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The Matrix Reloaded: How Sensing the Extracellular Matrix Synchronizes Bacterial Communities

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In response to chemical communication, bacterial cells often organize themselves into complex multicellular communities that carry out specialized tasks. These communities are frequently referred to as biofilms, which involve the collective behavior of different cell types. Like cells of multicellular eukaryotes, the biofilm cells are surrounded by self-produced polymers that constitute the extracellular matrix (ECM), which binds them to each other and to the surface. In multicellular eukaryotes, it has been evident for decades that cell-ECM interactions control multiple cellular processes during development. While cells both in biofilms and in multicellular eukaryotes are surrounded by ECM and activate various genetic programs, until recently it has been unclear whether cell-ECM interactions are recruited in bacterial communicative behaviors. In this review, we describe the examples reported thus far for ECM involvement in control of cell behavior throughout the different stages of biofilm formation. The studies presented in this review have provided a newly emerging perspective of the bacterial ECM as an active player in regulation of biofilm development.

In natural settings, bacterial cells are most often found in the form of multicellular aggregates commonly referred to as biofilms (1, 2). Thus, bacterial cells are similar to many other living cells, which are capable of unicellular existence but generally reside within multicellular communities. Biofilms offer their member cells several benefits, such as improved attachment to hosts and more-efficient access to nutrients (3). For example, the oligotrophic bacterium *Caulobacter crescentus*, which lives in a particularly nutrient-deficient environment, uses a biofilm-related adhesion mechanism to improve its access to organic matter in its habitat (4). In contrast to these benefits, cells in the interior part of the biofilm may experience oxygen deficiency (5–7). The three-dimensional (3D) structure of the biofilm has been suggested to relieve this stress. For example, channels formed below the ridges and wrinkles within bacterial colonies may facilitate diffusion of fluids, nutrients, and oxygen (8–11). The arrangement of cells in the biofilm structure might expose the cells to different levels of oxygen, nutrients, and quorum-sensing molecules, thereby affecting the genetic programs they express (5, 12, 13).

Elucidation of the pathophysiology of bacterial biofilms bears significant clinical relevance, as biofilms account for over 80% of microbial infections in the human body (14). In a biofilm, microorganisms can be up to 1,000 times more resistant to antibiotics than planktonic (free-living) bacteria and can more effectively evade the immune system (15–19), rendering them extremely difficult to eradicate. For instance, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* cause persistent biofilm infections that incur significant costs and morbidity (3, 20). The ability of *Vibrio cholerae*, which is the agent of the diarrheal disease cholera, to create a biofilm is important for its survival in the host environment as well as in aquatic ecosystems that serve as its reservoir (21, 22). On the other hand, *Bacillus subtilis* biofilms can be beneficial, as they colonize plant roots and play a key role in protecting the host from fungal and other bacterial infections (23, 24).

Given their ubiquity and importance in the microbial world, it is hardly surprising that biofilms have attracted the attention of the scientific community. Identification of the fundamental principles and molecular mechanisms underlying microbial multicellularity is the focus of much research. One common principle is the production of extracellular substances that, upon assembly, constitute an extracellular matrix (ECM) (25). Although the ability to generate an ECM appears to be a common feature of multicellular bacterial communities, there is remarkable diversity in the means by which these matrices are constructed. The different types of bacterial ECMs are discussed in more detail in the first section of this review.

Biofilm formation can be seen as a developmental process in which various genetic programs are activated in a specific order in different subpopulations of cells for the proper establishment of a functional structure (9, 10, 12, 26–29). Others have suggested that this apparent coordination is triggered by temporally distinct exposure of cell subpopulation to specific microenvironments (12). In this model, the timed activation of the genetic programs is triggered by the various degrees of exposure to oxygen, nutrients, or signaling molecules and is not due to a dedicated inherent developmental program. Importantly, while independent observations of biofilm formation in the spore-forming bacterium *B. subtilis* favored the developmental model (29–33), it does not necessarily apply to other bacteria.

In considering the developmental model, it is tempting to speculate that the bacterial ECM is involved in regulation of genetic programs in designated subpopulations of cells in the biofilm. In multicellular eukaryotes, it has been evident for decades that cell proliferation, cell migration, tissue morphogenesis, and

