Environmental Influences on Biofilm Development

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Abstract Bacterial biofilms are found under diverse environmental conditions, from sheltered and specialized environments found within mammalian hosts to the extremes of biological survival. The process of forming a biofilm and the eventual return of cells to the planktonic state involve the coordination of vast amounts of genetic information. Nevertheless, the prevailing evidence suggests that the overall progression of this cycle within a given species or strain of bacteria responds to environmental conditions via a finite number of key regulatory factors and pathways, which affect enzymatic and structural elements that are needed for biofilm formation and dispersal. Among the conditions that affect biofilm development are temperature, pH, O_2 levels, hydrodynamics, osmolarity, the presence of specific ions, nutrients, and factors derived from the biotic environment. The integration of these influences ultimately determines the pattern of behavior of a given bacterium with respect to biofilm development. This chapter will present examples of how environmental conditions affect biofilm development, most of which come from studies of species that have mammalian hosts.

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1 Introduction

In the past decade, substantial advances in the understanding of the genetic and physiological bases of biofilm formation have been made. Dramatic differences in gene expression patterns exist between planktonic and sessile cells, and indeed even between different stages of biofilm development (e.g., Sauer et al. 2003). Nevertheless, the environmental and genetic factors that promote the transition from planktonic to sessile communities are only beginning to be understood in a few model organisms (reviewed by Stanley and Lazazzera 2004). It is clear that different species and even strains of bacteria can exhibit unique patterns of response to the environment. What environmental conditions predispose various species of bacteria to initiate a given biofilm? How are the molecular genetic, biochemical, and structural elements that mediate biofilm development regulated in response to environmental conditions? The following sections describe some of the environmental influences on biofilm development in the context of the molecular genetics and biochemistry of the biofilm development cycle (Fig. 1).

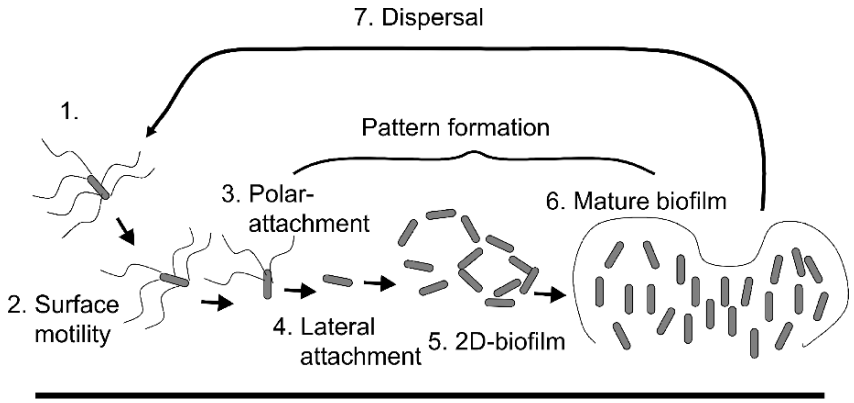

Fig. 1 A model for biofilm development. Planktonic cells (*1*) use motility to approach and swim on a surface (*2*). Upon interacting with the substratum by a pole, cells can become reversibly attached, which may allow for sampling of the environment before committing to a sessile lifestyle (*3*). Next, cells become laterally attached to the surface, involving adhesins such as PGA or LapA (*4*). During this time, the attachment of cells begins to create a two-dimensional biofilm, which in *E. coli*, exhibits distinct periodicity in cellular distribution (*5*). The biofilm grows in thickness as more cells are incorporated into its structure. Extracellular polysaccharides and other substances are produced, resulting in more firmly attached cells within an extracellular matrix. The architecture of the biofilm may be modified by production of surfactant and release of attached cells (*6*). In response to environmental or physiological clues, cells may be released from the matrix and return to a planktonic state, thus completing the developmental cycle (*7*). The entire process of biofilm development is dynamic and is influenced by numerous environmental factors

2 Surface Factors and Hydrodynamic Effects

Virtually any material that comes into contact with fluids containing bacteria is a substrate for biofilm formation. The roughness, chemistry, and presence of conditioning films affect attachment of bacterial cells to a surface. While rough surfaces are readily colonized because shear forces are diminished and surface area is increased in rougher surfaces (Donlan 2002), studies have indicated that nondomesticated strains of at least some species seem to colonize smooth surfaces equally as well (Donlan and Costerton 2002). Studies have also demonstrated that microorganisms typically attach more rapidly to hydrophobic surfaces such as plastics than to hydrophilic glass or metals (reviewed by Donlan 2002). For instance, hydrophobic substrata promote biofilm formation by most clinical isolates of *Staphylococcus epidermidis* (Cerca et al. 2005). Hydrophobic interactions between the cell surface and the substratum may enable the cell to overcome repulsive forces and attach irreversibly (Donlan 2002). A notable exception is that *Listeria monocytogenes* forms biofilms more rapidly on hydrophilic than on hydrophobic surfaces (Chavant et al. 2002).

Submerged surfaces adsorb solutes and small particles, including bacteria (Geesey 2001). Studies dating back to the 1940s showed that glass surfaces adsorb nutrients from sea water, with consequent effects on metabolic activity associated with bacterial attachment (e.g., ZoBell 1943). Furthermore, the metabolic activities of bacteria associated with a surface cause temporal and spatial changes in the three-dimensional chemical gradients at the liquid-solid interface (Geesey 2001; Rani et al. 2007). When surfaces exposed to fluid environments adsorb proteins, coatings or conditioning films are formed that alter the surface properties and affect attachment of bacteria (Dunne 2002; Murga et al. 2001; Tieszer et al. 1998). For example, the proteinaceous conditioning films called acquired pellicles that develop on tooth enamel within the oral cavity are colonized within hours by Gram-positive cocci (Donlan and Costerton 2002; Rickard et al. 2003). The surface of a central venous catheter is in direct contact with the bloodstream and becomes coated with platelets, plasma, and tissue proteins including albumin, fibrinogen, fibronectin, and laminin (see the chapter by R.M. Donlan, this volume). This coating acts as a conditioning film that is colonized by organisms such as *Staphylococcus aureus*, which adheres to fibronectin, fibrinogen, and laminin via large surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (see the chapter by M. Otto, this volume; Mack et al. 2007; Patti et al. 1994).

Fluid flow or hydrodynamics influences biofilm structure and can have dramatic effects on the type of biofilm that is formed. Physical properties of biofilms such as cell density and strength of attachment can be affected by fluid sheer (reviewed by Stoodley et al. 2002 a, 2002b; van Loosdrecht et al. 2002). Furthermore, biofilms grown under low flow conditions may form isotropic structures, whereas higher unidirectional flow may produce filamentous cells or groupings of cells with evidence of directionality (Stoodley et al. 1999, 2002a). *Pseudomonas*

aeruginosa biofilms grown under high shear were more strongly attached than those grown under lower shear (Stoodley et al. 2002b). Others speculate that turbulent flow may enhance bacterial adhesion and biofilm formation by impinging cells on the surface (Donlan and Costerton 2002). In contrast, rolling of entire staphylococcal microcolonies over surfaces has been observed in biofilms grown under turbulent flow, perhaps allowing mature biofilms to colonize new surfaces downstream (Hall-Stoodley and Stoodley 2005; Rupp et al. 2005). Similarly, *Escherichia coli* attachment to mannose-coated surfaces via the type 1 fimbrial adhesive subunit, FimH, is shear-dependent. At low shear, the cells tended to roll over the surface; however, as shear was increased, they became more firmly attached (Anderson et al. 2007; Thomas et al. 2004). Weak rolling adhesion at low shear force allows for cells to spread out and colonize more surface area than under high shear stress, where cells remain in tight microcolonies. Thus, preferred sites of colonization may be those with the necessary flow to maintain a stable interaction between the bacteria and host proteins (Isberg and Barnes 2002). In a study of *E. coli* biofilm formation under flow, fluid flow altered the spatial organization of cell attachment patterns (Agladze et al. 2003). While these and other studies document the important role of hydrodynamics in biofilm development and structure, little is known about the possible molecular genetic responses to fluid flow.

