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Biofilms: the matrix revisited

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Microbes often construct and live within surface-associated multicellular communities known as biofilms. The precise structure, chemistry and physiology of the biofilm all vary with the nature of its resident microbes and local environment. However, an important commonality among biofilms is that their structural integrity critically depends upon an extracellular matrix produced by their constituent cells. Extracellular matrices might be as diverse as biofilms, and they contribute significantly to the organization of the community. This review discusses recent advances in our understanding of the extracellular matrix and its role in biofilm biology.

Biofilms: matrix-encased microbial communities

It is now widely recognized that in natural settings bacterial cells are most often found in close association with surfaces and interfaces, in the form of multicellular aggregates commonly referred to as biofilms. This proclivity towards multicellularity makes bacterial cells similar to many other types of living cells: capable of unicellular existence and yet generally residing within multicellular communities. Biofilms offer their member cells several benefits, of which protection from environmental insults and assaults is foremost [1]. Given their ubiquity and importance in the microbial world, it is hardly surprising that biofilms have attracted the attention of the scientific community. However, simple observation of naturally occurring biofilms, which almost invariably house a mixture of species, reveals that biofilms are as diverse as their constituent microbes; for example, the streaming biofilms formed on submerged rocks in acid mine drainage [2] are strikingly different from the plaque formed on air-exposed surfaces of teeth [3]. Will the study of these diverse biofilms provide insight into the fundamental principles and molecular mechanisms underlying microbial multicellularity? An affirmative answer is beginning to emerge from molecular genetic studies of single-species biofilms formed in the laboratory. It is now appreciated that the formation and maintenance of structured multicellular communities critically depends upon the production of extracellular substances that, in conglomerate, constitute an extracellular matrix [4]. Although the ability to make an extracellular matrix appears to be a common feature of multicellular communities, there is a remarkable diversity in the means by which these matrices are constructed. This diversity becomes readily apparent as we review what has been learned in recent years with regard to the extracellular matrices of bacterial biofilms.

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Biofilms in the laboratory

There are myriad laboratory conditions that favor biofilm formation. As a consequence of their ease of manipulation, and in an effort to maximize reproducibility from laboratory to laboratory, four general systems have been routinely used by different investigators (Figure 1). For the study of submerged biofilms the flow cell has been and continues to be the gold standard [5]. Experimental setups that use flow cells are particularly amenable to observation through confocal scanning laser microscopy (CSLM). Studies of biofilms grown in flow cells have provided us with the familiar image of the submerged biofilm consisting of pillars and mushroom-like structures separated by water-filled channels. Submerged biofilms also have been studied in batch culture under conditions of no flow, mainly because of the rapidity with which large numbers of these samples can be grossly analyzed in microtiter dishes [6]. This experimental system has been exploited to carry out high-throughput screens to identify genes that are involved in biofilm formation and maintenance. Floating biofilms, or pellicles, that form at the liquid–air interface of standing cultures represent yet another type of biofilm [7,8]. The lack of a solid surface on which to initiate growth imposes a greater requirement for self-organization on the constituent cells, and the lack of rapid fluid flow at the air-exposed surface enables the formation of more complex structures. Finally, the bacterial colonies that grow on the surface of agar-solidified media, for decades the workhorses of the bacterial geneticist, are now recognized as a most practical object for the study of biofilms. Colonies can vary widely in morphology, and there is a clear correlation between highly structured morphologies and the ability of a cell to produce an extracellular matrix. Electron micrographs of structured colonies show that they are composed of cells surrounded by an extracellular matrix [8,9] (Figure 2). Mutants defective in the production of extracellular matrix are still able to form colonies, but these tend to be smooth and flat. Similar to the biofilms that form on the walls of microtiter dish wells, pellicles and colonies are amenable to high-throughput screens for identification of genes involved in biofilm formation and maintenance [10,11]. To date, no single experimental approach has adequately fulfilled all of the needs of the biofilm investigator; rather, each method has complemented the others, and the accumulated knowledge gained from their combined application has provided new insights into the nature of biofilms.

