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BIOFILMS AS COMPLEX DIFFERENTIATED COMMUNITIES

P. Stoodley,[1] K. Sauer,[1,2] D. G. Davies,[2] and J. W. Costerton[1]
[1]*Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717; e-mail: bill_c@erc.montana.edu*
[2]*Department of Biology, State University of New York, Binghamton, Binghampton, New York 13902*

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■ **Abstract** Prokaryotic biofilms that predominate in a diverse range of ecosystems are often composed of highly structured multispecies communities. Within these communities metabolic activities are integrated, and developmental sequences, not unlike those of multicellular organisms, can be detected. These structural adaptations and interrelationships are made possible by the expression of sets of genes that result in phenotypes that differ profoundly from those of planktonically grown cells of the same species. Molecular and microscopic evidence suggest the existence of a succession of *de facto* biofilm phenotypes. We submit that complex cell-cell interactions within prokaryotic communities are an ancient characteristic, the development of which was facilitated by the localization of cells at surfaces. In addition to spatial localization, surfaces may have provided the protective niche in which attached cells could create a localized homeostatic environment. In a holistic sense both biofilm and planktonic phenotypes may be viewed as integrated components of prokaryote life.

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INTRODUCTION

In just two decades we have learned that biofilms comprise highly structured matrix-enclosed communities (10) whose cells express genes in a pattern (51) that differs profoundly from that of their planktonic counterparts. Because direct observations show that biofilms constitute the majority of bacteria in most natural (8) and pathogenic (11) ecosystems, it seems unwise to continue to extrapolate from planktonic cultures in studies of these systems. A new mindset is clearly required because direct observations of structural complexity and unequivocal demonstrations of one or more distinct biofilm phenotypes presage a new concept in which biofilms are seen as complex differentiated communities. Observations of biofilms formed in pure cultures of the gamma proteobacteria group of bacteria and of mixed species biofilms in natural ecosystems show a basic organization in which cells grow in matrix-enclosed microcolonies separated by a network of open water channels. The importance of these complex structures, which are seen in direct observations of living biofilms by scanning confocal laser microscopy (SCLM), is that they demonstrate a level of differentiation that requires a sophisticated system of cell-cell signals and a degree of cellular specialization. The simple maintenance of open water channels in multispecies biofilms requires interspecies signaling to direct growth and exo-polysaccharide production away from the channels. We have barely begun to decipher the system of environmental cues and phenotypic responses that shape the multispecies microbial communities that predominate in most ecosystems, but the communities themselves bear witness to a remarkably complex developmental process. This developmental process is unique in biology in that it involves the coordinated activity of several relatively small prokaryotic genomes, rather than one or more large and coordinated eukaryotic genomes, to produce a functional multicellular community. This notion alters the perceived position of bacteria in the hierarchy of living things because the single cells we have studied so acidulously in planktonic cultures are actually members of coordinated multicellular communities whose complexity and sophistication are only now being appreciated.

STRUCTURE AND DIFFERENTIATION IN BIOFILMS

Claude Zobell first noted the preference of marine bacteria for growth on surfaces (66), and Costerton's group has extended this observation to freshwater systems and to a variety of microbial ecosystems, including those on the surfaces of

eukaryotic tissues (11). This concept of preferential growth on surfaces included no implications of complex biofilm structure, and as late as 1987 (7), biofilms were perceived (and pictured) as simple "slabs" of matrix material in which sessile bacterial cells were randomly embedded. In this early biofilm era, the question that was often asked and never answered was, "How do the deeply embedded cells in the biofilm have access to nutrients, including oxygen?" This pivotal question was answered, and the modern biofilm era began, when the first SCLM images of living biofilms (35) showed that sessile bacteria grow in matrix-enclosed microcolonies interspersed between open water channels as shown in Figure 1. The basic biofilm structure presented in Figure 1 is a computer-assisted compilation of confocal images of living fully hydrated biofilms formed by pure cultures of several proteobacteria and by natural mixed-species populations in natural ecosystems, such as mountain streams. This basic microcolony and water channel architecture is affected by shear forces and by many other factors discussed below, but its main consequence is that water from the bulk phase is entrained into channels and can therefore deliver nutrients deep within the complex community. Even in