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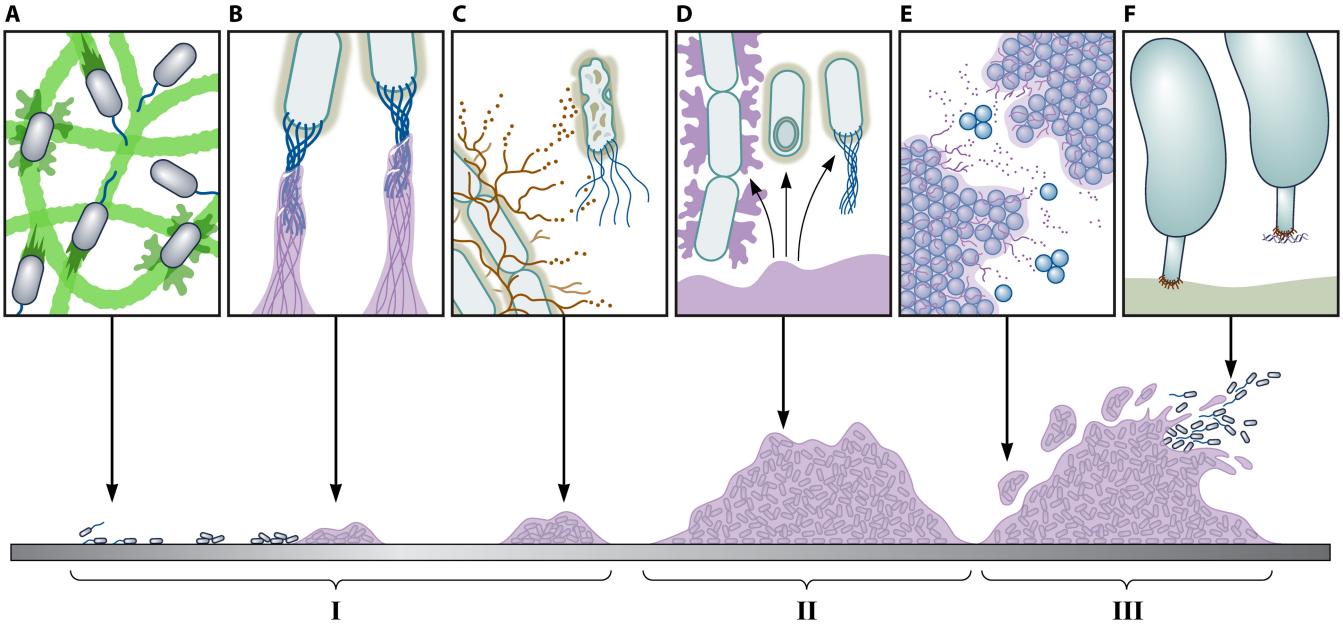

FIG 1 Signals from the ECM during biofilm development. (Bottom panel) Scheme of the different stages of biofilm development. (I) Attachment, monolayer formation, and aggregation. (II) 3D structure development and patterning. (III) Dispersal. (Top panel) (A) *P. aeruginosa* PAO1 deposits trails of high local concentrations of ECM (in green) that attract other cells and induce further ECM production (114, 128). (B) In *B. subtilis*, inhibition of the rotation of the flagella, e.g., by the viscous environment of the ECM, induces ECM production (150, 151). (C) TasA (brown), a structural amyloid in *B. subtilis* ECM, can be toxic to vegetatively growing *B. subtilis* cells (80). (D) During *B. subtilis* biofilm development, ECM induces emergence of different cell subpopulations: motile cells, ECM-producing cells, and sporulating cells (31). (E) In *S. aureus* PSMs, peptides (purple) can create structural amyloid fibers (85) but have a destabilizing effect on the biofilm in their monomeric form (176). (F) In *C. crescentus* biofilms, eDNA induces dispersal by inhibiting the reattachment of the mature stalked cell by binding to the exopolysaccharides of the holdfast (dark brown) (175).

homeostasis all depend on cell-ECM interactions (34). Such interactions involve adhesion receptors (e.g., integrins [35]), intercellular adhesion complexes (e.g., tight junctions [36]), and extracellular polysaccharides (e.g., glycosaminoglycans [37]), which activate cellular receptors. In contrast, the bacterial ECM has long been thought to function merely as a passive extracellular scaffold that holds the biofilm together and protects resident cells from environmental stresses.

In this review, we first describe the structural roles of different exopolymeric substances that constitute the ECM of various biofilms. Then, we focus on studies that demonstrate the role of some of these ECM components in regulating genetic programs of the biofilm cells (Fig. 1). The examples presented in this review are divided according to the three stages of biofilm formation (38), i.e., (i) attachment, exploration of the surface, monolayer formation, and aggregation, (ii) 3D structure development and patterning, and (iii) dispersal.

ECM COMPONENTS IN BACTERIAL BIOFILMS

The most extensively studied biofilm ECM components are carbohydrate-rich polymers (i.e., extracellular polysaccharides or exopolysaccharides), proteins, and nucleic acids (25).

Exopolysaccharides. Exopolysaccharides generated by bacteria have been recognized to significantly impact bacterial virulence and promote capsule formation. Various genetic analyses have provided strong evidence that biofilm exopolysaccharides play a fundamental structural role in different bacterial species. Mutants defective in the production of exopolysaccharides display severe defects in biofilm formation and in achieving complex biofilm architecture. Common bacterial exopolysaccharides include cellulose (39–43) and the staphylococcal polysaccharide intercellular adhesin (PIA) (44). PIA-related polymers are produced by *Staphylococcus epidermidis* and *S. aureus* (45, 46) and by several Gram-negative bacterial species (47–52). In *S. epidermidis* and *S. aureus*, PIA synthesis is mediated by the *icaADBC* operon (53–55). The PIA molecule contains both positive and negative charges, which are important for its adhesive properties (44). Many staphylococcal isolates lack *ica* and are still capable of producing biofilms, indicating alternative routes of biofilm formation (see below).

Many bacterial species are capable of producing several different exopolysaccharides, simultaneously or differentially, as a function of environmental factors or the genetic background of the specific strain. For instance, the ECM of *P. aeruginosa* biofilms can contain three exopolysaccharides: alginate, Psl, and Pel (56).

Alginate is a polymer of manuronic acid and guluronic acid, and its synthesis is mediated by the *alg* operon, as well as by 12 additional genes (57). *P. aeruginosa* isolates from lungs of cystic fibrosis (CF) patients overproduce alginate, resulting in a mucoid colony phenotype. Alginate is not essential for biofilm formation, as the laboratory strains of *P. aeruginosa* PAO1 and PA14 do not produce alginate and are capable of forming a submerged biofilm in a microtiter plate assay (58). However, mutants in alginate biosynthesis form biofilms with an altered architecture compared to the parental strains (59, 60).