3 Approach and Initial Attachment to the Surface

3.1 Motility and Chemotaxis

Although both motile and nonmotile species form biofilms, in motile species, the ability to move using flagella or pili is generally required for efficient cell-to-surface attachment. Microscopic observations indicate that motility promotes both initial interaction with the surface and movement along it (O'Toole and Kolter 1998; Pratt and Kolter 1998). However, there are reports suggesting that motility may only be important for biofilm formation under certain conditions (McClaine and Ford 2002). Motility may be needed to overcome the repulsive forces generated between cellular and abiotic surfaces and to permit favorable cell-surface interactions required for attachment (Geesey 2001). However, flagellar motility is not essential for initial adhesion and biofilm formation when the cell is equipped with an efficient adhesin (Jackson et al. 2002b; Prigent-Combaret et al. 2000; Wang et al. 2004). Furthermore, steric hindrance and/or movement caused by a flagellum can destabilize cellular attachments. Accordingly, motility genes are repressed after the bacterium attaches to the surface (Prigent-Combaret et al. 1999). Another example of the complex influence of environmental conditions on motility and biofilm development is the finding that while twitching motility via type IV pili appears to be needed for *P. aeruginosa* biofilm formation (O'Toole and Kolter 1998), overstimulation of twitching by the chelation of iron with lactoferrin, a component

of innate immunity, prevents this bacterium from establishing productive surface contacts and forming biofilm (Singh et al. 2002).

Surface motility is widespread among flagellated Gram-negative bacteria. When it involves groups of long, hyperflagellated cells, moving as an organized mass, it is referred to as swarming motility. In *P. aeruginosa*, swarming motility is regulated through Rhl quorum sensing, while swimming is not. In recent studies, Rhl-dependent quorum sensing and nutritional conditions determined whether a flat, uniform biofilm or a structured biofilm was formed (Shrout et al. 2006). In contrast to motility, chemotaxis is not required for *E. coli* biofilm development in batch cultures (Pratt and Kolter 1998). However, in topologically constrained environments, chemotaxis may be important for assembling a quorum of cells that can initiate biofilm development (Park et al. 2003).

Expression of the genes involved in flagellum synthesis, motility, and chemotaxis in *E. coli* occurs in a hierarchical fashion, permitting ordered synthesis and assembly of the flagellum components (e.g., Macnab 2003; Soutourina and Bertin 2003). The master regulator $FlhD_2C_2$ is a DNA-binding protein that is directly or indirectly required for expression of all other motility and chemotaxis genes, over 50 in total. These are expressed from at least 15 operons, clustered at several regions on the chromosome. Expression of the *flhDC* operon serves as a pivotal point for integrating environmental signals (Fig. 2). Its expression is controlled by numerous regulators including H-NS, Crp, EnvZ-OmpR, CsrA, QseBC, LrhA, and RcsCDB, which sense environmental conditions such as osmolarity (H-NS, EnvZ-OmpR), envelope stress (RcsCDB), nutritional conditions (Crp), or quorum sensing (QseBC).

In *E. coli*, high osmolarity and acetyl-phosphate levels inhibit *flhDC* expression and motility through the phosphorylation and subsequent binding of OmpR to the *flhDC* promoter region (Shin and Park 1995). The synthesis of flagella is also controlled by growth temperature: cells are not flagellated at 42°C, perhaps because of competition for the heat shock chaperones DnaK, DnaJ, and GrpE, which are needed for flagellum gene expression (Shi et al. 1992). Furthermore, *flhDC* and flagellum biosynthesis are regulated by catabolite repression, i.e., activated by the cyclic AMP-Crp complex, and are repressed by the nucleoid-associated protein H-NS (Silverman and Simon 1974; Soutourina et al. 1999). Overall, stressful conditions such as high concentrations of salts, sugars, or alcohols, high temperature, both low and high pH, or conditions of blocked DNA replication inhibit flagellum biosynthesis (Maurer et al. 2005; Shin and Park 1995; Soutourina et al. 2002).

The Csr (carbon storage regulator) system of *E. coli* also controls motility and flagellum biosynthesis. The RNA binding protein CsrA positively regulates *flhDC* expression by binding to the untranslated leader and stabilizing this mRNA (Wei et al. 2001). Although much information has been obtained concerning the regulatory circuitry and mechanisms of this complex system (e.g., Romeo 1998; Suzuki et al. 2002, 2006; Weilbacher et al. 2003), the environmental signals are still somewhat obscure. At the present time, it is evident that quorum sensing via SdiA and environmental pH affect the expression of noncoding RNA antagonists that sequester CsrA (Babitzke and Romeo 2007; Suzuki et al. 2002; Mondragon et al. 2006). Importantly, while CsrA activates motility, its dominant role in biofilm formation

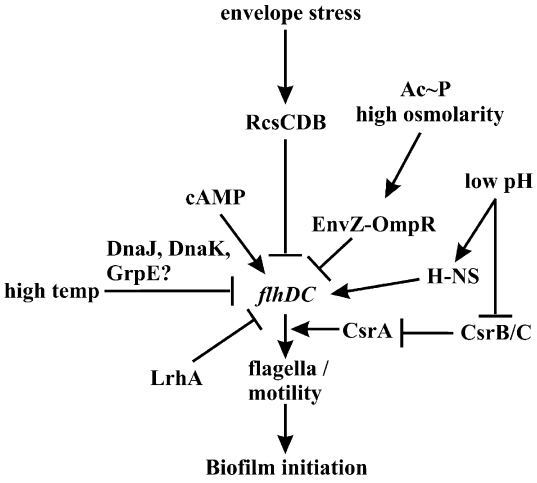

Fig. 2 Regulation of *E. coli* motility. The *flhDC* operon encodes a DNA binding protein ($FlhD_2C_2$) that serves as a central regulatory point to initiate the motility and chemotaxis cascade of gene expression, which is needed for optimal biofilm formation. Stressful conditions such as high concentrations of salts, sugars, or alcohols, high temperature, both low and high pH, or conditions of blocked DNA replication inhibit flagellum biosynthesis. The RcsCDB phosphorelay system, which somehow is activated by envelope stress, represses *flhDC*. Acetyl-phosphate and high osmolarity activate the EnvZ-OmpR two component signal transduction system, which represses *flhDC*. The heat shock chaperones DnaK, DnaJ, and GrpE are needed for flagellum gene expression, but may be limiting at high temperatures. In addition, *flhDC* transcription is under catabolite repression and is activated by cAMP-Crp. The RNA binding protein CsrA activates *flhDC* expression by binding to the untranslated leader and stabilizing this mRNA. However, the main effect of CsrA on biofilm formation is to repress expression of the adhesin PGA (see Fig. 5). LrhA, a LysR-type transcriptional regulator, represses motility as well as expression of type 1 fimbriae. In various species, c-di-GMP, which is synthesized by GGDEF domain-containing proteins and is degraded by EAL domain proteins, inhibits flagellum-based motility

is to repress expression of the polysaccharide adhesin PGA of *E. coli* K-12 (e.g., Wang et al. 2005) and overall it acts as a strong repressor of biofilm formation (Jackson et al. 2002a).