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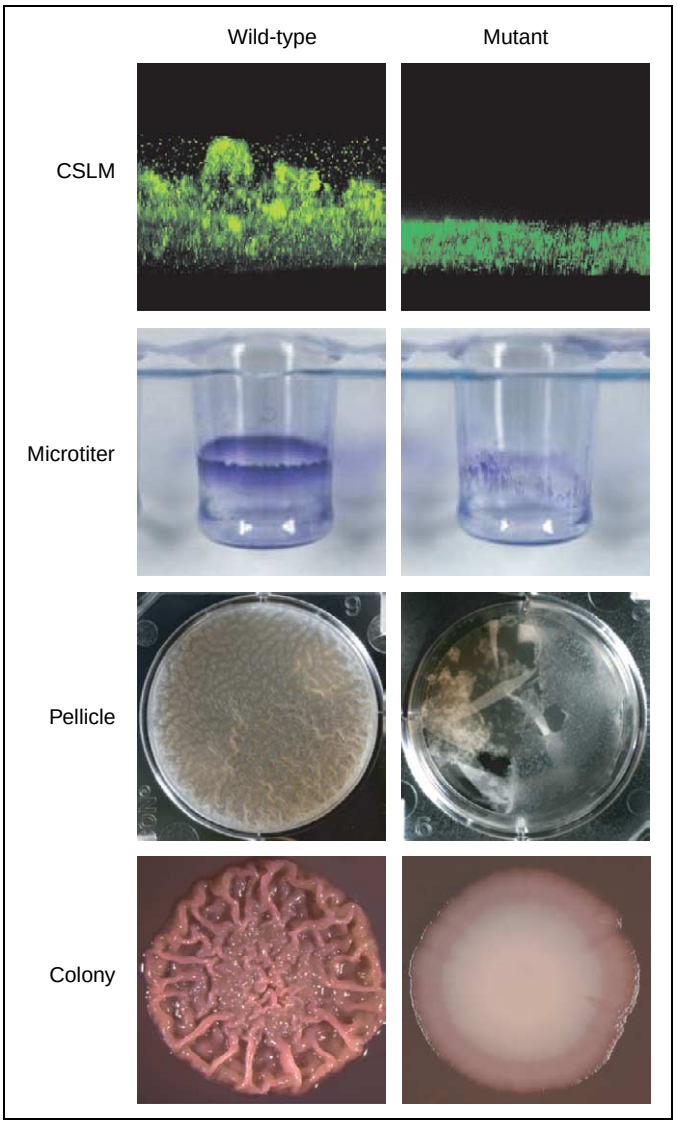

Figure 1. Approaches used in the study of biofilms. Four approaches in common use are represented by images of wild-type and mutant biofilms formed and visualized under typical conditions. Flow cell biofilms are usually analyzed using confocal scanning laser microscopy (CSLM). The biofilms shown in the top panels were produced by *Vibrio cholerae* strains that constitutively express the green fluorescent protein, and were visualized using CSLM (the biofilms are in profile). 'Microtiter' biofilms are formed on the surfaces of microtiter dish wells under standing culture conditions (no medium flow), and they are visualized using a non-specific dye (crystal violet); the biofilms shown here were formed by *Escherichia coli* strains. A 'pellicle' is a biofilm formed at the air–liquid interface of a standing culture; those shown here were produced by *Bacillus subtilis* strains. The 'colony' images are of structured communities formed by *Pseudomonas aeruginosa* strains spotted at high cell density on agar containing Congo Red dye.

