Biofilm Development

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ABSTRACT During the past decade we have gained much knowledge about the molecular mechanisms that are involved in initiation and termination of biofilm formation. In many bacteria, these processes appear to occur in response to specific environmental cues and result in, respectively, induction or termination of biofilm matrix production via the second messenger molecule c-di-GMP. In between initiation and termination of biofilm formation we have defined specific biofilm stages, but the currently available evidence suggests that these transitions are mainly governed by adaptive responses, and not by specific genetic programs. It appears that biofilm formation can occur through multiple pathways and that the spatial structure of the biofilms is species dependent as well as dependent on environmental conditions. Bacterial subpopulations, e.g., motile and nonmotile subpopulations, can develop and interact during biofilm formation, and these interactions can affect the structure of the biofilm. The available evidence suggests that biofilm formation is programmed in the sense that regulated synthesis of extracellular matrix components is involved. Furthermore, our current knowledge suggests that biofilm formation mainly is governed by adaptive responses of individual bacteria, although group-level activities are also involved.

INTRODUCTION

Experimental approaches primarily focused on genetic and microscopic techniques have laid the foundation for our current models of bacterial biofilm formation. This work has enabled researchers to define biofilm formation as a process that consists of specific stages. The biofilm developmental cycle is believed to include (i) initial attachment of microbes to a surface or each other, (ii) formation of microcolonies, (iii) maturation of the biofilm, and (iv) dispersal of the biofilm (e.g., reference 1). The different biofilm stages include bacterial physiology and phenotypic responses suggestive of the existence of a unique biofilm biology which is not found for planktonic bacteria.

The switch from the solitary planktonic bacterial lifestyle to the communal biofilm lifestyle involves a change in the bacteria so that they initiate the production of adhesins and extracellular matrix compounds which interconnect them in the biofilm. The extracellular biofilm matrix serves as a scaffold that has an essential cell-to-cell connecting and structural function in biofilms and plays a role in a number of processes including cell attachment, cell-to-cell interactions, and antimicrobial tolerance (2–7). The biofilm matrix that is produced by the bacteria contains mainly polysaccharides, proteins, and extracellular DNA (8).

This article focuses on several issues of biofilm formation, including initiation of biofilm formation, development of biofilm structure, formation of biofilms through multiple pathways, bacterial motility during biofilm formation, roles of quorum-sensing in biofilm formation, subpopulation development and interactions, biofilm formation governed by adaptive responses, and termination of biofilm formation in response to environmental cues.
BIOFILM FORMATION INITIATES IN RESPONSE TO SPECIFIC ENVIRONMENTAL CUES

Recent work in a number of bacterial species, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Vibrio cholerae*, *Yersinia pestis*, *Escherichia coli*, *Salmonella enterica*, *Burkholderia cenocepacia*, *Bacillus subtilis*, and *Clostridium difficile*, indicates that initiation of biofilm formation occurs in response to an increase in the level of the intracellular second messenger c-di-GMP (9–24). Synthesis and degradation of c-di-GMP in the bacteria is accomplished by two distinct classes of proteins with opposing enzymatic activities (see reference 25 for a review). Diguanylate cyclases harboring GGDEF domains synthesize c-di-GMP from two GTP molecules, whereas phosphodiesterases harboring EAL or HD-GYP domains degrade c-di-GMP. Sensory domains are frequently associated with the GGDEF and EAL/HD-GYP domain proteins, translating diverse environmental cues into c-di-GMP levels. The bacteria usually produce several different c-di-GMP diguanylate cyclases and phosphodiesterases, and evidence is accruing that they work in separate c-di-GMP circuits (26). Our current knowledge suggests that a number of different environmental cues and transducer mechanisms can lead to increases in local pools of c-di-GMP, which in turn can activate the production of adhesins and extracellular matrix products (e.g., references 27, 28). For example, in *P. aeruginosa* the WspA protein was found to be a membrane-bound receptor that detects a signal associated with contact to a surface (27, 29, 30). The signal is mediated to the histidine kinase WspE, which catalyzes phosphate transfer to the response regulator-diguanylate cyclase WspR, which in turn produces c-di-GMP. Synthesis of biofilm matrix products such as the CdrA adhesin and the Psl, Pel, and alginate exopolysaccharides in turn is positively regulated by c-di-GMP in *P. aeruginosa* (18, 31).