The temporal control of flagellum biogenesis also involves the Rcs phosphorelay and acetyl-phosphate (Fredericks et al. 2006). The Rcs (regulator of capsule synthesis) phosphorelay activates genes required for capsular biosynthesis and membrane proteins (Boulanger et al. 2005), while repressing genes required for flagellum biogenesis (Francez-Charlot et al. 2003). The Rcs regulon is thought to be activated by surface contact and envelope stress; however, the exact nature of the signal remains unknown (reviewed by Majdalani and Gottesman 2005).

Recent studies implicate the ubiquitous bacterial secondary messenger c-di-GMP (3′-5′-cyclic diguanylic acid) as a central regulator of motility and biofilm formation in diverse Gram-negative species. In general, this nucleotide, which is synthesized by GGDEF domain-containing proteins and is degraded by EAL or HD-GYP

domain proteins, affects the transition from planktonic to sessile communities by promoting the production of adhesins and exopolysaccharides and inhibiting flagellum- and pilus-based motility (reviewed in Jenal and Malone 2006; Ryan et al. 2006). While c-di-GMP metabolizing proteins often contain sensory domains (e.g., PAS, GAF, CheY-like, and REC), only a few environmental cues are known or suspected to influence c-di-GMP metabolism, and with the exception of cellulose synthase, the mechanism of action of this nucleotide is unknown. As in the case of Csr regulation, c-di-GMP generally has opposite effects on biofilm formation and motility, consistent with the idea that while motility facilitates initiation of biofilm formation, it may be detrimental at later stages.

3.2 Surface Sensing?

Are bacteria able to sense contact with a surface and respond by expressing adhesins? The Cpx signaling system in *E. coli* has provided some circumstantial evidence for surface sensing. Cpx is a two-component system composed of CpxA, a sensor kinase/phosphatase, and CpxR, a DNA-binding response regulator (Raivio and Silhavy 1997). Studies by Otto and Silhavy (2002) showed that a *cpxR* mutant strain forms altered cell-surface interactions in comparison with the wild type strain and that Cpx-regulated gene expression is enhanced by surface attachment. The mechanism of surface sensing is unknown and may be indirect. Studies indicate that the Cpx system responds to misfolded proteins in the periplasm (Danese and Silhavy 1998). In a microtiter plate assay for biofilm formation, *cpxA* mutants that apparently have lost the phosphatase activity of the CpxA protein formed biofilm with less biomass than wild type strains (Dorel et al. 1999). This was due to decreased transcription of the curlin-encoding gene *csgA* (described Sect. 3.3.1). In uropathogenic *E. coli*, Cpx responds to misfolded pyelonephritis-associated P pilin subunits in the periplasm. In turn, DNA binding by CpxR, in conjunction with other transcription factors, induces transcription from the *papB* and *papI* promoters (Hung et al. 2001). Finally, transcriptome analysis in *E. coli* K-12 showed that *cpxP* is highly expressed in biofilms and affects biofilm structure (Beloin et al. 2004). Whether attachment to a surface leads to denaturation of certain envelope proteins and mediates the proposed surface-sensing by Cpx remains to be determined.

3.3 Environmental Effects on Surface Attachment Proteins

Bacteria make extensive use of proteinaceous extracellular fimbriae or pili, which permit them to establish surface contacts that promote biofilm formation. Fimbriae are generally under complex regulatory controls, often involving multiple physiological and/or environmental inputs. The following discussion presents some examples in which the environmental conditions and genetic regulation of fimbriae

of *E. coli* and its relatives have been examined, illustrating the complexity of the regulatory networks involved in biofilm formation.

3.3.1 Curli

Proteinaceous extracellular fibers called curli were first observed in *E. coli* (Olsen et al. 1989) and have been shown to mediate adhesion, colonization, and biofilm formation in this and other species. In *Salmonella* spp., curli are also known as thin aggregative fimbriae (Romling et al. 1998). In *E. coli*, curli promote both initial adhesion and cell-cell interaction (Prigent-Combaret et al. 2000). A variety of environmental isolates of *E. coli* form biofilms according to their ability to express curli (Castonguay et al. 2006). Curli synthesis in *E. coli* is dependent on at least six genes located in the divergently transcribed *csgBA* and *csgDEFG* operons. CsgD activates transcription of the *csgBA* operon, which encodes CsgA, the structural subunit that is secreted outside of the cell, where CsgB nucleates it into a fiber (Barnhart and Chapman 2006).

Expression of curli is activated under conditions of low temperature; microaerophilic conditions; low nitrogen, phosphate, and iron; low osmolarity; and slow growth or starvation (Gerstel et al. 2003; Maurer et al. 1998; Olsen et al. 1993a, 1993b; reviewed in Barnhart and Chapman 2006) (Fig. 3). These features imply that curli are produced in the external environment, as opposed to in the mammalian host. However, in addition to abiotic surfaces, curli mediate bacterial binding to extracellular matrix proteins such as fibronectin and laminin (Barnhart and Chapman 2006), suggesting that they may be produced in anticipation or preparation for host attachment and colonization. Other studies have indicated that within a biofilm, curli fimbriae may be expressed at 37°C (Kikuchi et al. 2005). Of note, curli are not expressed in many laboratory strains of *E. coli*, due to silencing of the *csgD* promoter (Hammar et al. 1995).

Curli expression responds to environmental conditions through at least three different phosphorelay signaling systems. The EnvZ-OmpR two-component regulatory system activates *csgD* transcription and thereby promotes production of curli fimbriae and stable cell-surface interactions at low osmolarity (Vidal et al. 1998; Prigent-Combaret et al. 2001). However, in conditions of low osmolarity, there is a reduced level of the active response regulator, phosphorylated OmpR, due to the decreased kinase/phosphatase ratio of EnvZ (Cai and Inouye 2002). This would seem to suggest that an increase in osmolarity should result in higher *csgD* transcription and curli biosynthesis. However, high osmolarity has a negative effect on transcription of the curli genes (Prigent-Combaret et al. 2001). This apparent contradiction can be reconciled by the observation that the Cpx pathway, which represses transcription of curli, is induced by high osmolarity and masks OmpR activation (Prigent-Combaret et al. 2001). Whereas CpxR represses *csgD* in high salt concentrations, the nucleoid-associated protein H-NS mediates *csgD* repression in high sucrose, independently of CpxR (Jubelin et al. 2005). Activation of the Cpx pathway by curlin accumulation also results in the repression