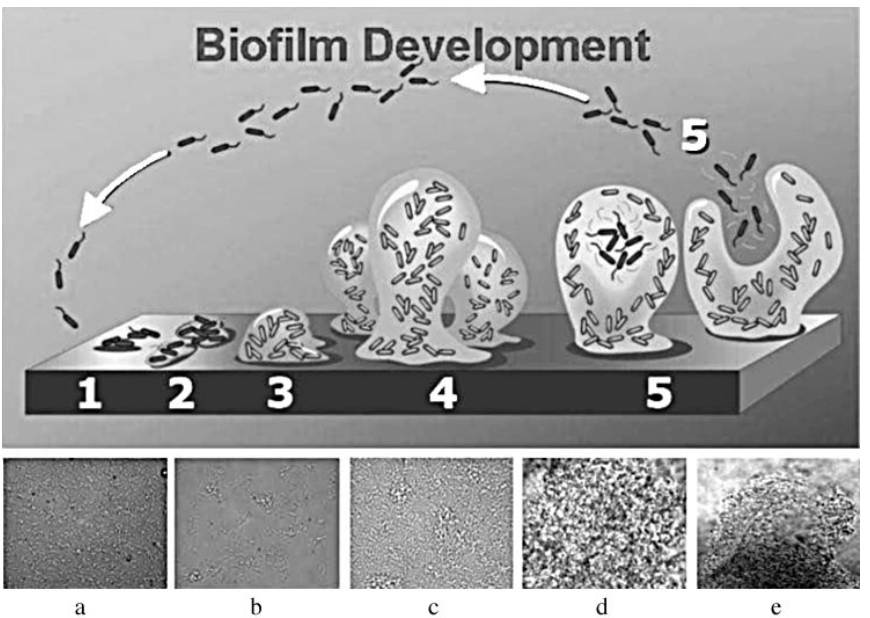

Figure 1 Diagram showing the development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered "irreversible" attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The *bottom panels* (a-e) show each of the five stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum.

thick biofilms, such as the subgingival plaque seen in periodontitis, the functional microcolony architecture is maintained because each structurally distinct microcolony is anchored on the tooth surface and each moves independently in response to shear forces. The exchange of nutrients facilitiated by this biofilm architecture enables biofilm communities to develop considerable thickness and complexity while keeping individual cells (some of which are physiologically specialized) in optimal nutrient situations in many locations within the biofilm.

Initial Processes in Biofilm Formation and Subsequent Structural Differentiation

Biofilm formation can occur by at least three mechanisms. One is by the redistribution of attached cells by surface motility (12, 30). Results from O'Toole & Kolter (45) on studies of *Pseudomonas aeruginosa* mutants suggest that type IV pili-mediated twitching motility plays a role in surface aggregation for this organism. A second mechanism is from the binary division of attached cells (25). As cells divide, daughter cells spread outward and upward from the attachment surface to form cell clusters, in a similar manner to colony formation on agar plates. A time-lapse video illustrating this type of aggregation can be found at the ASM MicrobeLibrary ("Growth and Detachment of Biofilm Cell Cluster in Turbulent Flow," www.microbelibrary.org/Visual/page1.htm). A third mechanism of aggregation is the recruitment of cells from the bulk fluid to the developing biofilm (58a). The relative contribution of each of these mechanisms will depend on the organisms involved, the nature of the surface being colonized, and the physical and chemical conditions of the environment. In oligotrophic environments mature biofilms may consist of little more than a sparse covering of cells with relatively little structural complexity. Biofilms can take over 10 days to reach structural maturity, based on microscopically measured physical dimensions and visual comparison (25, 53). For this reason we must be careful not to misinterpret biofilm retardation in the initial events of attachment, which is caused by a particular gene knockout, with the total suppression of biofilm development.