In addition to c-di-GMP-mediated regulation, biofilm formation is also regulated via small regulatory RNAs (sRNA) in many bacterial species (32). For example, work with *P. aeruginosa* suggests that synthesis of the exopolysaccharides Psl and Pel is induced in response to environmental signals sensed by the sensor kinases LadS and RetS and the sensor kinase/response regulator pair GacS/GacA (33–38). Phosphorylation of GacA by GacS is antagonized by the RetS sensor kinase and stimulated by the LadS sensor kinase. After phosphorylation of GacA by GacS, GacA induces transcription of the RsmY and RsmZ sRNAs. The RsmY and RsmZ sRNAs bind to and reduce the activity of the RsmA protein, which otherwise inhibits expression of a number of genes, including those encoding Psl and Pel polysaccharide production. In addition, sensor kinases and response regulators encoded by the *bfi*SR, *bfm*SR, and *mif*SR genes are evidently involved in the regulation of *P. aeruginosa* biofilm formation (39). Evidence was provided that the BfiSR system regulates biofilm formation by affecting synthesis of the CafA ribonuclease, which controls the level of the RsmZ sRNA (40). Moreover, the protein SagS was shown to contribute to the motile-to-sessile switch and act in concert with BfSR to initiate *P. aeruginosa* biofilm formation (41).

Moscoso et al. (42) provided evidence that a *P. aeruginosa* retS mutant contains increased levels of c-di-GMP, indicating a link between sRNA-mediated and c-di-GMP-mediated biofilm regulation in *P. aeruginosa*.

THE ARCHITECTURE AND ORGANIZATION OF BIOFILMS ARE SPECIES DEPENDENT

Although microcolonies are the basic unit in most biofilms, the structure of the microcolonies can vary greatly depending on the biofilm-forming bacterial species. For example, it has been demonstrated that under identical conditions in a flow chamber *P. putida* forms loose protruding microcolonies (Fig. 1A), whereas *Pseudomonas knackmussii* (formerly termed *Pseudomonas* sp. B13) forms spherical microcolonies (Fig. 1B) (43). Moreover, when the two *Pseudomonas* species were grown together in dual-species biofilms, they still formed their characteristic microcolony structures (Fig. 1C), apparently without affecting each other (43). The architecture and organization of the three different biofilms are therefore dependent on the biofilm-forming bacterial species. Multiple factors are involved in the formation of particular structures in biofilms, and currently the mechanisms underlying the differences in biofilm structure displayed by *P. putida* and *P. knackmussii* is not known. However, in the case of *P. putida*, biofilm formation in flow chambers is mainly governed by the large adhesive protein LapA (9, 10, 44), whereas for other pseudomonads, such as *P. aeruginosa*, biofilm formation in flow chambers is mainly dependent on the exopolysaccharides Psl and Pel (7, 45, 46). Differences between the extracellular matrix components that interconnect bacteria in biofilms may give rise to different structures of the microcolonies.
As described above, different bacterial species may form different biofilm structures under identical conditions. In addition, the same bacterial species may form different biofilm structures under different environmental conditions. For example, Klausen et al. (47) demonstrated that P. aeruginosa forms mushroom-shaped microcolonies when it grows in flow chambers that are irrigated with glucose medium, whereas it forms flat biofilms when it grows in flow chambers that are irrigated with citrate medium (Fig. 2). Moreover, the structure of an established biofilm can change in response to a change in nutritional conditions. Nielsen et al. (48) studied biofilm formation in flow chambers of a mixture consisting of P. knackmussii and Burkholderia xenovorans (formerly termed Burkholderia sp. LB400). These bacteria have the potential to interact metabolically because P. knackmussii can metabolize chlorobenzoate produced by B. xenovorans when grown on chlorobiphenyl. When the dual-species biofilm was fed with medium containing chlorobiphenyl, mixed-species microcolonies consisting of associated P. knackmussii and B. xenovorans bacteria were formed. In contrast, when the mixture was fed citrate, which can be metabolized by both species, the two species formed separate microcolonies. After a shift in carbon source from a citrate medium to a chlorobiphenyl medium, movement of the P. knackmussii bacteria led to a change in the spatial structure of the biofilm from the separate microcolonies toward the mixed-species microcolonies.