Genetic studies using these biofilm-generating systems have revealed many of the extracellular components of biofilms, and have helped to define the stages of biofilm formation [1]. Initiation of biofilm formation is characterized by the interaction of cells with a surface or interface as well as with each other. Once enough cells have aggregated, the biofilm begins to mature through the production of an extracellular matrix, which contributes greatly to the final architecture of the community. Despite the universal presence of extracellular matrices in biofilms, it is clear that there is enormous diversity in their composition and in the timing of their synthesis. This diversity is readily apparent at several levels. For instance,

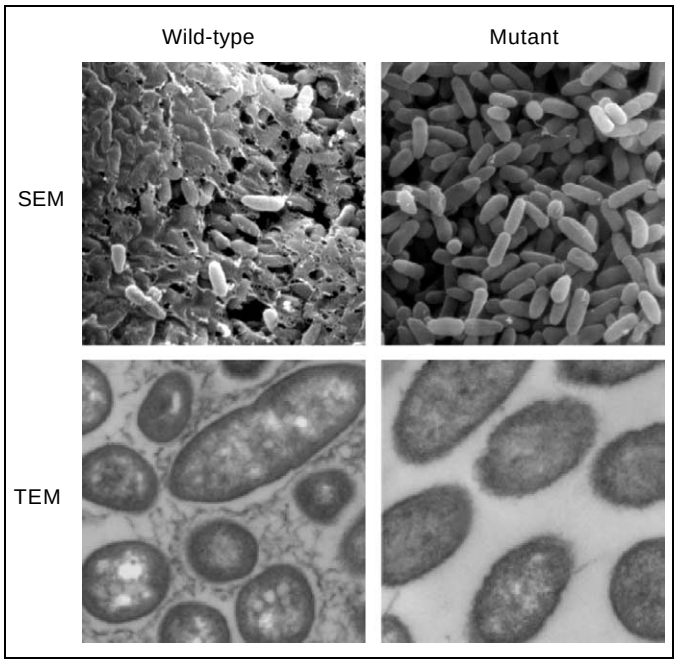

Figure 2. Visualization of the extracellular matrix by electron microscopy. Biofilms formed by wild-type *Pseudomonas aeruginosa* and a matrix-deficient mutant were analyzed by scanning electron microscopy (SEM), and by transmission electron microscopy (TEM) after staining with ruthenium red.

biofilms formed by different species are usually easily distinguished from each other. Similarly, there can be great variation in the properties of biofilms formed by different strains of a single species. Quite importantly, dramatic differences in biofilm architecture can result from even small changes in environmental conditions (Figure 3). Such differences in biofilm structure appear to reflect differences in the composition of the extracellular matrix. Extracellular polysaccharides and proteins have been shown to be key components of the matrix [4,12]. However, there are other known components of the extracellular matrix. For example, a recent report indicates that extracellular DNA plays an important role in the establishment of biofilm structure [13]. Also, dead cells have been observed in some biofilms, suggesting that cell detritus can be considered part of the extracellular matrix [14,15]. Studies of the roles played by DNA and dead cells in biofilms represent exciting new directions for future studies of biofilm matrices. However, for the purposes of this review, we limit our discussion to the extracellular matrix components that have been most extensively studied: carbohydrate-rich polymers, and proteins.

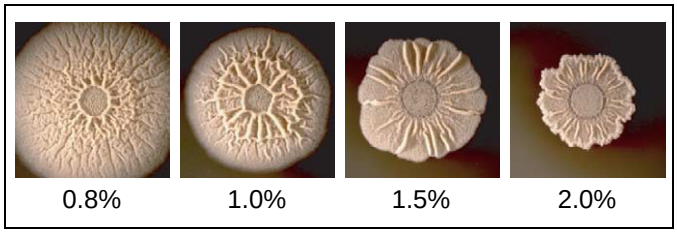

Figure 3. Biofilm structure varies with environmental conditions. *Bacillus subtilis* strain 3610 was spotted at high cell density on medium solidified by different concentrations of agar.