In *P. putida* and *Escherichia coli* it has been shown that the activity of cells in the centers of the cell clusters diminished as the clusters grew larger, but their activity could be restored by the addition of a more readily utilizable carbon source, indicating that cell activity in the interior of the clusters may be controlled by nutrient availability (52). DeBeer et al. (17) demonstrated that the channels surrounding the cell clusters could increase the supply of oxygen (and other nutrients) to bacteria within the biofilm, thus relating structure to function. The biofilm structure appears to be largely determined by the production of slime-like matrix of extracellular polymeric substances (EPS), which provides the structural support for the biofilm (21). The EPS is composed of polysaccharides, proteins, and nucleic acids. Nivens et al. (42) found that mucoid strains of *P. aeruginosa* produced more structurally differentiated biofilms than nonmucoid strains and more specifically that O-acetylation of alginate (a principal component of the EPS of mucoid *P. aeruginosa* strains) was

also required for structural development. Henzter et al. (24) found that they could induce structural complexity in a normally flat undifferentiated wild-type nonmucoid strain of *P. aeruginosa* biofilm by causing the overexpression of alginate. These alginate overproducing biofilms formed mound- and mushroom-shaped cell clusters separated by water channels similar to those formed by the wild-type mucoid strains (42). The nonmucoid PAO1 wild-type strain produced a flat biofilm in the Hentzer (24) study, but a heterogeneous biofilm structurally differentiated into mushroom-shaped cell clusters in the Davies (16) study. Hentzer et al. attribute this difference to differing nutrient conditions. The connection between EPS formation and differentiated biofilm structure is supported by Danese et al. (13) and Watnick et al. (60), who reported that colonic acid and EPS were required for the development of complex structures in *E. coli* K-12 and *Vibrio cholerae* O139 biofilms, respectively. These studies also showed that disruption of EPS not only resulted in less structural complexity but also conferred greater susceptibility to antimicrobial agents, while induced EPS overproduction had the opposite effect.

Influence of Hydrodynamics on Biofilm Structure and Material Properties

While the structure of biofilms is clearly influenced by a number of biological factors such as twitching motility, growth rate, cell signaling, and EPS production, the physical growth environment may also play a significant role in the determination of biofilm structure. Most laboratory biofilms are grown under low laminar flow conditions and the towers and mushroom-shaped microcolonies, and the patterns they form on the surface are generally isotropic i.e., there is no obvious evidence of directionality (Figure 1*a*,*c*). However, under higher unidirectional flows the influence of the increased shear becomes apparent and the biofilm cell clusters become elongated in the downstream direction to form filamentous streamers (55). Filamentous biofilms, or mats, commonly occur in both archeal and bacterial biofilms growing in fast-flowing environments such as hot springs (49b) or acid mine drainage runoff (20a). Filamentous biofilm streamers can also be formed by nonsheathed species such as *P. aeruginosa*, which grow as single cells in flask culture (49b, 55). The streamers are attached to the substratum by an upstream "head," while the downstream "tails" can freely oscillate in the flow (54a). In vitro studies of the development of biofilm streamers showed that the streamers became increasingly elongated over time, and scanning electron micrographs of in vitro *P. aeruginosa* biofilms show that the streamers thin along the tail until there is only a small chain of single cells at their tips ("*Pseudomonas aeruginosa*—Biofilm Streamers Growing in Turbulent Flow," ASM MicrobeLibrary, http://www.microbelibrary.org).

In addition to influencing biofilm structure, fluid shear also influences the physical properties of biofilms such as density and strength. A study by Liu & Tay (37) found that biofilms grown at higher shear were smoother and denser than those grown at low shear. Stoodley et al. (54) reported that *Desulfovibrio* spp.