Similar observations were made by Wolfaardt et al. (49), who studied a microbial mixture capable of degrading the herbicide diclofop. When this mixture was grown in flow chambers irrigated with diclofob, a highly structured biofilm with specific patterns of intergeneric cellular coaggregation was formed. But when the mixture was grown on tryptic soy broth (TSB), a biofilm lacking variation in thickness and structure was formed. After a shift in carbon source from TSB to diclofob, it took TSB-grown biofilms only two days to acquire the typical structure of diclofob-grown biofilms. Although the organisms and the nature of the metabolic interactions in the study (49) were unknown, this study provided substantial evidence that structure development in biofilms is dependent on nutritional conditions and that the structure of an established biofilm can change in response to a change in nutritional conditions.

BACTERIAL MOTILITY MAY BE AN INTEGRATED PART OF BIOFILM FORMATION

The studies described above suggest that bacterial migration is involved in the structural changes that can occur in biofilms in response to a change in nutritional conditions. However, it appears that bacterial migration can be important for the structural development of biofilms also under stable environmental conditions.

FIGURE 1 Confocal laser scanning microscopy (CLSM) images showing spatial structures in flow-chamber-grown 5-day-old biofilms formed by (A) Gfp-tagged (green fluorescent) P. putida, (B) Gfp-tagged P. knackmussii, and (C) a mixture of Gfp-tagged P. putida and DsRed-tagged (red fluorescent) P. knackmussii. Bars, 20 μm. Adapted from reference 43 with permission from the American Society for Microbiology. doi:10.1128/microbiolspec.MB-0001-2014.f1
The available evidence suggests that *P. aeruginosa* forms flat biofilm in flow chambers irrigated with citrate medium because the bacteria migrate extensively during the initial phase of biofilm formation and do not settle to form microcolonies (47). *P. aeruginosa pilA* mutants (deficient in biogenesis of type IV pili) were shown to form protruding microcolonies in flow chambers irrigated with citrate medium, indicating that the motility that occurs in citrate-grown *P. aeruginosa* wild type biofilms is driven by type IV pili (47). In flow chambers irrigated with glucose medium *P. aeruginosa* forms two distinct subpopulations in the initial phase of biofilm formation: a nonmotile subpopulation that forms initial microcolonies and a motile subpopulation that initially migrates on the substratum (50). Formation of the mushroom-shaped structures in the glucose-grown biofilms evidently involves colonization of the initial microcolonies by bacteria from the migrating subpopulation that subsequently form mushroom caps on top of the initial microcolonies, which then correspond to the stalk of the mushroom-shaped structures (50). In glucose-grown *P. aeruginosa* biofilms containing a mixture of Cfp-tagged pilA mutants and Yfp-tagged wild type bacteria, mushroom-shaped structures that contain pilA mutants in the stalk and wild type in the cap are formed (50) (Fig. 3). In addition, mushroom-shaped structures that contain wild type bacteria in both the stalk and cap can also be found (Fig. 3). These findings provide evidence that bacterial migration may be an integrated part of biofilm formation.

**FIGURE 2** CLSM micrographs acquired in 5-day-old *P. aeruginosa* PAO1 biofilms grown on (A) glucose minimal medium and (B) citrate minimal medium. The central pictures show top-down fluorescence projections, and the flanking pictures show vertical sections. Bars, 20 μm. Adapted from reference 47 with permission from Wiley-Blackwell publishing. doi:10.1128/microbiolspec.MB-0001-2014.f2

**BIOFILM FORMATION CAN OCCUR THROUGH MULTIPLE PATHWAYS**

As described above, the formation of biofilm structures is both species-specific and dependent on environmental conditions, suggesting that biofilm formation can occur through multiple pathways. Under similar conditions the mechanisms underlying biofilm formation for closely related species such as *P. aeruginosa* and *P. putida* may be very different. As indicated above, the formation of *P. putida* biofilm in flow chambers is mainly dependent on the large adhesive surface protein LapA (9, 10, 44), whereas the formation of *P. aeruginosa* biofilm in flow chambers usually depends on exopolysaccharides such as Psl and Pel (7, 45, 46). However, it is also conceivable that a specific bacterial species may form biofilm through different pathways under different conditions. *P. aeruginosa*, for example, can produce a number of biofilm matrix products, including the Psl, Pel, and alginate polysaccharides; extracellular DNA; and the type IV pili, Cup, CdrA, LecA, LecB, and Fap protein components (2, 31, 45, 51–57). It may be expected that some *P. aeruginosa* strains selected for under specific
conditions will produce some of these biofilm matrix components in higher quantities than other strains and that the biofilm pathways will vary accordingly.