Carbohydrate-rich polymers in the matrix

The fact that bacteria produce extracellular polysaccharides has been recognized for decades, largely because these polymers significantly impact bacterial virulence. Extracellular polysaccharides have been classified as capsular polysaccharides or exopolysaccharides, the distinction between the two being operational: when bacteria are grown in shaken liquid culture and then harvested by centrifugation, extracellular polysaccharides that remain cell-associated are referred to as the capsule, whereas those remaining in the supernatant are referred to as exopolysaccharides. However, when considering structured communities such as biofilms, this distinction is not so easy to discern. In addition, many of the extracellular polysaccharides produced in biofilms are insoluble and not easily separated from the cells, complicating the precise determination of their chemical structures. Nevertheless, genetic analyses strongly support the notion that in many different bacterial species extracellular polysaccharides play an important role in determining a biofilm's architecture.

The ability of some bacteria, most notably *Gluconacetobacter xylinus,* to produce extracellular cellulose has long been recognized [16]. Studies of biofilms and bacterial multicellular behaviors over the past decade strongly suggest that many other species of bacteria are capable of producing cellulose. In the case of *Salmonella typhimurium* and *Escherichia coli*, cellulose has been shown to be a crucial component of the extracellular matrix [10,11]. In several other species, the presence of genes homologous to the *G. xylinus bcs* (bacterial cellulose synthesis) genes strongly suggests that they too are able to produce cellulose [16]. In *G. xylinus* the activity of cellulose synthase is positively regulated by cyclic-di-GMP. The synthesis and degradation of this molecule are carried out by a diguanylate synthase (Dgc) and a phosphodiesterase (PdeA), respectively [17]. The genes encoding these enzymes in *G. xylinus* share sequence similarity with each other as well as with apparent homologs that are widely distributed throughout the eubacterial world. The protein products of these genes contain characteristic repeated sequence motifs, which are referred to as GGDEF and EAL domains because of their amino acid compositions [18]. The fact that several GGDEF-containing proteins have been shown to complement *G. xylinus* mutants lacking diguanylate synthase might lead one to believe that such proteins are universally involved in activating cellulose synthase. However, many bacterial genomes encode one or more proteins with GGDEF and EAL domains but do not encode any known homolog of cellulose synthase. For example, the *Vibrio cholerae* genome does not appear to encode a cellulose synthase, but it has many genes encoding GGDEF and EAL proteins. Mutations in at least three of these genes result in severe alterations of the biofilm matrix [19–21]. Thus, it appears that cyclic-di-GMP is a widespread activating molecule that regulates the production of exopolysaccharide components of the extracellular matrix including, but perhaps not limited to, cellulose. Because GGDEF and EAL proteins generally mediate signal transduction [22], the presence of several GGDEF- and EAL-encoding genes in a single genome suggests the possibility that several environmental signals can be integrated into a matrix-producing response through a single secondary messenger (cyclic-di-GMP). It should be noted, however, that GGDEF and EAL proteins can be involved in cell processes other than biofilm formation [23].

In addition to cellulose, other previously characterized polysaccharides are now recognized as important components of biofilm extracellular matrices. One example is the staphylococcal polysaccharide intercellular adhesin (PIA), which is produced by enzymes encoded by the *ica* locus [24]. *Staphylococcus epidermidis* and *Staphylococcus aureus* produce PIA or the related poly-N-acetyl glucosamine (PNAG) polymer, both of which depend upon the *ica* locus for their synthesis, serve as adhesins, and are required for biofilm formation [25–27]. Now it appears that PIA-like polymers are made by several Gram-negative bacterial species. For instance, a PIA-like polymer was recently shown to play a role in biofilm formation by *E. coli* strain MG1655 [28]. The genes that encode this polymer in *E. coli* are designated *pga*, and they share sequence similarity with the staphylococcal *ica* genes. *Yersinia pestis* also appears to synthesize a PIA-like polymer that plays a role in transmission of the microbe through its insect vector. *Y. pestis* is transmitted to humans through the bites of fleas whose digestive systems are blocked by bacterial biofilms. In a model system, *Y. pestis* and the closely related *Yersinia pseudotuberculosis* were shown to block feeding by *Caenorhabditis elegans,* by making a biofilm that covered the worm's mouth [29]. This block of the digestive system required exopolysaccharide production by enzymes encoded by the *hms* locus, which shows sequence similarity to the *ica* locus. Thus, in this case biofilm formation appears to function as a bacterial defense against predation by invertebrates, with the added advantage of aiding in transmission.