(an anaerobic sulfate-reducing bacteria) and *P. aeruginosa* biofilms grown at higher shear stresses were more rigid and stronger than those biofilms grown at lower shear. It is yet unclear if the increased density and strength of biofilms exposed to higher shear stresses are regulated at the genetic level, occur through selection processes (i.e., only those cells that produce strong EPS remain attached and divide), or are determined by purely physical mechanisms (i.e., the alignment of EPS polymers that would be expected to occur when subjected to unidirectional shear). The deformation of laboratory-grown biofilms in response to normal or shear loading demonstrate that biofilms are viscoelastic and the viscoelastic properties were influenced by multivalent cations, presumably due to cross-linking of polymers in the EPS matrix (31, 54, 55). Preliminary evidence suggests that when biofilms are subjected to loads over short periods of time (seconds) they behave elastically, but over longer time periods they behave like viscous fluids. This may explain the fluid-like flow of biofilm ripples along the walls of a glass flow cell that was revealed by time-lapse microscopy (53, 56). These data and behaviors can be modeled using principles of associated polymer viscoelastic systems (29a), suggesting that the material properties of biofilm are largely determined by the EPS. Thus it appears that the EPS determines both the structure and cohesive strength of biofilms (21). Since it has been shown that alginate can protect mucoid *P. aeruginosa* FRD1 biofilm cells from exposure to ultra-violet radiation, it is possible that there was early selection pressures for biofilms colonizing wetted surfaces exposed to ultra-violet radiation to produce protective EPS. The biomechanics of biofilms are an important factor in linking structure to function and may ultimately help answer the question of whether biofilms are coordinated entities that actively shape their structure or whether they are merely aggregates of cells that are passively shaped by the chemistry and physics of the environment.

PHENOTYPIC DIFFERENTIATION DURING BIOFILM DEVELOPMENT

As in many processes of differentiation in many other biological systems, the differentiation process that transforms small groups of adherent bacteria into a thick matrix-enclosed biofilm community on a colonized surface was first perceived as a series of morphological changes. The individual adherent cells that initiate biofilm formation on a surface are surrounded by only small amounts of exopolymeric material, and many are capable of independent movement (45) by means of pilus-mediated twitching or gliding (Figure 1, *stage 1*). These adherent cells are not yet "committed" to the differentiation process leading to biofilm formation, and many may actually leave the surface to resume the planktonic lifestyle. During this stage of reversible adhesion (38) the bacteria exhibit several species-specific behaviors, which include rolling, creeping, aggregate formation, and "windrow" formation (30), before they begin to exude exopolysaccharide and adhere irreversibly (Figure 1, *stage 2*). Davies & Geesey (15) showed that in *P. aeruginosa* the cluster

of genes responsible for alginate production is upregulated within 15 min of the cell's initial contact with the colonized surface and that this genetic event initiates the process of biofilm formation. As biofilms mature they develop the basic microcolony/water channel architecture that is now well recognized in natural and in in vitro biofilms (Figure 1, *stage 3*), and many cells alter their physiological processes (e.g., grow anaerobically) in response to conditions in their particular niches. Individual microcolonies may detach from the surface or may give rise to planktonic revertants that swim or float away from these matrix-enclosed structures, leaving hollow remnants of microcolonies or empty spaces that become parts of the water channels (Figure 1, *stages 4 and 5*). Additionally, whole microcolonies may naturally break away from the biofilm (detach without any obvious perturbation to the system), although the mechanisms behind this phenomenon are yet unclear. These processes are not necessarily synchronized throughout the whole biofilm but are often localized so that at any one time a small area on the surface may contain biofilm at each developmental stage.

Differentiation in biofilm development has been explored in increasing detail since the 1980s. Studies from this period have resulted in various models that characterize biofilm development as a process of adaptation and changing genetic regulation. In a recent review, O'Toole et al. (44) describe biofilm development as a process of microbial development, not unlike that observed in fruiting-body formation by *Myxococcus xanthus* and sporulation in *Bacillus subtilis*. A number of investigations have been directed at determining the degree to which gene regulation during biofilm development controls the switch from planktonic to biofilm growth. Brözel and coworkers (3) monitored changes in global gene expression in attached *P. aeruginosa* cells and found more than 11 genes whose levels were altered during various stages of attachment. Whiteley et al. (61) used DNA microarrays to analyze gene expression of *P. aeruginosa* grown in chemostats and as a biofilm on submerged gravel substrata, and 73 genes that showed differences in regulation when compared with planktonic bacteria were identified. Prigent-Combaret et al. (48) carried out a screen in *E. coli* K-12 and found attachment-related changes in the regulation of 38% of the generated *lacZ* gene fusions (out of 446 clones). In a separate study by Sauer et al. subtractive hybridization of *P. putida* biofilms grown in continuous culture in tubes and planktonic bacteria in chemostats revealed more than 30 operons that were altered within 6 h following attachment (50). It is difficult to extrapolate to a universality of these changes in phenotypic expression in response to quite different growth strategies, but several examples are now available to illustrate these changes in several species.