Whitchurch et al. (2) reported that the presence of DNase in the medium prevented biofilm formation by the *P. aeruginosa* PAO1 laboratory strain in microtiter trays and flow chambers, suggesting that extracellular DNA is an important matrix component in these biofilms. Addition of DNase to young flow-chamber-grown *P. aeruginosa* PAO1 biofilms led to dispersal but did not disperse mature biofilms, probably due to production of increasing amounts of other biofilm matrix components during biofilm maturation. In contrast, Nemoto et al. (58) found that mature biofilms formed by four independent clinical *P. aeruginosa* isolates could be dispersed by DNase treatment, suggesting that extracellular DNA is the primary cell-to-cell interconnecting compound in mature biofilms formed by these *P. aeruginosa* strains. In addition, Murakawa (59, 60) investigated the chemical composition of the biofilm matrix from 20 clinical *P. aeruginosa* isolates and found that the biofilm matrix from 18 strains consisted primarily of DNA, while 2 strains with a mucoid phenotype produced slimes composed primarily of alginate.

Differences in biofilm structure may give rise to different phenotypic responses, e.g., to antibiotic treatment. Many types of biofilms display a remarkable increased tolerance to antimicrobial treatment compared to planktonic bacteria (61). The mechanisms that contribute to biofilm-associated antimicrobial tolerance include restricted antimicrobial diffusion, differential physiological activity, induction of specific tolerance mechanisms, and persister cell formation (62). However, similar phenotypic responses to antibiotic treatment may have different underlying conditions even for biofilms formed by the same species and displaying similar structure. For example, tolerance to tobramycin in *P. aeruginosa* PA14 biofilms can be caused by periplasmic glucans that bind tobramycin and prevent cell death most likely by sequestering the antibiotic (63). Synthesis of the periplasmic glucans requires the *ndvB* gene in *P. aeruginosa* PA14, and biofilms formed by a *P. aeruginosa ndvB* mutant were found to be much more sensitive to tobramycin than wild type biofilms. In contrast, the *ndvB* mutant and wild type showed no difference in tobramycin sensitivity when grown in planktonic culture. Reverse transcriptase PCR provided evidence that the *ndvB* gene was expressed specifically in *P. aeruginosa* PA14 biofilms and not in planktonic cells (63). However, microarray analysis has provided evidence that *ndvB* is expressed at the same low level in biofilm and planktonic cells of *P. aeruginosa* PAO1 (64), and therefore the *ndvB*-mediated mechanism appears to be restricted to specific *P. aeruginosa* strains. However, *P. aeruginosa* PAO1 biofilms also display tolerance to tobramycin treatment, and in this case extracellular DNA seems to be of primary importance because it binds tobramycin and shields the antibiotic (65).