Pel and Psl, the other two exopolysaccharides produced by *P. aeruginosa*, play redundant roles in defining ECM structure (61). Psl is composed of mannose, rhamnose, and glucose and is synthesized by the gene products of the *pslA-O* operon (62–65). Pel is

a glucose-rich polymer, and its synthesis is mediated by the *pelA-G* operon (66, 67). As the PAO1 and PA14 laboratory strains do not produce alginate, they rely on Pel and Psl production for their biofilm formation (58). However, the ECM of PAO1 contains mostly Psl, while PA14 cannot produce Psl and its primary exopolysaccharide is Pel (61, 67–69). In addition to its structural role, Psl is specifically important for surface attachment (61, 70).

The biofilm of the *B. subtilis* soil bacterium is strengthened by several exopolysaccharide polymers as well. These exopolysaccharides are produced by the *epsA-O* operon and are composed of glucose, galactose, and *N*-acetylgalactosamine (71). Colonies of mutants in the *epsA-O* operon are not as structured and wrinkled as the wild-type colonies (72). When *B. subtilis* is grown in sucrose-rich growth medium, it is also capable of producing levan, a fructan biopolymer, via the *sacB-yveB-yveA* levansucrase tricistronic operon (73).

Proteinaceous components. The proteinaceous components of the biofilm ECM are especially intriguing. We first focus on bacterial functional amyloids. Amyloids are insoluble fibrous aggregates of proteins that contain parallel beta sheets, first identified in human neurodegenerative diseases (74). In bacteria, however, amyloid fibers were found to be the major proteinaceous component of the microbial ECM and are produced by both Gram-negative and Gram-positive bacterial species. In Gram-positive bacteria, the best-characterized functional amyloids are the TasA amyloid fibers, produced by *B. subtilis* (75–77). The TasA amyloid fibers are attached to the cell wall and mediate cell-to-cell adhesion in conjunction with other extracellular components (76). TapA, an additional protein encoded by the *tapA-sipW-tasA* operon, is a minor extracellular component incorporated within the TasA fibers (78). SipW, the product of the third gene in the *tapA-sipW-tasA* operon, is a signal peptidase that processes both TasA and TapA to their mature forms (79–82). SipW also promotes adherence of *B. subtilis* to surfaces (75, 83, 84). This second function of SipW is independent of its function in TasA and TapA maturation and involves a distinct signal peptidase domain (84). Functional amyloids are surprisingly common structural ECM components in a variety of bacterial biofilms, including *S. aureus*, *Pseudomonas* species, *Salmonella enterica* serovar *enteritidis*, and *E. coli* (85–89).

Amyloid fiber proteins, like TasA, show self-assembly properties. BslA, another protein component in the ECM of *B. subtilis*, also self-polymerizes into a structural element (90, 91). BslA is a cell surface-associated amphiphilic protein which is reminiscent of hydrophobin of filamentous fungi (90). BslA forms a hydrophobic coat on the surface of *B. subtilis* biofilms that may enhance protection against environmental insults (90).

However, not all proteinaceous components of a biofilm are functional amyloids. Cell-cell and cell-host tissue contacts within a biofilm can be mediated by surface adhesins. Adhesins are surface proteins of bacteria that promote the adhesion of cells to the host tissues. Among them are *S. aureus* biofilm-associated proteins Bap and SasG (92) and fibronectin binding proteins FnBPA and FnBPB (93). Intriguingly, FnBPA and FnBPB have been shown to stimulate integrin signaling and actin rearrangements in host cells, supporting the notion of ECM-cell signaling between bacteria and their host (94). In *V. cholerae*, the ECM is composed of three structural proteins—RbmA, RbmC, and Bap1 (95–97)—and the exopolysaccharide *Vibrio* polysaccharide (VPS). Berk et al. (98) simultaneously visualized these proteins during biofilm development in a chambered cover glass. Interestingly, the proteins exhibited distinct localization patterns and were not distributed uniformly throughout the biofilm. During the development of the biofilm structure, Berk et al. noted that the cells arranged in defined clusters and were encapsulated by Bap1 and RmbC, which interacted with the VPS exopolysaccharide. In contrast, RmbA was found only within the cell clusters and was essential for their formation. Taken together, these observations indicate that each structural biofilm protein in *V. cholerae* plays a specific role.

eDNA. Extracellular genomic DNA (eDNA) was found to be an important structural component in many bacterial biofilms (99, 100). Addition of DNase to growing or mature biofilms of various bacterial species results in inhibition of biofilm formation or disruption of the established biofilms (101). In *S. aureus*, *S. epidermidis*, *V. cholerae*, and *P. aeruginosa* PAO1, the DNase-driven disruption of established biofilms was dependent on biofilm age; young biofilms were more sensitive to DNase than older biofilms (102–105). Thus, eDNA is important for the structure of the young biofilm but its role is later taken over by other exopolymers (101). The chemical nature of the long, charged DNA molecule is thought to modulate the cell surface properties and to promote cell-to-cell and cell-to-surface adhesion (100). In addition, eDNA was shown to interact with other ECM components, such as exopolysaccharide and protein components, which has been suggested to add to structure stability (101). For instance, type IV pili (T4P) in *P. aeruginosa* can bind to eDNA and in this manner may mediate cell attachment to the eDNA scaffold (106). eDNA is released to the ECM from lysed cells or via eDNA secretion mechanisms suggested to exist in certain species (99). In *S. aureus*, the mechanism that controls cell lysis was shown to be analogous to the bacteriophage holin-antiholin system (107). Holins are membrane proteins that facilitate access of endolysins to the cell wall, leading to peptidoglycan cleavage and to cell lysis (108). Antiholins are proteins that inhibit holin activity. The *S. aureus* CidA and LrgA proteins act as holin and antiholin, respectively (109–113). Deletion of the *cidA* gene or the *lrgAB* operon results in altered cell lysis and disrupted biofilm structure, mediated by a change in the amount of released eDNA (102, 113).