Irreversible Attachment

Once attachment to a surface has been affected, by reversible attachment, the bacteria must maintain contact with the substratum and grow in order to develop a mature biofilm. This change from reversible to irreversible attachment was noted as early as 1943 by Zobel, and it has been characterized by Characklis (5) as the

transition from a weak interaction of the cell with the substratum to a permanent bonding, frequently mediated by the presence of extracellular polymers. Early investigators did not appreciate the possibility of surface transduction as a mechanism for inducing irreversible attachment, but recent investigations have gone a long way to suggest that profound physiological changes may accompany the transition to permanent attachment at a surface. One means of transition from reversible to irreversible attachment is mediated by type IV pili. Twitching motility is a mode of locomotion used by *P. aeruginosa* in which type IV polar pili are believed to extend and retract, propelling bacteria across a surface. In *P. putida*, irreversible attachment to a surface was shown by Sauer & Camper (50) to induce a surface-regulated switch from flagella-based motility to type IV pili–based twitching motility, as shown by differential gene expression and immunoblot analyses. Twitching motility is speculated by O'Toole & Kolter (45) to be involved in the formation of microcolonies. The authors suggest that interactions of bacteria with one another at a surface, forming groups of cells, help to strengthen the degree of attachment to the surface. Working with *Staphylococcus epidermis*, Gerke et al. (22) showed that adherent cells produce a polysaccharide intercellular adhesin that bonds the cells together and facilitates the formation of microcolonies and the maturation of the biofilm.

The hallmark of bacterial biofilms that segregates them from bacteria that are simply attached to a substratum is that biofilms contain EPS that surround the resident bacteria. Microbial EPS are biosynthetic polymers that can be highly diverse in chemical composition and may include substituted and unsubstituted polysaccharides, substituted and unsubstituted proteins, nucleic acids, and phospholipids (64). Among the best-characterized of all EPS is the bacterial product alginate, which has been shown to be involved in biofilm formation by *P. aeruginosa* in pulmonary infections and in industrial water systems. The production of alginate by *P. aeruginosa* has been regulated in response to a variety of environmental factors. The activation of a critical alginate promoter, *algD*, has been show to take place during nitrogen limitation, membrane perturbation induced by ethanol, and when cells were exposed to media of high osmolarity (20). The *algC* promoter is activated by environmental signals such as high osmolarity, and this activation is dependent on the presence of the response regulator protein AlgR1 (65). These experiments have been performed in liquid medium and on agar-based medium and have not been duplicated for biofilm bacteria, but they hint that environmental activation of alginate genes may take place in bacterial biofilms. Because the activities of alginate synthesis enzymes are difficult to detect, reporter constructs have been used to detect alginate production, and the *algC* gene has been shown to be regulated within 15 min of attachment to a substratum (15). This indicates that the production of alginate is an early event in the formation of a biofilm by *P. aeruginosa*, and we presume that it forms the structural and mechanical framework required for biofilm maturation. Although the term "irreversible attachment" was originally used to distinguish processes in the early events of biofilm formation, it implys that biofilms are rigidly "cemented" to surfaces. Time-lapse

microscopy showing the dynamic motion of single cells over surfaces by twitching motility, within biofilm microcolonies by flagellar motility, the flow of entire microcolonies along surfaces, and the continual detachment of single cells and entire microcolonies from mature biofilms (each of which are discussed in this review) indicate that this term may need to be revised.