### QUORUM-SENSING CAN PLAY IMPORTANT ROLES IN BIOFILM FORMATION

In agreement with the fact that bacteria are closely associated in biofilms, quorum-sensing has been shown to play a role in biofilm formation for various bacterial species, e.g., *P. aeruginosa* and *B. cenocepacia*. Davies et al. (66) showed that a quorum-sensing-defective mutant strain of *P. aeruginosa* formed flat and undifferentiated...
biofilms in flow-chambers under conditions where the wild type strain formed biofilms with large mushroom-shaped structures. In addition, Hentzer et al. (67) demonstrated that acylated homoserine lactone analogues, which inhibit quorum-sensing, affected P. aeruginosa biofilm formation in flow chambers. Subsequent studies provided further evidence for the importance of quorum-sensing signaling in the structural development of P. aeruginosa biofilms (e.g., references 4, 68). Quorum-sensing plays a role in the generation of extracellular DNA in P. aeruginosa biofilms (69). In addition to a small amount of extracellular DNA present in the initial phase in P. aeruginosa biofilms, release of a large amount of extracellular DNA occurs at a later stage of P. aeruginosa biofilm formation, regulated via the Pseudomonas quinolone signal (PQS)-based quorum-sensing system (69). Evidence has been provided that the release of DNA in P. aeruginosa biofilm occurs as a consequence of lysis of a small subpopulation of the bacteria (69). In agreement with a role for PQS-based quorum-sensing in the generation of extracellular DNA that functions as a biofilm matrix component, P. aeruginosa pqs mutants were found to have defects in biofilm formation, and the thin biofilms that were formed by the pqs mutants contained little extracellular DNA (4, 68–70). The mechanism involved in PQS-mediated DNA-release is unknown, but evidence has been provided that PQS can act as a cell-sensitizing pro-oxidant (71), which might cause lysis of a subpopulation of the P. aeruginosa bacteria during biofilm formation.

In addition to extracellular DNA, quorum-sensing signaling controls the production of the biosurfactant rhamnolipid, which was shown to be important for biofilm formation by P. aeruginosa (72–74). Furthermore, the production of the P. aeruginosa LecA and LecB lectins was shown to be regulated by quorum-sensing (75), and the lectins were shown to play a role in P. aeruginosa biofilm formation (56, 57). Quorum-sensing signaling in P. aeruginosa also controls the production of siderophores such as pyoverdine and pyochelin, which are also of importance for biofilm formation (76).

Although quorum-sensing evidently controls the production of many factors which are important for P. aeruginosa biofilm formation, the role of quorum-sensing appears to be conditional, because quorum-sensing mutant strains evidently form biofilms indistinguishable from wild type biofilms under some conditions (77). A more definitive requirement for quorum-sensing in biofilm formation seems to be the case for B. cenocepacia. Inhülsen et al. (78) and Schmid et al. (79) identified three main factors which potentially link quorum-sensing signaling to B. cenocepacia biofilm formation: type-1 fimbriae, the BclACB lectins, and the large surface protein BapA. These factors are all under quorum-sensing regulation in B. cenocepacia and were investigated in detail for their roles in biofilm formation. Deletion of neither the type-1 fimbriae genes nor the adjacent chaperone-usher transport system genes affected biofilm formation by B. cenocepacia in microtiter trays or flow chambers, indicating that type-1 fimbriae are not essential for biofilm formation by B. cenocepacia on abiotic surfaces. Deletion of the bclACB operon did not result in any significant difference in biofilm formation in microtiter trays compared to the wild type strain. In contrast, the deletion of bapA, as well as the adjacent genes encoding a putative ABC transporter system responsible for the secretion of BapA, resulted in a significant reduction in biofilm formation in microtiter trays and flow chambers. Downregulation of BapA expression through a rhamnose-inducible promoter in the bclACB mutant resulted in a defect in biofilm formation compared to the corresponding wild type strain, with reduced bapA expression, suggesting that lectins are important in surface colonization when BapA is limiting and that BapA is the major factor for biofilm formation.

In addition to the fimbriae, lectins, and BapA, quorum-sensing also seems to regulate the production of extracellular DNA in B. cenocepacia biofilms. McCarthy et al. (80) provided evidence that four-day-old B. cenocepacia wild type biofilms grown in flow chambers contained substantial amounts of extracellular DNA as a part of the biofilm matrix. In contrast, biofilms formed by a quorum-sensing-defective B. cenocepacia mutant never passed the initial microcolony stage, and the thin biofilms showed little staining by propidium iodide, indicating that extracellular DNA was not generated in large quantities (80).