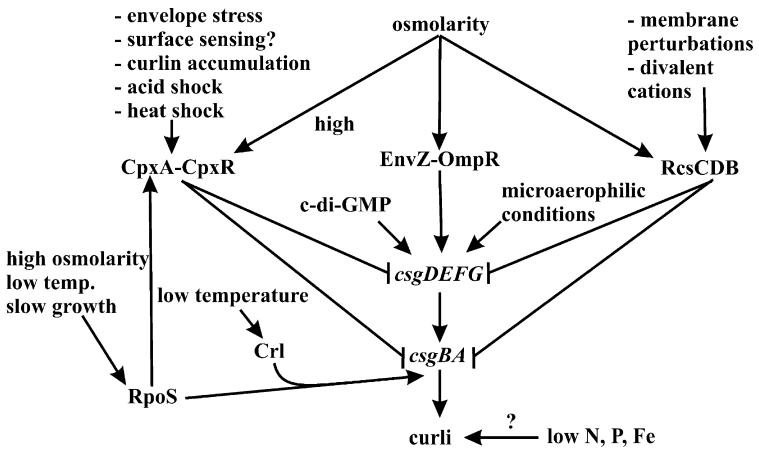

Fig. 3 Conditions affecting curli fimbriae in *E. coli*. Curli fimbriae aid in biofilm formation in certain *E. coli* strains and related species, and are produced through the expression of the divergent operons *csgDEFG* and *csgBA*. CsgD is a DNA-binding protein necessary for transcription of *csgBA*, which encodes the nucleation factor and pilin for curli fimbriae, respectively. Other Csg proteins are involved in pilus biogenesis. Both OmpR-P (activator) and CpxR-P (repressor) can simultaneously occupy the *csgDEFG* promoter. EnvZ-OmpR promotes *csgDEFG* transcription at low osmolarity, while CpxA-CpxR represses this operon under envelope stress and high osmolarity. H-NS has multiple effects on these pathways, one of which is to repress *csgD* in high sucrose, independently of CpxR. The RcsCDB phosphorelay system, which controls synthesis of capsule and flagella, also represses curli in response to membrane perturbations and high osmolarity. c-di-GMP activates production of both curli and cellulose in response to uncharacterized stimuli. Low temperature, nitrogen, phosphorus or iron limitation, slow growth, and microaerophilic conditions promote curli production. RpoS, in conjunction with Crl, activates transcription of the *csgBA* promoter in response to several of these conditions

of the *csgD* and *csgB* operons (Prigent-Combaret et al. 2001). In addition, the RcsCDB phosphorelay system, which controls the synthesis of capsule and flagella, also represses expression of curli (Vianney et al. 2005). A comprehensive model in which EnvZ-OmpR, Cpx, and Rcs regulate *csgD* transcription and curli gene expression in response to changes in osmolarity has been proposed (Jubelin et al. 2005).

Transcription from the *csgD* promoter is also regulated by other global transcription factors, including *rpoS*, *crl*, and *hns* (Romling et al. 1998). The stationary phase sigma factor RpoS (σ^s) directly activates transcription of the *csgBA* promoter in response to slow growth or other stresses (Hengge-Aronis 2002). The small protein Crl, which is preferentially expressed at low temperature and in stationary phase, interacts with the σ^s subunit and apparently promotes curli production by strengthening the association of σ^s with core RNA polymerase to enhance transcription initiation at *csgBA* (Bougdour et al. 2004). The protein H-NS has both direct and indirect effects on curli, depending on the environmental

conditions (Jubelin et al. 2005). Apparently, integration host factor (IHF), H-NS, and OmpR form a nucleoprotein complex with the *csgD* promoter, resulting in elevated expression under microaerophilic growth conditions (Gerstel et al. 2003).

The regulatory nucleotide c-di-GMP (which is produced in response to complex regulatory cues) activates the production of both curli and cellulose in certain *E. coli* strains and in *Salmonella enterica* serovar Typhimurium (e.g., Kader et al. 2006; Weber et al. 2006). Curli and cellulose together produce a strong biofilm matrix facilitating attachment to hydrophilic and hydrophobic surfaces (Zogaj et al. 2003).

3.3.2 Type 1 Fimbriae

Type 1, or mannose-sensitive, fimbriae are rigid, 7-nm-wide and approximately 1-μm-long, rod-shaped surface structures found on the majority of *E. coli* strains and are widespread among the Enterobacteriaceae (Schembri et al. 2001). Type 1 fimbriae are important in the colonization of various host tissues by *E. coli* and in biofilm formation on abiotic surfaces (see the chapter by J.K. Hatt and P.N. Rather, this volume; Pratt and Kolter 1998). The FimH adhesive protein expressed on the tip of type 1 fimbriae binds to glycoproteins, including natural ligands such as uroplakins on urinary epithelial cells in urinary bladders and immunoglobulin A or mucin in intestines and lungs (e.g., Mulvey et al. 2000). A typical type 1 fimbriated bacterium has 200-500 peritrichously arranged fimbriae (Lowe et al. 1987).

Production of type 1 fimbriae requires a polycistronic operon comprising the seven structural genes (*fimAICDFGH*) and two monocistronic operons encoding the site-specific recombinases FimB and FimE. Transcription of type 1 fimbriae genes is phase variable due to FimB- and FimE-mediated inversion of a 314-bp DNA fragment that contains the promoter for the polycistronic *fim* operon (Klemm 1986). Within a cell population, type 1 fimbriae expression is activated at body temperature and is repressed by high osmolarity and low pH. These effects are mediated through altered switching frequency of the *fim* operon promoter (e.g., Gally et al. 1993; Schwan et al. 2002). Although the environmental signals remain to be shown for LrhA, this transcriptional regulator represses motility and chemotaxis genes and represses production of type 1 fimbriae by altering phase variation (Blumer et al. 2005). Furthermore, the alarmone ppGpp (guanosine 3′, 5′-bispyrophosphate), which is produced in response to amino acid or carbon starvation, activates expression of type 1 fimbriae and biofilm formation in uropathogenic *E. coli* through its role in expression of the FimB recombinase (Aberg et al. 2006). Acetyl-phosphate activates production of type 1 fimbriae, perhaps by serving as a phosphodonor for the FimZ response regulator (discussed in Wolfe et al. 2003). Acetyl-phosphate accumulates at the transition to stationary phase in the presence of excess carbon and/or the lack of oxygen (Wolfe 2005). Thus, the production of type 1 fimbriae on cells is complex; it is governed by nutritional status and repressed by stresses such as low pH, low temperature, and high osmolarity.

3.3.3 Antigen 43 and Related Proteins

Antigen 43, encoded by the *flu* locus, is an autoaggregation factor produced by many *E. coli* strains. It was originally discovered for its ability to cause bacterial aggregation (reviewed in Klemm et al. 2006). Antigen 43 is a member of the self-associating autotransporter (SAAT) group of proteins consisting of a signal peptide for transfer across the inner membrane, a translocator domain, and a secreted passenger domain. SAATs, including Ag43, TibA, and AIDA (adhesin involved in diffuse adherence), can interact with each other to cause formation of mixed bacterial aggregates. These proteins are anchored directly to the outer membrane and protrude only approximately 10 nm from the surface, resulting in closer cell-cell interactions than those seen with curli or other fimbriae. Expression of bulky surface structures that protrude beyond this distance in the bacterial envelope (e.g., type 1 pili or capsules) interferes sterically with Ag43-mediated aggregation (Klemm et al. 2006).