A theme that emerges with regard to carbohydrate-rich components of the extracellular matrix is that individual strains are often able to produce several different extracellular polysaccharides. Recent developments in our understanding of the extracellular polysaccharides produced during *Pseudomonas aeruginosa* biofilm formation underscore this point. For many years alginate was generally believed to be the major constituent of the extracellular matrix in *P. aeruginosa* biofilms. This concept probably arose from studies of mucoid strains isolated from patients with cystic fibrosis; in such strains mucoidy was shown to be due to the overproduction of alginate [30]. However, biofilms formed by non-mucoid strains appear to contain little if any alginate [31]. Furthermore, when genes required for alginate biosynthesis (e.g. *algD*) were disrupted in non-mucoid strains there was no detectable change in the structure of biofilms formed in flow cells or in colony morphology [8,31]. By contrast, recent genetic and biochemical analyses have revealed two distinct carbohydrate-rich polymers that make significant contributions to *P. aeruginosa* biofilm structure. The synthesis of PEL, a glucose-rich polymer, is mediated by the *pelA-G* genes [8], whereas the synthesis of PSL, a mannose-rich polymer, is mediated by the *pslA-O* genes [32–34]. All *P. aeruginosa* strains tested to date carry the *pel* genes,

but expression of these genes varies greatly among strains; in particular, the commonly used strains PAK and PA01 do not express these genes strongly under common laboratory conditions [8]. By contrast, the *psl* genes are present in some but not all strains; specifically, strain PA14 lacks *pslA-D* [33]. Finally, in some instances both PEL and PSL have been detected in the extracellular matrices of *P. aeruginosa* biofilms. Thus, strain-to-strain variation in both gene content and expression levels has been observed and might in part account for the great diversity of biofilm phenotypes that is evident when different strains are studied.

Quorum sensing and extracellular polysaccharide production

Recent developments in biofilm research suggest a connection between population density and extracellular polysaccharide production, albeit in an unexpected way. The initial recognition of a relationship between quorum sensing and biofilm architecture in *P. aeruginosa* generated great enthusiasm because it provided direct evidence for the role of extracellular signaling in biofilm development [35]. The dramatic effects on biofilm thickness and shape that resulted from the inability to produce the N-(3-oxododecanoyl)-L-homoserine lactone signal suggested major alterations in the extracellular matrix. Unfortunately, the quorum-sensing-regulated genes involved in matrix production in *P. aeruginosa* have eluded detection thus far. However, rhamnolipid production, which is under quorum-sensing control, does play a role in shaping the biofilm architecture by keeping the water channels of the biofilm open during maturation of the matrix [36]. By contrast, when a broad approach was used to identify quorum-sensing-regulated genes, the *pel* and/or *psl* genes were not revealed as direct targets [37]. Whether production of either of these polymers is in any way regulated as a function of population density remains to be determined. Thus, in *P. aeruginosa* the molecular mechanisms underlying the connection between quorum sensing and biofilm architecture remain mysterious.