The different components of the ECM, i.e., exopolysaccharides, proteins, and eDNA, were recently demonstrated to participate in several nonstructural roles, which are discussed in length in the following sections.

ECM SIGNALING DURING BACTERIAL EXPLORATION OF THE SURFACE

In many cases, bacterial biofilms are surface associated and are suggested to require a stimulus-response mechanism to coordinate between surface attachment and ECM production. Thus, it is not surprising that bacteria developed numerous ECM-derived signaling mechanisms that induce biofilm initiation (Fig. 1, panel I).

In the *P. aeruginosa* PAO1 strain, the presence of exopolysaccharide Psl was found to act as a signal that activates the production of bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) (114). c-di-GMP is a second messenger prevalent in numerous bacterial species (115). It is a key regulator in the planktonic-to-biofilm switch, mediated by activation of biofilm production and repression of motility (115–117). Intracellular c-di-GMP concentrations are tightly regulated by the counteractive action of diguanylate cyclases that produce c-di-GMP from two GTP molecules and of phosphodiesterases that break it down to 5′-phosphoguanylyl-

(3′-5′)-guanosine (116). There are 41 predicted diguanylate cyclase and phosphodiesterase genes in *P. aeruginosa* PAO1, many of which contain domains known to participate in signal sensing and transduction (118). Two important c-di-GMP receptors are involved in biofilm formation: FleQ and PelD (119, 120). FleQ is a repressor of the *pelA-G* and *pslA-O* operons, which drive exopolysaccharide synthesis, and of *cdrA*, which encodes an adhesin (119, 121, 122). Upon c-di-GMP binding, FleQ repression is relieved, leading to induction of the *pelA-G*, *pslA-O*, and *cdrA* operons. PelD is part of the biosynthetic pathway of the Pel polysaccharide (120). Its binding to c-di-GMP was found to be essential for Pel production (120). Thus, through binding to FleQ and PelD, high levels of c-di-GMP lead to induction of ECM production, while low levels lead to repression (123, 124). Interestingly, Irie et al. (114) found that the presence of Psl leads to increased intracellular c-di-GMP levels. Both overexpression of Psl, under the control of an arabinose-inducible promoter, and addition of purified Psl from another biofilm enhanced c-di-GMP levels by up to 2-fold (114). Irie et al. showed that Psl-induced c-di-GMP elevation depended on the activity of SadC and SiaD, two diguanylate cyclases, which have been previously established as critical for biofilm maturation (125, 126). As mentioned above, high c-di-GMP levels lead to activation of ECM production. Thus, these findings present a positive-feedback loop in which sensing of the newly produced ECM components leads to activation of further ECM production. The fact that Psl can act when applied exogenously demonstrates that it can amplify ECM production in neighbor cells that are not yet producing ECM.

Apart from its amplificatory function, Psl was found to play a critical role in directing the cells at the forefront of the *P. aeruginosa* PAO1 biofilm. During the initial stages of microcolony formation in flow cells, *P. aeruginosa* PAO1 cells deposit trails of Psl as they move along the surface, using T4P twitching motility (127, 128). In twitching motility, T4P extend, attach to the surface, and retract, leading to cell progression in the retraction direction (129, 130). Using cell-tracking algorithms, Zhao et al. (128) demonstrated that these Psl trails influence the surface motility of cells that later encounter these trails, encouraging them to follow the same routes. Δ*pslD* mutant cells explore much more of the surface than wild-type cells, as they lack Psl traffic signs. On the other hand, a Psl-overproducing mutant is limited to fewer trajectories as a result of the higher local concentration of Psl. Furthermore, Zhao et al. showed that, after cell division, the tendency of daughter cells to stay near the mother cell depends on the presence of Psl. Cells of the Δ*pslD* mutant strain left the mother cell more frequently than wild-type strain cells, while cells of the ECM-overproducing mutant left the mother cell less frequently. Deletion of *pilA*, the gene that encodes the T4P structural protein, resulted in a reduction of the surface area that was explored. This behavior indicates that bacterial microcolony initiation is self-organizing and that local concentrations of Psl are used to induce positive-feedback loops. Cells then tend to reside in areas with significant Psl accumulation. Together with the aforementioned evidence that high Psl concentrations serve as a signal to produce more Psl, those results imply that, during the initial phase of biofilm formation, a group of pioneer cells locally increases ECM concentrations to attract more cells that later contribute to the ECM production and serve as the founding population of the microcolony. Note that both studies used the PAO1 *P. aeruginosa* laboratory strain, which produces mainly Psl. As mentioned above, PA14, the other well-studied *P. aeruginosa* laboratory strain, does not produce Psl, and its primary exopolysaccharide is Pel. It will be interesting to study whether PA14 shows similar behavior given the suggested structural redundancy between the two exopolysaccharides (61) or, alternatively, whether cell guidance is a unique property of Psl.

