examine changes in protein and gene expression in *P. aeruginosa* PAO1 over a 12 day period. Five distinct stages of biofilm development were characterized. Protein extracts separated by two-dimensional gel electrophoresis were used to monitor phenotypic differences during each stage of biofilm development. A total of 57 biofilm-associated proteins were identified that differed from the planktonic profile. Of these, 23 differentially expressed (mostly upregulated) proteins were further analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) and found to be associated with metabolic processes such as amino acid and carbon metabolism and lipid biosynthesis. Membrane proteins and superoxide dismutase were also identified. In PAO1 biofilms grown for six days, only 40% of the proteins expressed were identical to PAO1 planktonic (chemostat grown) cells. At days 9 and 12, the percentage of identically expressed proteins increased to 60% suggesting that multiple phenotypes were expressed over time.

Differential gene expression in a five day *P. aeruginosa* PAO1 biofilm was studied using DNA microarray technology [15••] and showed that relatively few genes (~1%) were differentially expressed in planktonic and biofilm cells. Specifically, flagella and pilin genes were downregulated in biofilm cells, as was the gene for the σ factor RpoS. An *rpoS* mutant selected for further study, showed not only enhanced biofilm formation but also increased resistance to the antibiotic tobramycin. In another study by Sauer and Camper [16••] biofilm-associated *Pseudomonas putida* was compared with planktonic cells using both a proteomic approach and subtractive cDNA hybridization. Gene expression data demonstrated changes in genes associated with flagellar and type IV pili components, polysaccharide biosynthesis, amino acid metabolism and an outer membrane protein, NlpD. Proteomic analysis showed differential expression of proteins largely required for amino acid metabolism and NlpD. Whereas DNA microarrays provide a sensitive, but transient snapshot of gene expression, proteomics reflects the expression of more stable gene products. Both approaches suggest that biofilm bacteria differ from planktonic cells but the degree to which they differ will require further application of these techniques to the study of biofilms.

In situ gene expression can also be evaluated microscopically by the use of green fluorescent protein (GFP) fusion reporter constructs. This technique, although limited in the number of genes that can be tracked in a single experiment, has the advantage of being able to look at both the temporal and spatial distribution of genes in biofilms. Tolker-Nielson *et al.* [9] studied the dynamics of two species of *Pseudomonas* biofilm *in situ*, each tagged with a different fluorescent protein (*gfp* and *dsRed*) by microscopically tracking cells over time. Initially, separate microcolonies (green or red) were observed, but after three days microcolonies contained cells of both species, suggesting that the biofilm contained both sessile and motile populations at the same time. Taken together, these studies suggest that the initial downregulation of flagella is transitory and motile bacteria are present in biofilms at later stages of biofilm development. These studies indicate that although molecular methods show extraordinary promise, *in situ* techniques (combined with reporter gene technology) remain a fundamental component of biofilm investigation.

Genetic regulation of biofilm formation

Increasing evidence suggests that biofilm formation is influenced by complex regulatory pathways. O'Toole *et al.* [17•] examined the initial events in biofilm development using a *crc* mutant of *P. aeruginosa* and attributed the observed attenuated biofilm formation to a defect in

type IV pili expression/function. Crc is a global carbon metabolism regulator and its role in influencing biofilm development via type IV pili expression was thought to be due to sensing nutritional cues. In another study in *E. coli*, the effect of a global carbon regulator (CsrA) was found to have a profound effect on biofilm formation [4••]. In this study, multiple genetic and nutritional conditions were found to affect biofilm formation, suggesting that biofilm development is multifactorial and regulation of this response is complex. Disruption of *csrA* increased biofilm formation compared with the isogenic parent and overexpression of CsrA was inhibitory in *E. coli* K12 and in pathogenic isolates. The disruption of *csrA* enhanced biofilm formation even when extracellular appendages shown to be important for biofilm development in knock-out experiments were absent, perhaps implicating *csrA* regulation in several biosynthetic processes. The primary effect of *csrA* appeared to be as a regulator of glycogen metabolism and the authors speculate that glycogen may be the principal carbon/energy source for stationary-phase biosynthesis of adhesion factors.