Biofilm Maturation

The next phase of biofilm development, maturation, results in the generation of complex architecture, channels, pores, and a redistribution of bacteria away from the substratum (16). In a recent study, mature biofilms of *P. aeruginosa* were shown to have a radically different protein profile from planktonic bacteria grown in chemostats (51). As much as 50% of the detectable proteome (over 800 proteins) was shown to have a sixfold or greater difference in expression. Of these, more than 300 proteins were detectable in mature biofilm samples that were undetectable in planktonic bacteria. The identified proteins fall into five major classes: metabolism, phospholipid and LPS-biosynthesis, membrane transport and secretion, as well as adaptation and protective mechanisms (51). In a separate study, Whiteley et al. (61) used DNA microarray technology to evaluate mature biofilms and compared these to chemostat cultures of *P. aeruginosa*. Just over 70 genes were shown to undergo alterations in expression in this study. Among those genes detected to be upexpressed in mature biofilms were genes encoding proteins involved in translation, metabolism, membrane transport and/or secretion, and gene regulation. These researchers also observed that the sigma factor *rpoH* was upexpressed and that the sigma factor *rpoS* was downexpressed in mature biofilms.

Cell-Cell Communication During Biofilm Formation

McLean et al. (39) have shown that acyl HSL autoinducers are detectable in naturally occurring biofilms, suggesting that biofilm communities in nature contain populations that are able to undergo cell density-dependent gene regulation. In 1998, Davies et al. (16) showed that *P. aeruginosa* PAO1 requires the *lasI* gene product $3OC_{12}$-HSL in order to develop a normal differentiated biofilm. In this study, it was observed that bacteria knocked out in the quorum-sensing inducer gene *lasI* produced biofilms that were only 20% as thick as those produced by the wild-type organism. In addition, these mutants grew as continuous sheets on the substratum, lacking differentiation and not demonstrating evidence of matrix polymer. By contrast, the wild-type organism formed characteristic microcolonies composed of groups of cells that were separated by intervening matrix polymer and separated from one another by water channels. When the autoinducer $3OC_{12}$-HSL was added to the medium of growing *lasI* mutant bacteria, these cells developed biofilms that were indistinguishable from the wild-type organism. These results indicated that $3OC_{12}$-HSL was responsible for the complex architecture observed in mature biofilms produced by *P. aeruginosa*. Spatial analysis revealed that the genes *lasI* and *rhlI* were maximally expressed in biofilm cells located at the

substratum (18). Whereas *lasI* gene expression was found to decrease over time with increasing biofilm height, *rhlI* expression remained steady throughout biofilm development but occurred in a lower percentage of cells.

Since biofilm architecture is presumed to be influenced by matrix polymer, we can hypothesize that autoinduction is at least partly responsible for regulation of EPS synthesis in *P. aeruginosa*. In support of this hypothesis, it has been demonstrated that the alginate genes *algC* and *algD* are induced in *P. aeruginosa* strain 8830 by $3OC_{12}$-HSL and by $3OC_4$-HSL (D.G. Davies & J.W. Costerton, unpublished data). These observations indicate the possible role of quorum sensing as a signal transduction system by which *P. aeruginosa* may initiate the production of alginate and possibly other types of EPS. In a separate study Olvera et al. (43) have observed that *algC* is necessary for the production of rhamnolipid. The transcription of rhamnolipid has been shown to be dependent on activation by the quorum-sensing gene product *rhlI* (46), further indicating that *algC* transcription is regulated at some level by bacterial quorum sensing. In addition to activation of multiple factors by autoinduction, it has recently been shown that *P. aeruginosa* is able to repress the transcription of *lasI* in response to RsaL, an 11-kDa protein whose gene lies downstream from *lasR* (19). This protein has been shown to suppress LasB production and presumably all other factors regulated by the lasI quorum-sensing system. This finding demonstrates a further level of control of quorum sensing in *P. aeruginosa* and suggests that cell-to-cell communication is highly complex.