In a different study, Gloag et al. (131) showed that eDNA promotes cell migration through interconnected channels in *P. aeruginosa* biofilms. In this experimental system, *P. aeruginosa* cells created channels on the surface of the semisolid substrate, which was solidified by the addition of gellan gum. The channels assembled into a network that facilitated colony expansion. The formation of the channel network was achieved by the coordinated movement of leader groups followed by cells that traced the same trails. eDNA was found to be necessary for this tracing, and addition of DNase caused the leader groups to remain isolated and hampered the construction of the channel network and colony expansion. Moreover, DNA strands were found to be aligned with the direction of movement of the cells, possibly aiming the cells in the correct direction. The cells moved using T4P twitching motility. T4P were shown to bind DNA, and it was suggested that T4P eDNA binding was responsible for the directed movement of cells. Importantly, the involvement of T4P-eDNA interaction in *P. aeruginosa* PAO1 biofilm structure in flow cells was demonstrated previously (132–134). When grown in flow cells with glucose minimal medium, *P. aeruginosa* creates typical mushroom-shaped structures, with a stalk and a cap (132). In these structures, eDNA was found to be located at the stalk (135). The formation of the cap required T4P-dependent migration of a subpopulation of cells (133), which was also shown to depend on the presence of eDNA at the stalk (134). In these studies, eDNA plays a role in guiding cell migration within the biofilm structure. Similarly to the Psl example mentioned above, ECM signals, laid down by the cells in the colony frontline, direct the cells in the back, allowing the colony to expand to new territories.

ECM-guided cell migration resembling that of *P. aeruginosa* was observed in the bacterium *Myxococcus xanthus* (136). *M. xanthus* is a Gram-negative soil bacterium, demonstrating multiple types of social behaviors, and thus is used as an important model of bacterial development (137). One of these social behaviors is social motility (S-motility), which occurs during growth on nutrient-rich medium, on which *M. xanthus* cells collectively migrate, creating a swarm. S-motility was found to be driven by T4P twitching motility (138, 139). Interestingly, during swarming, *M. xanthus* cells seem to follow the same routes as precedent cells, resembling *P. aeruginosa* (140). *M. xanthus* T4P participate in S-motility by binding exopolysaccharides that are produced during swarming, causing retraction of the pili (136). These exopolysaccharides cover the cell surface, and in mutants lacking them, S-motility is inhibited (136, 141). Thus, movement is possible only when T4P attach to adjacent cell surface exopolysaccharides. It was suggested that a portion of the surface exopolysaccharide is shed during cell migration (142), possibly creating trails that subsequent cells follow. In the biofilm state of *M. xanthus*, also termed the fruiting body, the exopolysaccharides may create a network between cells, which might facilitate T4P twitching motility (143, 144). Interestingly, production of exopolysaccharides depends on the presence of assembled T4P (145, 146). This might imply a positive-feedback loop similar to Psl-induced ECM production in *P. aeruginosa* (147).

Recently, an example of posttranslational self-amplification of

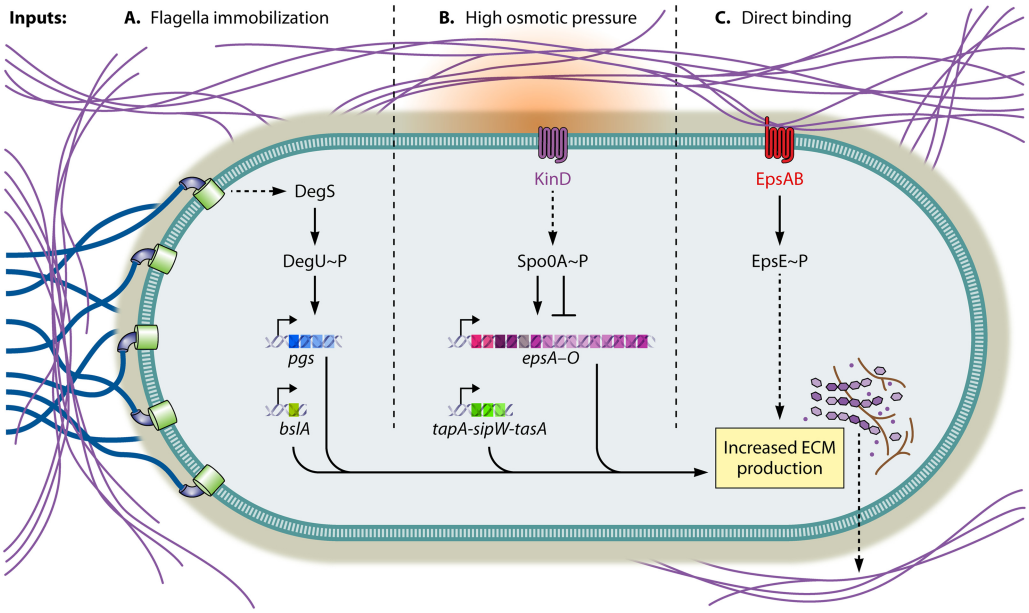

FIG 2 ECM-cell signaling induces positive-feedback in ECM production. There are three ECM-derived signals that induce ECM production in *B. subtilis* biofilms as follows. (A) Disruption of flagellar rotation, which can occur in the viscous ECM environment, causing DegS-dependent phosphorylation of DegU and induction of the *pgs* operon, yielding γ-PGA production, and of *bslA*, which forms the biofilm hydrophobic coat (150, 151). (B) The KinD kinase senses high osmotic pressure and phosphorylates Spo0A. Phosphorylated Spo0A leads to the activation (low Spo0A~P) or deactivation (high Spo0A~P) of the *epsA-O* operon for production of exopolysaccharides and of the *tapA-sipW-tasA* operon, inducing TasA amyloid fiber production (163–165). (C) EpsA-specific binding to *B. subtilis* exopolysaccharides causes inhibition of EpsAB autophosphorylation and phosphorylation of EpsE, which may then lead to increased ECM production (148).