In *V. cholerae* the connection between quorum sensing and biofilm architecture is better understood. The major extracellular polysaccharide expressed in the biofilms of this bacterium has been designated VPS (the genes responsible for its production being the *vps* genes) [9]. VPS production is negatively regulated by *hapR*; *hapR* mutants produce rugose colonies and form thick flow cell biofilms with narrow water channels [38–42]. The *hapR* gene encodes a transcription factor that is known to inhibit expression of *aphA* (a negative regulator of virulence) and promote expression of *hapA* (the structural gene for Hap, a metalloprotease with hemaglutinin activity) [38]. Importantly, expression of *hapR* itself is indirectly repressed by LuxO, a two-component response regulator that is most active under conditions of low cell density [39]. LuxO activity is controlled by at least two different quorum-sensing signals, but only the acyl-homoserine lactone CAI-1 (cholerae autoinducer 1) appears to play a significant role in biofilm formation [40]. Taken together, these observations suggest the following unexpected model: under conditions of low cell density, CAI-1 levels are low enough to permit LuxO-mediated repression of *hapR*, resulting in VPS production. Thus, this particular bacterium appears to initiate production of an extracellular matrix under conditions of low population density, presumably before the establishment of a multicellular community. Therefore, under biofilm-promoting conditions, planktonic *V. cholerae* cells might be expected to express a 'sticky' matrix that primes them for adherence to substrata as well as to other cells.

Alternative pathways: several routes to matrix production

It appears that most microorganisms are able to form biofilms through multiple pathways. From the earliest genetic studies of *Pseudomonas fluorescens* biofilm mutants it was already apparent that subtle alterations in environmental conditions could lead to suppression of biofilm-defective phenotypes [6]. This initial observation has now been extended to many other microbes. With respect to the extracellular matrix components of biofilms, two recent examples make the existence of alternative biofilm-formation pathways apparent. In the case of *V. cholerae*, the simple addition of Ca^{2+} activates a biofilm formation pathway that does not depend upon production of the VPS exopolysaccharide [43]. And in the case of *E. coli*, the PIA-like polymer mentioned above was discovered through a search for mutants in a genetic background in which the normal dependence on type 1 fimbriae, curli, colanic acid and motility for biofilm formation was suppressed by a mutation in the global carbon regulator *csrA* [28]. Thus, it appears that the ability to make different biofilms under different environmental conditions is a common trait in the microbial world.

Alternative pathways: the varied roles of pili

One mechanism by which biofilm formation can occur through different pathways depending upon environmental conditions is via differential expression of extracellular proteins. For example, in *E. coli,* expression of conjugative pili greatly accelerates initial adhesion and biofilm development in a flow cell by mediating non-specific cell-to-cell and cell-to-surface contacts [44], and this obviates the usual requirements for flagella, type I pili, curli and Ag43 [45]. As another example, enteroaggregative *E. coli* (EAEC) have been shown to form biofilms on glass and plastic when grown in media with high glucose and osmolarity; aggregative adherence fimbriae (AAF/II) are still required under these conditions, but type I pili, Ag43 and motility are dispensable [46].

There are also instances in which under different conditions the very same extracellular components play different roles within biofilms. This is the case for type IV pili in *P. aeruginosa*, which are known to mediate surface attachment and twitching motility, and have been shown to be differentially required for biofilm formation under different conditions [47–49]. In standing cultures of glucose minimal medium, mutants lacking type IV pili attach normally to abiotic surfaces but individual cells do not migrate towards each other and thus fail to form microcolonies [47]. In flow cell cultures using the same glucose minimal medium, microcolony formation occurs

through clonal growth, independent of type IV pili. However, in the later stages of biofilm formation cells do move across the substratum and onto the tops of pre-existing microcolonies in a type IV pili-dependent fashion [49]. By contrast, in a flow cell using citrate minimal medium, microcolony growth is followed by spreading across the substratum to form a uniformly flat biofilm; strains lacking pili form microcolonies but do not spread [48]. Thus, type IV pili-mediated surface migrations play an important role in biofilm formation, but the precise nature of that role and its impact on biofilm development varies with environmental conditions.