Ag43 expression undergoes phase variation controlled by OxyR and Dam (deoxyadenosine methylase). The cellular redox sensor OxyR represses Ag43 expression by binding to the *flu* promoter, while Dam activates Ag43 expression by methylating DNA that overlaps the OxyR binding (Wallecha et al. 2002; see the chapter by C. Beloin et al., this volume). OxyR plays an important role in sensing peroxides encountered during oxidative stress, although its oxidation state may not influence *flu* regulation (Wallecha et al. 2003). It activates protective measures, such as enzymes that detoxify reactive oxygen compounds or repair damage caused by them. Furthermore, Ag43-mediated cell aggregation confers protection from hydrogen peroxide killing (Schembri et al. 2003a). Ag43 and other SAAT proteins, including AIDA-I and TibA, also impair bacterial motility (Ulett et al. 2006). Several studies have indicated that Ag43 is induced specifically during biofilm growth, and its expression enhances *E. coli* biofilm formation (discussed in Klemm et al. 2004; Schembri et al. 2003b). In urinary track infections, Ag43 is expressed by *E. coli* cells that form biofilm-like structures within bladder cells (Anderson et al. 2003).

Finally, environmental pH affects antigen 43-mediated cellular aggregation, which occurs more rapidly as the pH decreases from 10 to 4 (Klemm et al. 2004). This strong effect of pH on cellular aggregation has been proposed to facilitate more rapid transit and thus improved survival in the stomach (Klemm et al. 2006).

4 Conversion from Temporary to Permanent Attachment: A Regulated Process?

During normal biofilm development, some species of bacteria bind to a surface reversibly or temporarily, followed by irreversible or permanent attachment. This phenomenon was first reported in the 1940s (reviewed in Stoodley et al. 2002a). Genes affecting this transition and biofilm development have been studied in *P. aeruginosa* and *Pseudomonas fluorescens* (Caiazza and O'Toole 2004; Hinsa et al. 2003) as well as in *E. coli* (Agladze et al. 2005). In these species, temporarily

attached cells interact with a surface by a cell pole, whereas permanently attached cells are associated via the lateral cell surface. Mutants of *P. fluorescens* that failed to produce a large adhesive protein (LapA) and *E. coli* mutants that fail to produce a polysaccharide adhesin (PGA, described below) were similarly defective in the conversion from temporary to permanent attachment (Agladze et al. 2005; Hinsa et al. 2003). In *E. coli*, the kinetics of this transition process was monitored with an assay developed for this purpose (Agladze et al. 2005).

Conversion from temporary to permanent attachment has been proposed to be a regulated process, perhaps allowing the cell to sample its local environment before committing to a sessile lifestyle (Caiazza and O'Toole 2004). Furthermore, because cell attachment in both monolayers and more mature biofilms of *E. coli* exhibit distinct, nonrandom spatial organization, it has been suggested that proximity to neighboring cells might govern the conversion to permanent attachment (Agladze et al. 2003; 2005). *E. coli* mutants lacking the polysaccharide adhesin PGA exhibited aperiodic cell distribution and no apparent cell-cell adhesion. In theory, formation of such patterns could be guided by a reaction-diffusion or Turing process (e.g., Maini et al. 2006), based on the sensing of a bacterially synthesized inhibitor of attachment. Validation of such hypotheses will require an understanding of the putative signals in the local environment that are being recognized, the putative signal transduction pathways through which this information flows, and a better appreciation of the biochemistry of temporary and permanent attachment processes.

5 Environmental Effects on Matrix Polysaccharides

A hallmark of prototypical biofilms is that they are composed of cells embedded within a complex matrix (reviewed in Branda et al. 2005; Sutherland 2001 a, 2001b). While polypeptides, nucleic acids, lipids, and a host of small molecules are often present in biofilm matrices, polysaccharide, which may include multiple different polymers, is often the main component (e.g., Morikawa et al. 2006; Schooling and Beveridge 2006; Steinberger and Holden 2005; Whitchurch et al. 2002). Due to their roles in cellular interactions with surfaces and their direct exposure to cells of the immune system, matrix polysaccharides have become topics of considerable interest. However, an understanding of these polymers is limited, even for the best studied biofilms. Certain polysaccharides influence biofilm architecture, ion selectivity, resistance to desiccation, and other properties, but probably do not function as biofilm adhesins per se. Acidic polysaccharides, such as alginate of *P. aeruginosa*, colanic acid and K antigens of *E. coli*, and capsular polysaccharides of *Pantoea stewartii* and *Xanthomonas campestris* may be considered in this class; they are not essential for biofilm formation and may even be inhibitory under certain conditions (Crossman and Dow 2004; Hanna et al. 2003; Schembri et al. 2004; Stapper et al. 2004; von Bodman et al. 2003; Wozniak et al. 2003). In contrast, other polysaccharides serve as adhesins that assist cell-surface and/or cell-cell attachment. The conditions and regulatory factors that promote the synthesis of

the latter polysaccharides drive biofilm formation. Polymers that fall into the latter category tend to be basic or neutral, and include β-1,6-*N*-acetyl-D-glucosamine polymers of staphylococci, *E. coli*, *Yersinia pestis*, *Bordetella* species, *Actinobacilli*, and *P. fluorescens* (Heilmann et al. 1996a, 1996b; Itoh et al. 2005; Litran et al. 2002; Maira-Wang et al. 2004; Parise et al. 2007; Kaplan et al. 2004), Psl and Pel of *P. aeruginosa* (Friedman et al. 2004; Jackson et al. 2004; Vasseur et al. 2005), cellulose, which is produced by many eubacteria (reviewed in Lasa 2006), and the extracellular D-glucans of *Streptococcus mutans* (Munro et al. 1995). Some examples that illustrate the complex regulation of poly-β-1,6-*N*-acetyl-D-glucosamine polymers in Gram-positive and -negative bacteria follow.

Poly-β-1,6-*N*-acetyl-D-glucosamine was discovered in *S. epidermidis* (Heilmann et al. 1996 a, 1996b; see the chapter by M. Otto, this volume) and later was found to serve as a biofilm adhesin in Gram-negative bacteria (Wang et al. 2004). This polymer is referred to as PIA (polysaccharide intercellular adhesin) or PNAG in *S. epidermidis* and *S. aureus*, respectively. Production of PIA/PNAG is dependent on the *ica* operon (*icaADBC*), which is regulated by a divergently transcribed gene (*icaR*) that encodes a transcriptional repressor, which responds to various environmental conditions (Conlon et al. 2002; Fig. 4).

Expression of the *icaADBC* operon is increased during growth in nutrient-rich or iron-limiting conditions and is induced by stressful stimuli such as heat, ethanol, and high concentrations of salt which increase *ica* expression and PIA production (Vuong et al. 2005 and references therein). The latter stressors are known to repress tricarboxylic acid (TCA) cycle activity, and the TCA cycle inhibitor fluorocitrate increases PIA production (Vuong et al. 2005). Furthermore, anaerobic conditions induce PIA production (Cramton et al. 2001). Subinhibitory concentrations of tetracycline and the semisynthetic streptogramin antibiotic quinupristin-dalfopristin enhance *icaADBC* expression nine- to 11-fold (Rachid et al. 2000). Ethanol induction of PIA synthesis is *icaR*-dependent (Conlon et al. 2002). Interestingly, glucose addition causes repression of *icaADBC*, but enhances PIA production, possibly via its precursor-product relationship with PIA (Dobinsky et al. 2003).