exopolysaccharide production was discovered in *B. subtilis* (148) (Fig. 2). EpsA and EpsB constitute a tyrosine kinase that was found to undergo autophosphorylation and remain inactive in the absence of a signal. However, in the presence of *B. subtilis* exopolysaccharides, autophosphorylation was repressed and the EpsAB kinase phosphorylated EpsE, a glycosyltransferase in the *B. subtilis* exopolysaccharide biosynthetic pathway. EpsE phosphorylation was suggested to promote production of exopolysaccharides. The binding of the EpsA extracellular domain to exopolysaccharides was found to be specific to *B. subtilis* exopolysaccharides. Interestingly, the extracellular domain of the *S. aureus* CapA (an EpsA homolog) bound specifically the *S. aureus* exopolysaccharide PIA. Similarly to the *P. aeruginosa* Psl, these findings present a mechanism for positive-feedback loop regulation of exopolysaccharide production, enabling amplification of the signal and the construction of the biofilm. The species specificity of the signal further emphasizes the idea of directed regulation.

Such regulation of glycosyltransferases is only one example of ECM signaling during biofilm development in *B. subtilis*. The production of exopolysaccharides creates a highly viscous environment (149), which can be sensed by the bacterial cell flagella. In *B. subtilis*, the attachment step was suggested to be governed and amplified by mechanosensing (Fig. 2). *B. subtilis* biofilm development requires the activation of three transcriptional regulators: ComA, Spo0A, and DegU (38). Recent studies showed that the disruption of flagellar rotation increased the DegU~P level (150, 151). Phosphorylated DegU triggers the biosynthesis of γ-polyglutamic acid (γ-PGA), a unique ECM polymer composed of glutamic acid and produced by the *pgs* operon (152–154). Deletion of either MotA or MotB, which together form the stator part of the flagellar motor, resulted in overproduction of γ-PGA and a mucoid colony phenotype. The same phenotype was achieved by other methods that hampered flagellar rotation, such as (i) mutations in MotB that disrupted proton flux through the motor, (ii) addition of specific antibodies against the flagellar filament, and (iii) overexpression of EpsE, which can function as a molecular clutch that separates the flagellar stator and rotor compartments (155, 156). Overproduction of γ-PGA required the presence of DegU and DegS (150), as well as of proteins involved in flagellar filament assembly (151). The results of the studies presented here suggest that hampering the rotation of the flagella during initial attachment results in altered gene expression. We propose that the flagella can sense the viscous ECM environment in later stages of microcolony formation by the same mechanism. Viscous environments are thought to increase flagellar motor torque (157), and a high viscosity level was shown to affect gene expression in *V. cholerae* (158, 159), *P. aeruginosa* (125, 160), and *Proteus mirabilis* (161, 162). In order to examine this hypothesis in *B. subtilis*, it will be interesting to measure the levels of γ-PGA production in cells grown in environments of various viscosities, perhaps by supplying different concentrations of ECM exopolysaccharides. This signaling pathway could constitute a positive-feedback loop, as the highly viscous environment of the ECM can halt the flagella. Halting the flagella then promotes production of more ECM, which further amplifies viscosity. Several mechanosensory mechanisms leading to initiation of biofilm formation have been described in numerous bacterial species, including *P. aeruginosa*, *C. crescentus*, and *V. cholerae*. A recent review summarizes the current knowledge of these mechanisms (157).

During the initial stages of biofilm formation, the population is still divided between motile planktonic cells and sessile surface or ECM-associated cells. As the biofilm matures, the motile cell pop-

ulation gradually diminishes and most of the cells become sessile. During the initial stages of *B. subtilis* biofilm formation, an unusual selection for ECM producers occurs. The amyloid fibers forming TasA, a structural component of the *B. subtilis* biofilm (76), also serve as a secreted antibacterial protein (80). Intriguingly, when *B. subtilis* cells were grown planktonically, they displayed sensitivity to TasA (80). Thus, TasA production may severely retard growth of planktonic motile cells in the initial stages of biofilm formation, shifting the population toward immobility and ECM association, which are crucial for further biofilm development. While this is a tempting speculation, the sensitivity of planktonic cells to TasA needs to be tested in the biofilm environment.

Overall, the examples described here present different and complementary mechanisms for ECM sensing. The sensing of *P. aeruginosa* Psl, and of *B. subtilis* exopolysaccharides by EpsAB, relies on a species-specific signal. The cells recognize and respond to the exact chemical properties of specific ECM components. In contrast, eDNA sensing by *P. aeruginosa* and the possible flagellar mechanosensing of the viscous environment in *B. subtilis* present signals that are more global. General signals may provide an advantage, as they can be sensed by many types of bacteria. The importance of such universal physical cues is prominent in multispecies biofilms in which various bacterial species must coordinate their behavior.

ECM SIGNALING DURING 3D BIOFILM STRUCTURE DEVELOPMENT

The effects of ECM signaling during biofilm development can be quite dramatic. Analysis of the spatiotemporal gene expression profiles of a *B. subtilis* ECM mutant lacking both exopolysaccharides and TasA fibers demonstrated alterations in the number and localization of motile cells, ECM producers, and sporulating cells within the mature colony (31) (Fig. 1, panel II, and Fig. 2). Specifically, reduced expression of the motility reporter was noted. The few cells that expressed the motility reporter were not localized at the base of the colony as observed in wild-type biofilms, suggesting that the ECM plays a critical role in regulating existence and localization of motile cells (31; N. Steinberg and I. Kolodkin-Gal, unpublished data). Interestingly, transcription of the two ECM operons, *epsA-O* and *tapA-sipW-tasA*, was dramatically increased in the ECM mutant (31), suggesting that the *B. subtilis* ECM serves a dual signaling role; it positively posttranslationally regulates ECM production, as mentioned in the previous section (148), and negatively regulates ECM production at the transcriptional level. ECM-driven negative feedback on its own production may play an important role in adaptation to the biofilm environment. In this manner, cells do not waste resources by constantly producing matrix, as a positive-feedback loop would predict. It would be interesting to test whether accumulation of sufficient matrix suppresses further matrix expression, thus overcoming the effects of the positive-feedback loop.