Altogether, *P. aeruginosa* is known to have 39 genes that are under the regulation of the *lasI*/*rhlI* quorum-sensing systems (62). In addition to cell-to-cell communication via acyl HSL-mediated quorum sensing, it has recently come to light that other communication systems are used by *P. aeruginosa*. Holden et al. (26) have discovered that cell-free extracts of cultures in which *P. aeruginosa* were grown contain diketopiperazines (DKPs), a cyclic dipeptide known to participate in cell-to-cell communication mostly in gram-positive bacteria. This compound has been shown to be capable of activating a LuxR biosensing system and swarming motility in *Serratia marcescens*. In addition, a third extracellular sensing system has been discovered in *P. aeruginosa* by Pesci et al. (47). In this system, *P. aeruginosa* produces a quinolone signaling molecule (2-heptyl-3-hydroxy-4-quinolone) that regulates LasB production via the *lasI*/*rhI* quorum-sensing system. This observation hints at a super-regulatory function for quinolones in bacterial communication.

Sauer et al. (51) have recently shown that quorum sensing does not account for all biofilm-specific protein production in *P. aeruginosa*. Analysis of protein patterns of irreversible attached (1 day) and planktonic cells revealed the presence of quorum-sensing independent but probably surface-induced differential expression of proteins. Fifty-seven unique protein spots were identified in 1-day biofilm protein patterns for both *P. aeruginosa* strain PAO1 and *LasI* minus strain PAO-JP1 that were absent in planktonic protein patterns and 48 protein spots that were unique for planktonic growth. These results imply that quorum sensing accounts for only a portion of the total number of genes whose regulation is altered during

the irreversible stage of biofilm development and that the physiological change in attached bacteria is not due solely to induction by PAI-1 quorum-sensing autoinducer (see 50). The implication of these results is that undiscovered biofilm regulons probably exist, that the role of quorum-sensing and other intercellular signaling mechanisms is unclear, and that these complex systems are under intercellular as well as extracellular control. The existence of these systems further suggests that interspecies communication is possible, as is evident from studies in which bioassays have been used to detect the presence of autoinducers from multiple species.

RpoS Activity During Biofilm Development

During stationary phase, gram-negative bacteria develop stress-response resistance that is coordinately regulated through the induction of a stationary-phase sigma factor known as RpoS (23). Biofilm bacteria are generally considered to show physiological similarity to stationary-phase bacteria in batch cultures. Thus, it is presumed that the synthesis and export of stationary-phase autoinducer-mediated exoproducts occur generally within biofilms. The stationary-phase behavior of biofilm bacteria may be explained by the activity of accumulated acyl HSL within cell clusters. The mechanism causing biofilm bacteria to demonstrate stationary-phase behavior is hinted at by the discovery that RpoS is produced in response to accumulation of the *rhlI* gene product in *P. aeruginosa* cultures (34). In a recent report, a similar relation was made, but rather than the RhlR-RhlI system influencing the expression *of* rpoS, it was demonstrated that RpoS regulated *rhlI* (63). In another study, Suh et al. (58) have shown that in *P. aeruginosa* PAO1 RpoS is responsible for a decrease in the production of exotoxin A, elastase A, LasA protease, and twitching motility. Additionally, it was found that *P. aeruginosa* FRD1 demonstrated a 70% loss in alginate synthesis when *rpoS* was inactivated.

Detachment as a Component of Biofilm Development

Detachment is a generalized term used to describe the release of cells (either individually or in groups) from a biofilm or substratum. Active detachment is a physiologically regulated event, but only a few studies (57) have been performed to demonstrate a biological basis for this process. Allison et al. (1) showed that following extended incubation, *P. fluorescens* biofilms experienced detachment, coincident with a reduction in EPS. In *Clostridium thermocellum* the onset of stationary phase has been correlated with increased detachment from the substratum (33). It has been postulated that starvation may lead to detachment by an unknown mechanism that allows bacteria to search for nutrient-rich habitats (44). This hypothesis is in agreement with recent observations by Sauer et al. (51) who compared two-dimensional-gel protein patterns to show that dispersing cells of *P. aeruginosa* are more similar to planktonic than to mature biofilm cells. This finding indicated that dispersing biofilm cells revert to the planktonic mode of growth; thus, the biofilm developmental life cycle comes full circle (51).