SarA is a global regulatory DNA-binding protein involved in expression of a variety of staphylococcal virulence genes. Transcription of *icaADBC,* which is essential for biofilm development in *S. aureus*, is activated by SarA binding (Tormo et al. 2005; Valle et al. 2003). In turn, *sarA* is activated by the stress response sigma factor, σ^B, which modulates responses to environmental stress and energy depletion. It is important to note that SarA, but not σ^B, is essential for biofilm development by *S. aureus* (Valle et al. 2003), suggesting that there are other means of activating *sarA* expression. σ^B also represses, possibly indirectly, *icaR* expression (Tormo et al. 2005), indicative of the complex interactions within this regulatory system.

The bacterial LuxS-dependent quorum-sensing systems are found in diverse species, and may permit bacteria to assess the overall microbial density of the environment (Schauder and Bassler 2001; Xavier and Bassler 2003). Biofilm formation in a *luxS* mutant strain of *S. epidermidis* was considerably enhanced, suggesting that the reaction product of the LuxS protein, autoinducer 2 (AI-2), represses *icaADBC* (Xu et al. 2006). Of note, quorum-sensing systems generally promote the

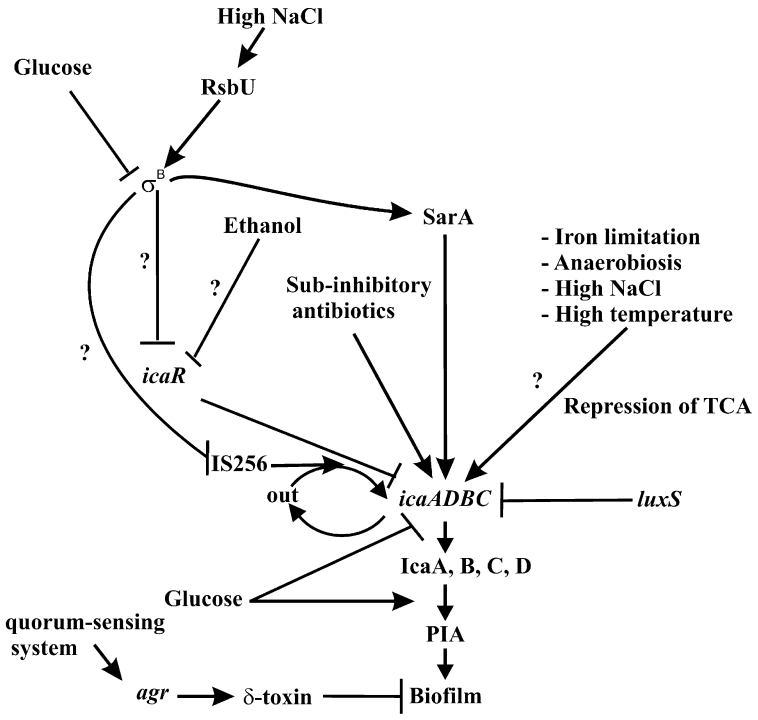

Fig. 4 Environmental influences on staphylococcal polysaccharide intercellular adhesin (PIA). The β-1,6-GlcNAc polymer PIA or PNAG, is required for cell-cell adhesion and biofilm formation in *S. epidermidis* and *S. aureus.* Production of PIA depends on *icaADBC* and is repressed by the divergently transcribed *icaR* gene. *icaABCD* expression is increased by growth in nutrient-replete, iron-limiting, anaerobic, and stress-inducing conditions. Several of these environmental conditions repress tricarboxylic acid (TCA) cycle activity. Subinhibitory concentrations of certain antibiotics also enhance *icaADBC* expression. IS256 causes phase variation by integrating into and excising from *icaADBC* or genes that affect its expression. The global regulator SarA activates transcription of *icaA* and is essential for biofilm development in *S. aureus*. In turn, *sarA* is activated by the general stress sigma factor σ^B, which also represses *icaR* and IS256 transposition. Glucose apparently represses *icaADBC* expression, but nevertheless enhances PIA production via a possible product-precursor relationship. The *agr* quorum sensing system negatively regulates biofilm development

expression of factors required for biofilm formation (Kirisits and Parsek 2006; Kong et al. 2006, Spoering and Gilmore 2006); although another example of quorum-sensing inhibition of biofilm formation is found in *Vibrio cholerae* (Hammer and Bassler 2003). A well-studied quorum-sensing system of *S. epidermidis* and *S. aureus*, *agr* (accessory gene regulator), also inhibits biofilm formation, but does not affect PIA levels (Vuong et al. 2003).

The IS256 insertion element is able to integrate into and inactivate or excise from *icaADBC* or genes that affect *ica* expression (e.g., *sarA*), thus constituting

a phase-variable mode of regulation (Conlon et al. 2004; Ziebuhr et al. 1999). Transposition of IS256, but not transcription of the transposase, is repressed by σ^B (Valle et al. 2007). Valle and colleagues believe that environmental stress conditions activate σ^B and decrease the generation of biofilm-negative variants, in line with evidence indicating that NaCl and other stressors induce *ica*-dependent biofilm formation. The authors of this study also speculate that the IS256 element may modulate biofilm dispersal by affecting the proportion of biofilm-negative variants in a biofilm.

In *E. coli*, an understanding of biofilm regulation preceded the discovery of the *pgaABCD* structural genes, which in turn led to the identification of novel regulatory genes for biofilm formation (Fig. 5). An initial observation was that the global regulatory gene *csrA* of *E. coli* dramatically represses biofilm formation (Romeo et al. 1993), a phenotype that could not be explained by any previously known adhesin (Jackson et al. 2002a). A genetic screen for factors that cause hyper-biofilm formation in the *csrA* mutant led to discovery of the *pgaABCD* locus, which encodes gene products similar to the glycosyltransferase IcaA (PgaC) (Gerke et al. 1998)

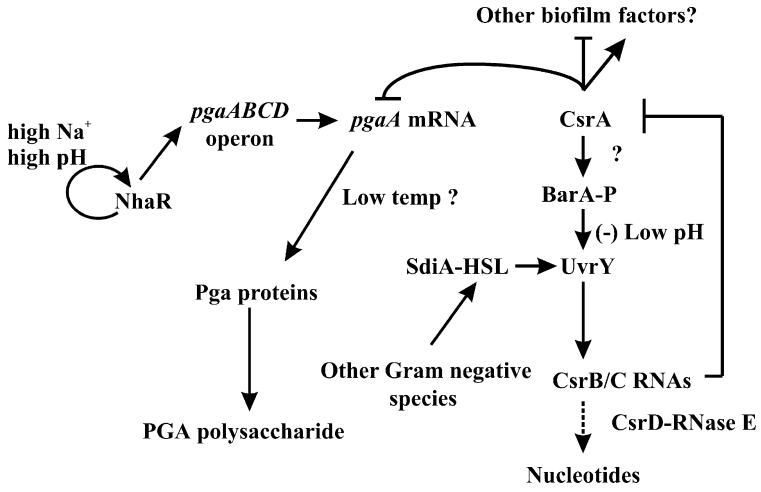

Fig. 5 Regulation of the biofilm adhesin PGA in *E. coli*. Poly-β-1,6-*N*-acetyl-glucosamine (*PGA*) synthesis is regulated at several levels. NhaR binds to the *pgaABCD* promoter and activates transcription in response to high pH or high sodium ion concentrations. CsrA protein binds to six sites in the leader of the *pgaABCD* transcript, which blocks ribosome binding and accelerates the turnover of this mRNA. Expression of *csrA* is activated as cultures approach the stationary phase, by unknown mechanism(s). In addition, CsrA is sequestered by the noncoding RNAs CsrB and CsrC. Transcription of these RNAs requires the BarA-UvrY two-component signal-transduction system, and CsrA itself. The signal for this system is not known, although BarA-UvrY signaling is blocked at low pH. SdiA activates *uvrY* transcription upon binding to *N*-acyl-homoserine lactones (*HSL*). *E. coli* does not produce HSL. Therefore, *csrB* and *csrC* transcription should be enhanced in the presence of Gram-negative species that produce such quorum-sensing compounds. CsrB and CsrC RNAs are degraded by a pathway involving a possible sensory protein, CsrD, and RNase E