Strikingly, virtually no sporulating cells were observed in the *B. subtilis* ECM mutant colony, at a time point at which wild-type biofilm had a high percentage of sporulating cells (31, 163). A mixture of the ECM mutant cells with sporulation mutant cells was capable of producing intact ECM, resulting in colonies with wild-type architecture and restored sporulation and suggesting that the ECM induces sporulation as a *trans*-acting signal.

What type of signal does the ECM convey that causes the observed effects? One attractive hypothesis claims that reduced ECM production supports increased cell growth. If the energy required to synthesize the ECM components is instead channeled toward other metabolic pathways, the ECM mutant cells might continue growing for longer periods of time, delaying the initiation of sporulation. However, when the total number of cells in the biofilm of the ECM mutant was compared to the number of cells present in the wild-type biofilm, over the course of development, there were significantly more cells in the biofilms formed by the wild-type strain than in those formed by the ECM mutant (163). When ECM mutant cells were visually compared to wild-type cells under the microscope, no differences in cell size were observed. Therefore, the delay observed in sporulation could not be explained by a prolonged period of growth of the ECM mutant cells (31). Can an ECM-generated chemical cue promote *B. subtilis* development and, more specifically, sporulation? Mutants with mutations in two different ECM components, the TasA protein and the exopolysaccharides, were both defective in sporulation when grown under biofilm-inducing conditions but not when grown in dispersed cultures (163). However, TasA fibers and exopolysaccharides have strikingly different chemistries; thus, it is not plausible that the ECM generates a chemical signal that regulates sporulation.

Another hypothesis as to the lack of specificity in the signal source is that a nonspecific physical cue, for instance, increasing osmotic pressure during the accumulation of ECM, might trigger sporulation. Rubinstein et al. (164) exploited unique properties of polymeric solutions to differentiate between the effects of multiple physical parameters. They tested the effects of both polyethylene glycol (PEG) and dextran over a range of molecular sizes and concentrations as well as various concentrations of the dextran subunit dextrose. The expression levels of ECM and sporulation genes strongly correlated with the osmotic pressure exerted by the various polymer solutions. Polymer solutions that exerted sufficient osmotic pressure complemented the sporulation defect of an ECM mutant *in trans*, as did purified polymers from *P. aeruginosa* ECM, added exogenously to the *B. subtilis* ECM mutants. Both sporulation and ECM production are regulated by the master regulator Spo0A. While low levels of Spo0A~P trigger ECM production, high levels trigger sporulation. In this case, the osmotic pressure exerted by both exopolysaccharides and amyloid fibers was shown to be sensed by KinD, as a *kinD* mutant did not respond to increased osmotic pressure (164). KinD is a membrane histidine kinase that phosphorylates Spo0A and was shown to affect both sporulation and matrix production, with activity redundant with that of KinC (163, 165). A deletion of *kinD* suppresses the sporulation defect of the ECM mutants (163), suggesting that KinD is the sensor of the ECM. Importantly, KinD has also been shown to specifically respond to a broad range of plant exopolysaccharides (166), as well as to several small molecules (167, 168). It is highly plausible that the specificity of this membrane kinase dramatically changes as a function of the mediator that it binds, such as the Med lipoprotein (169). Alternatively, it is plausible that KinD senses somewhat generic signals such as membrane stress, caused by increased osmotic pressure, antibiotics, or membrane interactions with high concentrations of polymers.

Taken together, the findings in *B. subtilis* biofilms present a case for several arguments. First, sensing the ECM may initiate a local positive-feedback loop by inducing a minor increase in the level of Spo0A~P, thereby promoting further ECM production. The gradual increase in ECM-driven local osmotic pressure can

drive neighboring bacteria to induce their own ECM production in mixed communities. When sufficient ECM is accumulated, further phosphorylation of Spo0A by KinD promotes sporulation and shuts off ECM production. Second, aside from sporulation, several severe defects in other developmental programs (e.g., competence and motility) were observed in these ECM mutants (31, 32). It is unlikely that these defects can be solely attributed to Spo0A-phosphorylation or to the structural role of the ECM. We suggest that several independent signaling pathways are activated by the ECM during the 3D patterning of *B. subtilis* biofilms.

Spatial arrangements of different cell types in the biofilm 3D structure were also demonstrated in *E. coli* and *P. aeruginosa* (170–172). Specifically, flagellated cells were observed at the bottom and exterior parts of *E. coli* biofilms grown on agar (170), as was observed in *B. subtilis* biofilms. In addition, ECM-producing cells, which produce Curli, the amyloid fiber of *E. coli*, were located at the top of the colony (170). In the context of this review, it will be interesting to examine the influence of the presence of each of the ECM components of *E. coli* and *P. aeruginosa* on the quantity and spatial organization of the different cell types in the biofilm.

ECM SIGNALING DURING THE LATE STAGES OF THE BIOFILM CYCLE

As the biofilm matures, resources become limited and waste products accumulate. Bacteria incapable of escaping the biofilm become trapped in what evolves into a death trap (173). Therefore, at a certain time, it is beneficial for the constituent cells of the biofilm to disperse (174). Effective probing of ECM maintenance level and density enables cells to determine the ideal time for dispersal (Fig. 1, panel III).