BACTERIAL SUBPOPULATIONS DEVELOP AND INTERACT DURING BIOFILM FORMATION

As described above, the different bacteria in mixed-species biofilms can engage in metabolic interactions, and this affects biofilm formation and the structure of the biofilm. However, it also appears that subpopulations develop and interact in mono-species biofilms and that these interactions affect biofilm formation and the structure of the biofilms. For example, the motile and nonmotile subpopulations that can develop during P. aeruginosa biofilm formation appear to interact in a number of ways. Yang et al. (4) provided evidence that
the PQS quorum-sensing system and genes necessary for pyoverdine synthesis are expressed only in the stalk part of the mushroom-shaped microcolonies that form in glucose-grown *P. aeruginosa* biofilms. PQS quorum-sensing controls the production of extracellular DNA in *P. aeruginosa* biofilms (69), and evidence was provided that release of extracellular DNA by the bacteria in the initial microcolonies (which subsequently constitute the stalk part of the mature mushroom-shaped microcolonies) is necessary for development of the mushroom-shaped microcolonies (4). Furthermore, evidence was presented that pyoverdine production in the initial microcolonies is necessary for iron uptake in the cap-forming subpopulation and for development of the mushroom-shaped microcolonies (4).

The propositions made by Yang et al. (4) were mainly based on genetic evidence. Experiments involving *P. aeruginosa* strains with *pqsA*-gfp and *pvdA*-gfp fluorescent reporters provided evidence that the genes required for PQS quorum-sensing and pyoverdine synthesis were expressed specifically in the stalk part of the mushroom-shaped microcolonies in *P. aeruginosa* biofilms. The three mutant strains *P. aeruginosa* pilA, *P. aeruginosa* *pvdA* (deficient in pyoverdine production), and *P. aeruginosa* *pqsA* (deficient in PQS quorum-sensing) were shown to be unable to form mushroom-shaped structures individually in single-strain biofilms. *P. aeruginosa* pilA mutants could only form the initial microcolonies because type IV pili are necessary for cap formation, and it appeared that the *P. aeruginosa* *pvdA* and *P. aeruginosa* *pqsA* mutant strains also had a defect in cap formation. However, mushroom-shaped structures with pilA mutants in the stalk and *pqsA* mutants in the cap were formed in *pilA/pqsA* mixed-strain biofilms. Likewise, mushroom-shaped structures with pilA mutants in the stalk and *pvdA* mutants in the cap were formed in *pilA/pvdA* mixed-strain biofilms. It appears that the pilA, *pvdA*, and *pqsA* mutants cannot form mushroom-shaped microcolonies individually in single-strain biofilms, but in *pilA/pqsA* mixed-strain biofilms and *pilA/pvdA* mixed-strain biofilms the subpopulations interact with each other, and together they form mushroom-shaped microcolonies.

**Biofilm Development**

**Biological Formation is Programmed in the Sense That Regulated Synthesis of Extracellular Matrix Components Is Involved, But It Is Also Governed by Adaptive Responses**

Observations of the mushroom-shaped microcolonies that can form in flow-chamber-grown *P. aeruginosa* biofilms have led to comparisons between *P. aeruginosa* biofilm microcolonies and the fruiting bodies that are formed by *Myxococcus* bacteria (66), and it has led to speculation about biofilm formation as a highly programmed developmental process with hierarchically ordered genetic pathways controlling attachment, microcolony formation, and microcolony maturation (81). The developmental model implies that the structure of biofilms has evolved to provide a specific function. For example, it has been proposed that the ability of *P. aeruginosa* to form mushroom-shaped microcolonies in biofilms has developed as a result of group-level evolution, because these structures give efficient nutrient supply to the bacteria and efficient removal of waste products (82). However, the available evidence suggests that the mushroom-shaped microcolonies are formed in the flow-chamber-grown *P. aeruginosa* biofilms mainly as a consequence of the special conditions in the flow chamber and the existence of motile and nonmotile subpopulations. Because of bacterial nutrient consumption, there is a decreasing nutrient gradient from the top to the bottom of the biofilm in the flow chamber (83). The motile bacteria that accumulate on the top of the microcolonies therefore have a growth advantage in comparison to the bacteria in the lower part of the biofilm and can proliferate to form the mushroom caps (84). As described above, extracellular DNA released by the nonmotile bacteria appears to be important for settling of the motile bacteria in the cap portion of the mushroom-shaped microcolonies (4). In addition, exopolysaccharide production, rhamnolipid surfactant production, quorum-sensing, and siderophores are also known to be involved in formation of the mushroom-shaped microcolonies (85). It is presently not understood why the *P. aeruginosa* population differentiates into nonmotile and motile subpopulations in the initial stage of biofilm formation in glucose-fed flow chambers and not in citrate-fed flow chambers.