Even the requirement for type IV pili in the initial stages of *P. aeruginosa* biofilm formation can be bypassed. A transposon mutagenesis screen starting with a strain that lacked type IV pili revealed the role of another class of pili, the CupA fimbriae, in mediating cell-to-surface interactions [50]. The CupA fimbriae have also been shown to be structural components of the extracellular matrices of mature pellicles [8]. Interestingly, the PAO1 genome is predicted to encode three *cup* clusters, but only one (the *cupA* cluster) is required for attachment to plastic and glass; the roles of the other *cup* gene clusters remain unknown.

The meeting of two matrices

Thus far, we have considered biofilms that form on abiotic surfaces and interfaces. What happens when a bacterial biofilm, with its extracellular matrix, meets mammalian tissue, with its own extracellular matrix? It has long been recognized that specific adhesins are present at the tips of Gram-negative fimbrial structures that interact with mammalian matrix components [51]. Discoveries in *Streptococcus parasanguis* indicate that similar strategies might also be used by Gram-positive bacteria. Fap1, which belongs to a family of adhesins known as MSCRAMM (microbial surface components that recognize adhesive matrix molecules), is a structural component of fimbriae that carry at their tips an additional adhesin called FimA, a member of the LraI protein family [52,53]. Thus, it appears that in Gram-positive bacteria constituents of the extracellular matrix can mediate direct interactions with mammalian matrix components.

Finally, it is interesting to note that MSCRAMM adhesins have been identified in Gram-negative bacteria, including pseudomonads that do not normally colonize mammalian tissue. LapA (large adhesion protein A), which contains two extensive repeat motifs that are characteristic of MSCRAMM adhesins, is a cell-surface protein that enables *P. fluorescens* to attach to plastic, glass and quartz sand [54]. Similarly, *Pseudomonas putida* depends upon its *lapA* gene for attachment to plastic, glass and corn seeds [55]. Given that *lapA* is present in numerous other non-pathogenic pseudomonads, it is probable that some MSCRAMM family members evolved in the context of surfaces found in the natural environment rather than within the mammalian host.

The matrix: speculation and routes to follow

We have attempted to provide a snapshot of our current understanding of the extracellular matrices of bacterial biofilms. Although much has been learned about the components of the matrices of bacterial biofilms, much work lies ahead. The great diversity of polysaccharide and protein components of the biofilm matrix is an emerging theme. Future studies will have to probe deeper into the molecular mechanisms that regulate the synthesis of the matrix. In closing, we offer a bit of speculation relating to matrix components, as stimulus for future research.

As others have already noted, the expression of extracellular matrix components that render cells adherent is often regulated through phase variation. Well-known examples include VPS expression in *V. cholerae* [9], type I pili [56] and Ag43 expression in *E. coli* [57], and the formation of small colony variants by *P. aeruginosa* [58]. It is plausible that in planktonic populations there is a selective advantage in maintaining some cells in a 'sticky' state (e.g. *V. cholerae* expressing VPS), so that they are primed for attachment to surfaces and initiation of biofilm formation. It is equally plausible that in biofilm communities there is a selective advantage in maintaining some cells in a 'non-sticky' state, so that they are more easily dispersed for colonization of new territory. But what about cases in which phase variation controls not a switch between 'on' and 'off' states, but rather a switch between states in which extracellular molecules are differentially expressed? Situations such as these, as epitomized by *Salmonella* flagellar phase variation [59], generally have been ascribed the function of enabling the individual cell to evade the host immune response. Undoubtedly there are certain instances in which this is the case. Nevertheless, it is also possible that expression of even subtly different extracellular molecules could change the surface properties of the cells and thereby promote the formation and maintenance of different types of multicellular communities. In this light, it will be interesting to investigate the impact of other forms of cell-surface diversity, such as O-antigen, flagellar or capsular variation, on biofilm phenotypes. Drawing a parallel from the recent demonstration that several mammalian virulence factors of *P. aeruginosa* have an ecological role *vis-a-vis* this bacterium's interaction with a fungus [60], it is possible that different surface antigens provide bacteria with selective advantages in different natural environments.

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