Dispersal triggered by an ECM polymer was demonstrated in *C. crescentus* biofilms, which are composed of swarmer cells and sessile stalked cells. The stalked cells have a polar adhesin composed of polysaccharides, called the holdfast, which is required both for permanent adhesion to surfaces and for biofilm formation. As cell death within the biofilm commences, eDNA accumulates. Berne et al. (175) have shown that eDNA inhibits submerged *C. crescentus* biofilm formation, in static cultures and in flow cells. When applied externally, the purified eDNA effectively blocked biofilm formation. This robust hindrance occurred both when the eDNA was purified from the spent medium and when genomic DNA was purified from *C. crescentus* cells. eDNA was found to specifically bind the *C. crescentus* holdfast. This ligand-receptor interaction specifically prevented the attachment of the newly produced stalked cells to one another and to the surface. When eDNA was added to established biofilms grown in flow cells, it did not cause dispersal. However, the biomass of the biofilm remained constant, indicating that the progeny of the attached cells dispersed. Specific targeting of nonattached cells in the mature biofilm stimulates dispersal without the simultaneous destruction of the biofilm. While eDNA-bound dispersing cells fail to efficiently attach to a surface, they can produce progeny swarmer eDNA-free cells that are capable of settling in a new environment.

In *S. aureus*, surfactant-like small peptides called phenol-soluble modulins (PSMs) were recently found to form functional amyloid fibers that have a structural role in the *S. aureus* biofilm (85). However, in their monomeric form, they were found to promote biofilm disassembly by reducing the surface tension (176). Distinguishing between the structural role of the PSMs and their role in dispersal of biofilms is challenging. Aggregation and disaggregation of PSMs are probably dynamic events occurring in parallel during biofilm formation. Periasamy et al. (176) suggested a possible role of PSMs in local biofilm dispersal in flow cells, where mutants incapable of producing PSMs formed thicker, denser, and smoother biofilms. In addition, after 72 h in flow cells, the wild-type strain showed waves of detachment and growth, while the biofilm of the PSM mutants remained thick. PSM expression was localized to specific areas of the biofilm, suggesting a signaling role in pattern formation. Together, these findings suggest regulation of biofilm dispersal via ECM components, where a structural element in the biofilm acts as a dispersal signal as well. PSMs were also suggested to play a role in regulating their own expression, though the underlying mechanism is still unknown (85).

CONCLUSIONS AND FUTURE DIRECTIONS: HOW CELL-ECM INTERACTIONS SHAPE MULTICELLULAR MICROBIAL COMMUNITIES

This review presents evidence from a number of studies demonstrating the pivotal role of the ECM in controlling gene expression and cell behavior in bacterial biofilms, throughout the various stages of biofilm formation and maturation.

During the initial stages of attachment, it is important for the pioneer cell population to create a positive-feedback loop that will lead to ECM and cell accumulation. As a biofilm is a multicellular structure, a bacterial cell that approaches a surface must signal other cells, or its daughter cells, to attach at locations close to its attachment site and to start producing ECM components. As local concentrations of ECM in the attachment area of the pioneer cells rise, they can be used as a signal for other cells.

This was demonstrated in *P. aeruginosa*, as Psl and eDNA trails being laid by the pioneer cells direct cell movement to these specific areas (128, 131). Psl in *P. aeruginosa*, and the *B. subtilis* exopolysaccharide, are specifically sensed by each of these bacteria and are used as a signal to increase ECM production, demonstrating a positive-feedback loop (114, 148). Another example is *B. subtilis*, which uses its flagella as mechanosensory organelles that might sense the ECM viscous environment; halting the flagellum leads to the induction of ECM production (150, 151).

When the *B. subtilis* biofilm develops, ECM probing is crucial for coordinating cell behaviors over large scales and to obtain a proper structure formation. When ECM components are absent, biofilm development is disrupted (31, 128, 131). Specifically, the size and the spatial organization of different cell type subpopulations in the biofilm are modified in ECM mutants compared to wild-type cells (31). The defects in cell behavior can be explained as a lack of ECM-derived regulatory cues. It will be interesting to examine the possible role of the ECM in regulation of cell localization and genetic program activation in other species (170–172).

As the biofilm reaches its last stages, ECM biochemistry, structure, and integrity serve as ideal probes of the physiological state of the biofilm. ECM structural components that either collapse or undergo active destruction during the dispersal stage may act as dispersing signals. PSMs that create structural amyloid fibers in *S. aureus* ECM (85) were shown to trigger dispersal in their monomeric form (176), suggesting that when the ECM is dismantled to its building blocks, the breakdown products may induce dispersal. eDNA, which functions as a structural component in many bacterial species (99, 100), was found to inhibit attachment of *C. crescentus* cells by binding to their attachment organelle, the stalk (175). Thus, eDNA from lysed cells can induce dispersal of the

new progeny of the biofilm cells, releasing them to search for new colonization sites.

In multicellular eukaryotes, numerous examples of ECM-driven signals crucial for determination of both cell behavior and correct tissue morphogenesis have been described. There is no apparent reason to think that bacterial ECM would differ in this aspect. ECM-derived signals may be a common feature of cell communities surrounded by an extracellular matrix, as such an arrangement allows probing of the local environment and dynamic adjustment of signals. The studies presented in this review are undoubtedly just the tip of the iceberg—many more examples of bacterial ECM signaling are sure to be discovered in the near future.

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