and the *N*-deacetylase IcaB (PgaB) (Vuong et al. 2004). The mechanism of CsrA in this regulation is to bind to the *pgaABCD* mRNA leader at six sites, including sites that overlap the Shine-Dalgarno sequence and initiation codon, and thereby prevent ribosome binding (Wang et al. 2005). Translational repression likely results in the observed destabilization of this transcript by CsrA. Transcription of the *pgaABCD* operon is activated by the binding of the LysR family protein NhaR to the sole promoter of this operon in response to high pH or high Na^+ (Goller et al. 2006). The biosynthesis of PGA is also regulated by c-di-GMP (Suzuki et al. 2006; A. Pannuri, C. Goller, and T. Romeo; Y. Itoh and T. Romeo, unpublished studies) and is increased at low temperature (Wang et al. 2005; unpublished data). The latter findings are reminiscent of regulation in the homologous *hmsHFRS* system of *Y. pestis* (Bobrov et al. 2005; Perry et al. 2004; Simm et al. 2005).

The *hmsHFRS* operon and *hmsT* are required for *Y. pestis* biofilm formation in the gut of the flea vector and are important in the transmission of plague (see the chapter by Hinnebusch and Erickson). Hms-dependent biofilm formation is optimal at low temperature. The levels of HmsH, HmsR, and HmsT proteins are lower at 37°C than at 26°C, and temperature-dependent degradation of HmsH, HmsR, and HmsT proteins seems to be responsible for the Hms^- phenotype at 37°C (Perry et al. 2004). Additionally, biofilm formation is stimulated by HmsT, a protein that synthesizes c-di-GMP, and is inhibited by HmsP, which likely degrades c-di-GMP (Bobrov et al. 2005).

6 Conditions and Factors Mediating Biofilm Dispersal

No doubt, there are times when it is advantageous for cells to be able to escape from a biofilm. Entrapment within the biofilm environment limits bacterial growth (e.g., Rani et al. 2007). Furthermore, the transcriptome of mature biofilm, on average, has been suggested to be more similar to that of stationary phase cells than of exponentially growing cultures, although many changes in gene expression appear to be biofilm-specific (Beloin et al. 2004; Sauer et al. 2002; Waite et al. 2005). In addition, the biofilm matrix may prevent or at least deter cells from fleeing deleterious conditions. Dispersal processes are of interest because of their potential to promote spread of bacteria in the environment and because of the possibility to exploit these processes to combat detrimental biofilms. Release of cells or clumps of cells from biofilm can be accomplished by constitutive low level sloughing as well as active dispersion in which a substantial proportion of the population synchronously exits the biofilm. Several different cellular patterns of biofilm dispersal or escape have been documented under microscopic examination (reviewed by Hall-Stoodley and Stoodley 2005). In addition, the dissolution of cell attachments by surfactant production in *Bacillus subtilis*, *P. aeruginosa* and *S. epidermidis* may help to shape biofilm architecture (Boles et al. 2005; Branda et al. 2001; Davey et al. 2003; Vuong et al. 2003).

Environmental conditions that influence biofilm dispersal include nutrient availability, oxygen levels, pH, and specific compounds (Gjermansen et al. 2005;

Jackson et al. 2002a; Sauer et al. 2004; Thormann et al. 2005; Table 1). Changes in nutrient availability are a well-recognized determinant of dispersal. This is not surprising, given the importance of nutrient acquisition to bacterial survival. For example, early studies revealed that introduction of a rich medium to a tightly-aggregated *Acinetobacter* biofilm that had been grown under low nutrient conditions led to a more open, widely dispersed cell arrangement (James et al. 1995). Although the molecular genetics of biofilm dispersal has lagged behind that of formation, recent breakthroughs have paved the way for understanding the dispersal process, from the detection of environmental cues to signal transduction circuitry to the biochemical activities responsible for dispersal.

Biofilm dispersion in *P. aeruginosa* is perhaps the most studied and best understood process. Sauer and coworkers examined the proteome of this bacterium during active dispersion and found expression patterns that more closely resembled those of planktonic cells than biofilm cells (Sauer et al. 2002). Specific carbon nutrients, including succinate and glutamate, were found to trigger immediate large-scale release of cells (Sauer et al. 2004). Genes for motility, ribosomal proteins, and phage Pf1 were induced in the dispersed cells, while cells remaining attached contained elevated transcripts for pilus production and anaerobic nitrogen respiration. The latter activity indicates that insufficient oxygen was available for complete aerobic metabolism of the added carbon substrate in this biofilm.

Table 1 Molecular genetics of biofilm dispersal processes in Gram-negative bacteria

Organism	Environmental cue	Signal transduction	Output	Reference
P. aeruginosa	Carbon nutrients	BdlA, c–di–GMP	Adhesins?	Morgan et al. 2006
P. aeruginosa	Nitric oxide	?	Phage induction, other?	Barraud et al. 2006
P. aeruginosa	Quorum sensing (*las/rhl*)	?	Phage induction	Purevdorj–Gage et al. 2005
P. putida	Carbon starvation	c–di–GMP?	LapA protein? Polysaccharide?	Gjermansen et al. 2005, 2006
X. campestris	Quorum sensing	Rpf signal pathway	β–1,4–Mannanase	Dow et al. 2003
S. oneidensis	Anaerobic conditions	c–di–GMP?	Polysaccharide?	Thormann et al. 2005, 2006
E. coli	Quorum sensing, other?	Csr system	PGA, other?	Jackson Wang et al. 2002a; et al. 2004, 2005
A. actinomycete mcommitans	?	?	PGA hydrolase (dispersin B)	Itoh et al. 2005; Kaplan et al. 2003, 2004

The above observations support the recent discovery that trace amounts of nitric oxide (NO) or a metabolite thereof mediates dispersal (Barraud et al. 2006). This product of anaerobic respiration facilitated the seeding dispersal of cells from mature biofilm. In this phage Pf1-dependent process, mature biofilm structures appear to liquefy internally, involving both cell death and release of viable cells, and leaving behind hollow, shell-like structures. In addition, exposure to NO dispersed immature biofilms without causing cell death. The normal resistance of biofilm cells to certain antibacterial agents reverted back to the planktonic, sensitive phenotype during this dispersion process. Sauer and coworkers recently identified a gene encoding an apparent chemotaxis protein, BdlA, which is crucial for nutrient dispersal of *P. aeruginosa* (Morgan et al. 2006). A mutant lacking this protein also exhibited increased adherence and increased c-di-GMP levels. The latter observation is consistent with a rapidly expanding role of this nucleotide in stimulating bacterial exopolysaccharide synthesis and enhancing adherence properties of cells (reviewed in Jenal and Malone 2006; Romling and Amikam 2006), and the correlation of degradation of this nucleotide with biofilm dispersal (e.g., Gjermansen et al. 2006; Morgan et al. 2006 Thormann et al. 2006). It is tempting to suggest that BldA might regulate c-di-GMP levels in response to, perhaps even by binding to NO. This is consistent with the observations that (1) nutrient-induced dispersal leads to increased anaerobic respiration and (2) the BdlA protein structure includes a PAS domain, which is typically involved in signal detection. Another consideration is that lung infections of cystic fibrosis patients by *P. aeruginosa* become anaerobic. How these observations might apply to this host environment is still an open question (discussed in Romeo 2006).