Several other papers have described regulatory pathways associated with adhesion to surfaces including the Cpx-signaling pathway in *E. coli* and *rel*A in *Listeria monocytogenes* [18,19]. To date, genetic regulation of biofilms appears to be impressively complex, perhaps because of redundancy or overlap in regulatory pathways. Prigent-Combaret *et al.* [20] found that the two-component regulatory systems OmpR/EnvZ and CpxRA converged (via positive and negative regulation of curli transcription) affecting *E. coli* biofilm formation.

Finally, biofilm formation has been correlated with conjugative plasmids. Ghigo [21•] found that the presence of plasmids induced biofilm formation and hypothesized that the high cell densities in biofilms favor higher rates of horizontal transfer of plasmid DNA.

Quorum sensing and biofilm structure

Davies *et al.* [22] demonstrated that quorum sensing (QS) molecules, normally associated with the regulation of virulence factors, could also regulate the development of complex mushroom structures in a *P. aeruginosa* PAO1 biofilm. The suppression of thicker, more complex biofilm structures has also been achieved using a synthetic furanone to inhibit QS [23]. Suppression of structural complexity may be related to deficiencies in twitching motility [24] or reduced extracellular polysaccharide (EPS) production [25], both of which were found in the *P. aeruginosa lasI* QS mutant used in the Davies study. However, in a study investigating the influence of alginate production on biofilm structure in PAO1, the WT control biofilm was flat [26] and appeared more like the QS *lasI* mutant biofilm reported by Davies *et al.* [22]. The difference between the structures of the WT in the two studies [22,26] was attributed to differences in media composition and may demonstrate the sensitivity of biofilm structure to experimental growth conditions. For example, the roughness of *Pseudomonas aureofaciens* biofilms was inversely related to the concentration of citrate in the growth medium [8] as predicted by mathematical models [27] (see Update).

The role of EPS in biofilm formation

Bacterial cells in biofilm microcolonies are held together by a slime-like matrix termed 'extracellular polymeric substances' or 'extracellular polysaccharides' which both abbreviate to EPS. The chemistry of the EPS is complex and includes polysaccharides, nucleic acids and proteins [28,29]. The polysaccharide polymer alginate, produced by mucoid *P. aeruginosa* strains, is the best-studied component of biofilm EPS and appears to play an important role in determining biofilm structure. The structural complexity of mushroom and mound structures, which developed in mucoid (WT) biofilms grown from the cystic fibrosis isolate *P. aeruginosa* FRD1, were suppressed in non-mucoid mutant strains, which formed flat, patchy biofilms [30]. Conversely, structural complexity was observed in a flat, non-mucoid WT *P. aeruginosa* PAO1 biofilm in an isogenic mutant where alginate was overexpressed [26].

Other EPS polysaccharides have a similar structural role in other species. Glucose and galactose-rich EPS produced by a rugose variant of *V. cholerae* O1 El Tor was required for complex structural biofilm development [31]. Interestingly, the increased production of EPS in flagellar mutants of *V. cholerae* 0139 correlated with reduced intestinal colonization in an infant mouse model [5]. Colanic acid, an EPS component of *E. coli* K12 biofilms, is variable in its association with structural changes in the biofilm [4••,32].

Finally, although polysaccharides are the best-studied component of EPS, the available data suggest a huge, but largely uncharacterized, diversity in EPS produced by different species under different growth conditions [29]. In part this is because of the difficulty in separation and extraction of EPS from bacterial cells and the complexities of analyzing polysaccharides, which can be highly branched with a wide variety of linkages and side groups. It has yet to be established if the nucleic acids and proteins found in EPS have a structural role or are merely remnants of lyzed cellular debris. However, the dissipation of microcolonies in young (<60 h) biofilms when exposed to DNase I suggests that extracellular DNA may play a structural role in the early events of biofilm formation [33]. Manipulation of the mechanical properties of EPS through enzymatic methods may prove a useful technology to remove or stabilize biofilms.