If biofilm formation is a highly programmed process, it would be expected that a core set of “biofilm genes” would be expressed in all biofilms of a given bacterium. In the case of *P. aeruginosa* a number of microarray analyses have been performed to monitor genes that are expressed during biofilm formation (e.g., 64, 86–88). However, the transcriptomic analyses performed by various research groups have failed to consistently identify specific biofilm regulons. The failure to detect common themes in the gene expression profile of *P. aeruginosa* biofilm cells suggests that biofilm formation is mainly governed by adaptive responses. This is in agreement with the fact that the structural development...
of *P. aeruginosa* biofilms and different mixed-species biofilms is dependent on the nutritional conditions and can change in response to changing nutritional conditions, as described above. However, formation of biofilms does require expression of biofilm matrix products. In the case of *P. aeruginosa* these biofilm matrix products include Psl, Pel, alginate, extracellular DNA, type IV pili, Cup, CdrA, LecA, LecB, and Fap (2, 6, 31, 45, 52–57). The synthesis of many of these products is positively regulated by the intracellular signaling molecule c-di-GMP (e.g., see reference 25 for a review). As mentioned previously, synthesis and degradation of c-di-GMP in the bacteria is accomplished by diguanylate cyclases and phosphodiesterases that contain sensory domains, enabling translation of diverse environmental cues into synthesis or degradation of c-di-GMP, which binds to downstream effector molecules and modulates their function, resulting in regulation of the production of different adhesins and biofilm matrix products. It therefore appears that biofilm formation is programmed in the sense that regulated synthesis of extracellular matrix components is involved. However, although researchers have defined a number of biofilm stages, there are currently no data that couple the initial c-di-GMP-regulated matrix production to specific stages occurring later during biofilm formation. In addition, there is currently no evidence that the different c-di-GMP signaling circuits are hierarchically ordered to control transitions through specific stages in biofilm formation. Biofilm-specific pathways may be limited to the initial regulation of adhesin and matrix production, whereas the successive stages of biofilm formation may be governed by adaptive responses.

**BIOFILM FORMATION IS LARGELY GOVERNED BY ADAPTIVE RESPONSES OF INDIVIDUAL BACTERIA, BUT GROUP-LEVEL ACTIVITIES ARE ALSO INVOLVED**

As mentioned above, biofilm formation can be viewed as a developmental process or as a process governed by adaptive responses of individual bacteria. Biofilm formation as a developmental process involves biofilm-specific genes that are part of hierarchically ordered pathways dedicated to controlling the transition through specific biofilm stages. Biofilm formation governed by adaptive responses involves the ability of the individual bacteria to regulate cellular adhesiveness and motility in response to micro-environmental cues. The developmental and adaptive response hypotheses can be distinguished in evolutionary terms, because the former involves selection of a given trait because of its benefit to the group, whereas the latter involves selection of a given trait because of its benefit to the individual bacterium. The apparently cooperative traits in biofilms can in many cases alternatively be explained by the fitness advantages of this behavior for the individual bacterium. For example, the formation of regular mushroom-shaped structures in *P. aeruginosa* biofilms via a pathway that involves type IV pili-dependent cellular migration may be a coordinated social process that creates biofilms with architectures which allow optimal circulation and nutrient supply to the population, or it may, because nutrient gradients develop in biofilms, be the result of chemotaxis of individual bacteria moving to a favorable nutrient-containing micro-environment.

The production of the cell-to-cell interconnecting components in biofilms may be a cost each bacterium pays to contribute to the social activity of creating a protective biofilm domicile, or it may simply increase the adhesiveness of single bacterial cells, allowing them to persist in the specific environment. Quorum-sensing-dependent production of extracellular DNA and lectins in *P. aeruginosa* biofilms (69, 75), as well as quorum-sensing-dependent production of surface protein, fim-briae, and lectins in *B. cenocepacia* biofilms (78), may be interpreted as group-level activities. However, instead of a means to regulate production of specific factors at the group level, quorum-sensing may be diffusion-sensing that enables the individual bacterium to determine whether secreted molecules rapidly move away from the cell (89). Yet the observed involvement of quorum-sensing in DNA release from a lysing subpopulation in *P. aeruginosa* biofilms, resulting in biofilm stabilization, may be interpreted as social behavior because the lysing cells that provide the DNA obviously are not themselves benefited directly.