There are parallels in other species that suggest dispersal processes that are related, though not identical, to those of *P. aeruginosa*. *Shewanella oneidensis* biofilm disperses rapidly under anoxic conditions and is likewise induced by an increase in c-di-GMP levels and possibly mediated via effects on exopolysaccharide production (Thormann et al. 2006). Is it possible that the cue for this process might not be the decrease of oxygen, but rather the production of NO or another product of anaerobic respiration? *Pseudomonas putida* responds to carbon starvation by inducing dispersal in a process that might involve c-di-GMP regulation, exopolysaccharide and a large proteinaceous adhesin that has also been studied in *P. fluorescens* (Gjermansen et al. 2005; 2006; Hinsa et al. 2003; 2006).

Studies in *E. coli* suggest that CsrA may facilitate dispersal (Jackson et al. 2002a). This RNA-binding protein, alternatively referred to as RsmA (repressor of stationary phase metabolites) in some species, posttranscriptionally represses production of the biofilm polysaccharide adhesin β-1,6-*N*-acetyl-D-glucosamine, or PGA, with dramatic effects on biofilm formation (Wang et al. 2005). The mechanism of CsrA in biofilm dispersal remains unknown, but could be based on inhibition of PGA synthesis if this polysaccharide is continuously removed by turnover or sloughing. The relatively slow rate of biofilm release (a few hours) that occurs in response to *csrA* induction suggests that the way in which CsrA affects dispersal may be different than in the preceding examples. CsrA activity is governed to a large extent by noncoding regulatory RNAs that sequester this protein, e.g., CsrB,

CsrC in *E. coli* (Gudapaty et al. 2001; Liu et al. 1997; Suzuki et al. 2002; Weilbacher et al. 2003). Thus, CsrA activity should increase as CsrB and CsrC synthesis decreases or their turnover increases. The environmental control of Csr RNAs is not well defined in any species. However, it typically involves transcriptional activation via BarA-UvrY or homologous two-component signal transduction systems, such as BarA-SirA, ExpS-ExpA, GacS-GacA, or VarS-VarA, and is connected to quorum-sensing pathways (Lenz et al. 2005; Suzuki et al. 2002; reviewed in Babitzke and Romeo 2007). Furthermore, while quorum-sensing systems often promote biofilm formation, they can activate biofilm dispersal in some species (e.g., Dow et al. 2003; Hammer and Bassler 2003; Yarwood et al. 2004).

7 Mixed Species Biofilms

The natural environments that most bacteria inhabit are typically complex and dynamic. Unfortunately, this complexity is not fully appreciated when growing organisms in monocultures under laboratory conditions. Biofilm communities associated with the plant rhizosphere (Ramey et al. 2004), intestinal mucosa (Eckburg et al. 2005), oral cavity and gingival crevices (Kroes et al. 1999; Kolenbrander 2000), and many other natural sites are inhabited by numerous different species in close proximity. Such environments are rich in biological stimuli to be processed by bacterial cells and used to direct biofilm development in response to changing conditions.

Studies using species-specific probes and microscopy have revealed complex spatial organization of species in natural biofilm communities (e.g., Bottari et al. 2006). Furthermore, co-culture experiments have demonstrated the importance of competition for nutrients and commensal metabolic networks in the dynamics of mixed-species biofilms (e.g., Christensen et al. 2002). Thus, spatial and metabolic interactions between species contribute to the organization of multispecies biofilms, and the production of a dynamic local environment (Battin et al. 2007; Tolker-Nielsen and Molin 2000). The distribution of cells and biomass in complex biofilms is influenced by the physiology of the organisms present, which in turn leads to the development of local nutrient gradients. Furthermore, mixed species biofilms can evolve rapidly and lead to stable interactions between species when driven by selective pressure for co-metabolism (Hansen et al. 2007). In the latter model system, a commensalistic relationship was established between *Acinetobacter* sp. strain C6 and *P. putida* KT2440 when the latter species evolved the ability to adhere and form biofilm close to *Acinetobacter* microcolonies, and thereby capture the metabolite benzoate.

Mutualistic relationships can also occur in mixed-species biofilms. For example, biofilm formation by *E. coli* PHL565 was synergistically enhanced by growth in mixed culture with *P. putida* MT2 (Castonguay et al. 2006). Particularly striking mutualistic effects on biofilm formation have been shown for species that inhabit dental plaque (e.g., Palmer et al. 2001). Conjugative plasmids have been demonstrated to induce

bacterial biofilm development in co-culture experiments (e.g., Ghigo 2001), and biofilm formation increases the chance for lateral gene transfer and thus the risk for interspecies gene transfer and the consequent spread of virulence factors and antibiotic resistance (e.g., Weigel et al., 2007). In fact, many examples of synergistic induction of biofilm formation were observed when a large collection of nondomesticated *E. coli* strains were individually cocultivated with a laboratory strain or with each other. This was most often precipitated by conjugal transfer of natural plasmids carried by the isolates.

Quorum sensing can have somewhat unpredictable effects on biofilm formation (Merritt et al. 2003; Schauder and Bassler 2001; Waters and Bassler, 2005; Bassler and Losick 2006). While many quorum-sensing systems are relatively species-specific, the autoinducer-2 based (or LuxS-dependent) quorum-sensing system is widespread among eubacteria and may serve as a universal language for these organisms. Despite its profound implications, the impact of interspecies communication on biofilm development is presently not well understood.

8 Conclusions and Outlook

Recent advances in our understanding of the biofilm development cycle have indicated that in most cases, it is a dynamic process in which common environmental factors such as nutritional conditions, temperature, oxygen tension and osmolarity have strong influences. We have begun to understand the factors and pathways that respond to environmental cues and regulate the surface transformations that drive the biofilm development cycle. The distinctive conditions that govern biofilm development for a given species can provide important clues to its natural ecology and life cycle and vice-versa. Many surprises lay in store, and the rules for biofilm development seem to be made to be broken. For example, the minimalist bacterium *Mycoplasma pulmonis* lacks any two component signal transduction system or recognizable global regulator. Nevertheless, it is able to modulate biofilm formation through slipped-strand mispairing of the gene for an adhesive surface protein (Simmons et al. 2007).

At the present time, there are many unanswered or partially answered questions concerning the influence of the bacterial environment on biofilm development:

1. Which steps in development are most important for regulation and how are these steps regulated? The transition from reversible to irreversible attachment would seem to be an important site for regulation to occur, but this remains to be shown.
2. While a variety of environmental influences on biofilm are now known in a few model organisms, information on their relative importance and integration is lacking.
3. Systematic analysis of gene expression by array studies has provided much information concerning gene expression patterns during biofilm development.

How are these genes regulated and which of these genes are critical for the development process?

4. How does the presence of other microorganisms and growth in association with eukaryotic hosts influence biofilm formation by a given species? This is a complex biological question that likely differs for each species of interest. Nevertheless, it is critical for the development of new therapeutic strategies and other applications.

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