The influence of hydrodynamics on biofilm structure

Molecular methods demonstrate that biofilm development and structure is clearly influenced by several genetically regulated factors, however, the physical forces acting on the biofilm can also influence structure. Laboratory biofilms are usually grown under low, laminar flow and the

patterns of microcolony formation are generally isotropic with no evidence of directionality. However, *in vitro* studies under high-shear, turbulent flow show that biofilm cell clusters tend to elongate in the downstream direction forming filamentous 'streamers', even in *P. aeruginosa* a species not usually associated with a filamentous phenotype [34]. Filamentous biofilms also commonly occur in nature in both bacterial and archaeal biofilms formed in fast-flowing environments in hot springs, marine hydrothermal vents and acid mine drainage runoff [35]. The magnitude of the fluid shear stress can also influence biofilm density and strength [36]. Stoodley *et al.* [37] found that an anaerobic marine *Desulfovibrio* spp. biofilm and *P. aeruginosa* biofilms were stronger when grown at higher shear stresses. Intriguingly, deformations to the biofilm structure caused by transitory variations in fluid shear demonstrated that mechanically these biofilms behaved as viscoelastic liquids [34,37,38] and can be modeled as polymer hydrogels [39]. This observation suggests that EPS not only determines the architecture, but also the strength and material properties of the biofilm. Biofilm mechanics is an emerging field in the study of biofilms and may provide new insights into the development, mechanical disruption or stabilization methods for biofilms.

Conclusions

We have discussed some of the factors influencing biofilm structure and development. Some common factors include extracellular components, such as flagella, pili, adhesins and fimbriae, and EPS. QS mechanisms (although perhaps via regulation of extracellular components), nutrient metabolism, and hydrodynamics (which will influence both nutrient exchange and physical shear) also emerge as factors consistently associated with biofilm development. Increasingly, global regulatory networks appear to play a fundamental role in biofilm formation. The broadly emerging concept of biofilms is one of a highly regulated developmental biological system; however, as within any rapidly developing field, specific details remain to be elucidated. This is not surprising given the complexity of biofilm regulation, which appears to be both temporally dynamic and spatially heterogeneous. A more detailed understanding of the complex roles of genetic and environmental factors in the attachment, development, and detachment of biofilms will ultimately lead to improved strategies for biofilm control.

Update

Heydorn and colleagues [40••] provide a systematic experimental approach that illustrates several points made in this review. In particular, this paper addresses some of the disparities in experimental systems on *P. aeruginosa* reported in the literature by consistently comparing biofilm development between different mutants of PAO1 under different nutrient and flow conditions in flow cells. Confocal microscopic analysis of the *gfp*-tagged strains was used to quantify biofilm development using an image analysis program COMSTAT. Multiple images were analyzed over three independent cycles of biofilm experiments to provide statistical rigor. Their results are discussed in the context of the current model of *P. aeruginosa* biofilm development and suggestions for modifying this model are presented. Their results suggest that twitching motility is not required for microcolony formation in *P. aeruginosa* biofilms and that cell–cell signaling via *lasI–lasR* QS was not required for the development of mature biofilms. The stationary phase σ factor RpoS was found to play a role. This study demonstrates that results may vary depending on flow, experimental duration, nutritional source and method of analysis. This paper also reflects that methodology is emerging that accommodates the complexities inherent in the investigation of biofilms.

The association of cell signaling with biofilm formation and structure is more established in other prokaryotic species. In experiments designed to identify proteins involved in matrix function, Kearns *et al.* [41••] identified a zinc metalloprotease *fibA* (fibril protein A) associated with the EPS matrix of the soil microorganism *Myxococcus xanthus*. FibA appears to affect aggregation patterns as the bacteria associate to form fruiting bodies. The matrix protein is proposed to be part of a sensory transduction mechanism involved in the regulation of chemotaxis. This paper demonstrates that cell–cell signaling is important in biofilm formation with this species.

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This paper addresses some of the different experimental systems used in the study of *P. aeruginosa* biofilms. Biofilm development between different mutants strains of PAO1 was monitored over a two week period in flow cells. As one of the problems inherent in flow cells is quantitative analysis, biofilm development and structure was analyzed using a computer program COMSTAT. Multiple images obtained over three independent cycles of biofilm experiments were examined for statistical analysis. This paper reflects emerging methodology that accommodates the complexities inherent in the investigation of biofilms.

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