**BIOFILM FORMATION TERMINATES IN RESPONSE TO SPECIFIC ENVIRONMENTAL CUES**

In agreement with the suggestion that biofilm formation is mainly governed by adaptive responses, and does not involve stable or meta-stable developmental changes, the bacteria in biofilms can at any stage sense environmental cues and terminate biofilm formation in response to specific signals. In the case of *P. putida*, for example, it was shown that established flow-chamber-grown biofilms can disperse completely within a few minutes in response to a shift from a medium with a carbon source to a medium without a carbon source or in response to a
stoppage in the flow of growth medium (10). Biofilm formation by P. putida in flow chambers is mainly governed by the large adhesive protein LapA (9, 44). Evidence has been provided that c-di-GMP signaling regulates biofilm formation in P. putida by controlling the presence of LapA on the cell surface (9). The presence of LapA on the cell surface is controlled by the proteins LapD and LapG in response to the intracellular level of c-di-GMP. The LapG protein is a periplasmic proteinase, and it is able to cleave LapA off the cell surface when it is not repressed. The LapD protein spans the cytoplasmic membrane and contains degenerate GGDEF and EAL domains which can bind c-di-GMP, and it regulates the activity of the LapG proteinase by repressing it when the intracellular level of c-di-GMP is high and derepressing it when the intracellular level of c-di-GMP is low. A c-di-GMP phosphodiesterase gene in P. putida, homologous to the P. aeruginosa bifA gene, has recently been identified as being essential for starvation-induced dispersal of P. putida biofilms along with the lapG gene, suggesting a link between P. putida BifA phosphodiesterase activity and LapG-mediated dispersal (90).

The work described above, together with structural and biochemical work done in P. fluorescens (91, 92), suggests that P. putida can rapidly terminate biofilm formation in response to starvation through the following mechanisms: (i) nutrient limitation leads to activation of the BifA phosphodiesterase via an unknown pathway; (ii) activation of the BifA phosphodiesterase leads to a decrease in the c-di-GMP level in the vicinity of the transmembrane LapD protein; (iii) the reduction in the c-di-GMP level causes dissociation of c-di-GMP from the LapD protein; (iv) dissociation of c-di-GMP from LapD causes derepression of the periplasmic LapG protease; (v) the derepressed LapG protease cleaves off the cell-associated LapA protein; (v) cleavage of the LapA protein leads to the release of the cells from the biofilm.

CONCLUDING REMARKS
During the past decade we have gained much knowledge about the molecular mechanisms that are involved in the initiation and termination of biofilm formation. In many bacteria, these processes appear to occur in response to specific environmental cues and result in, respectively, induction or termination of biofilm matrix production via the second messenger molecule c-di-GMP. In between initiation and termination of biofilm formation we have defined specific biofilm stages, but the currently available evidence suggests that these transitions are mainly governed by adaptive responses, and not by specific genetic programs. It appears that biofilm formation can occur through multiple pathways and that the spatial structure of the biofilms is species dependent as well as dependent on the environmental conditions. Bacterial subpopulations, e.g., motile and nonmotile subpopulations, can develop and interact during biofilm formation, and these interactions can affect the structure of the biofilm. The available evidence suggests that biofilm formation is programmed in the sense that regulated synthesis of extracellular matrix components is involved. Furthermore, our current knowledge suggests that biofilm formation is mainly governed by adaptive responses of individual bacteria, although group-level activities are also involved.

The realization that a number of separate c-di-GMP signaling circuits regulate the production of different biofilm matrix components in bacteria in response to specific environmental cues has been a major step forward in biofilm research. However, although we have defined a number of biofilm stages, there is currently no data that couple the initial c-di-GMP-regulated matrix production to specific stages occurring later during biofilm formation, and there is currently no evidence that the various c-di-GMP signaling circuits are hierarchically ordered to control the transition through specific stages in biofilm formation. Future research will show whether biofilm-specific pathways are limited to the initial and terminal c-di-GMP-mediated regulation of adhesin and matrix production, with the in-between stages of biofilm formation governed by adaptive responses, or whether the different c-di-GMP signaling circuits are hierarchically ordered to control the transition through specific stages in biofilm formation.

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