The transition from a flowing system to a batch culture system has been observed by many laboratories to result in biofilm detachment (14). We surmise that an increase in the concentration of an inducer molecule is responsible for the release of matrix polymer-degrading enzymes, which results in detachment of cells from the biofilm. One such example is found with the gram-positive organism *Streptococcus mutans*, which produces a surface protein releasing enzyme (SPRE) that mediates the release of cells from biofilms (36). Boyd & Chakrabarty (2) showed that overexpression of alginate lyase causes the degradation of alginate and produces biofilms that can be removed from surfaces by gentle rinsing. Cell density–dependent regulation may also trigger the release of matrix-degrading enzymes, allowing bacteria to disperse from a biofilm, when cell density reaches a high level in biofilm microcolonies (D.G. Davies, J.W. Costerton & H.M. Lappin-Scott, unpublished observations). Other investigators have demonstrated that homoserine lactones may play a role in detachment. Puckas et al. (49) reported that homoserine lactone production was negatively correlated with cell cluster formation in *Rhodobacter sphaeroides*, and Allison et al. (1) reported that the addition of *N*-acyl-C_6 homoserine lactone to *P. fluorescens* biofilms caused a reduction in biofilm and loss of exopolymer. The regulation of detachment events in microbial biofilms represents an important area for future research, and the new methods of analysis of gene expression promise to speed this process.

Biofilm Formation as a Developmental Process

As demonstrated above, biofilm development can be partitioned into at least four distinct stages. These are (*a*) reversible attachment, (*b*) irreversible attachment, (*c*) maturation, and (*d*) detachment. Detached cells are believed to return to the planktonic mode of growth, thus closing the biofilm developmental life cycle. A schematic overview is shown in Figure 1. These four biofilm developmental stages indicate significant episodes in the formation of a bacterial biofilm. Bacteria within each of the stages of biofilm development are generally believed to be physiologically distinct from cells in other stages. In a recent study, biofilm development in *P. aeruginosa* has been partitioned into four stages, and each stage was analyzed separately by two-dimensional polyacrylamide gel electrophoresis (51). The average difference in protein production between each developmental episode was 35% of detectable proteins. The transition from planktonic growth to the stage of irreversible attachment resulted in a 29% change in the production of detectable proteins. The transition from irreversibly attached cells to the stage of mature biofilms caused a change in the protein production of 40%, with the majority of proteins showing an increase in concentration. In contrast, the transition from mature-stage biofilm to the dispersion stage resulted in a reduction in 35% of detectable proteins. Cells during this stage of development had protein profiles that were more similar to planktonic cells than to mature-stage biofilm cells. The most profound differences were observed when planktonic cells were compared to mature biofilm cells, with more than 800 detectable proteins showing more than

a sixfold change, or to dispersing biofilm cells. Differences in the protein patterns of planktonic cells and mature and dispersing biofilm cells of a clonal population of *P. aeruginosa* were as profound as the difference for different but related *Pseudomonas* species grown at the same stage of development. Therefore, the authors conclude that *P. aeruginosa* displays at least three phenotypes, (*a*) planktonic, (*b*) mature biofilm, and (*c*) dispersion, during biofilm development (51).

SALIENT CHARACTERISTICS OF BIOFILM COMMUNITIES

Biofilms as Physiologically Integrated Microbial Communities

When we examine the mature communities that dominate particular microbial ecosystems, with some emphasis on systems with high levels of physiological efficiency (like the bovine rumen), we find that these communities are composed of complex multispecies biofilms. The biofilm mode of growth is optimal when bacterial communities must colonize insoluble nutrient substrates (e.g., cellulose), and it is especially useful when multistage digestive processes involve many different species whose cells can be stably juxtaposed within a communal matrix. The bovine rumen provides a particularly cogent example of a biofilm-driven ecosystem because fresh nutrient substrate is ingested continually, the primary cellulose degraders colonize it avidly, and physiologically cooperative species accrete to form an efficient digestive consortium. The efficiency of the digestive consortium is as dependent on microbial teamwork as it is on the activity of the primary cellulose degraders because the removal of the products of primary digestion (e.g., butyrate) drives the whole cellulolytic process (6). In the rumen ecosystem, some of the organisms that drive cellulose digestion by product removal live within the biofilms on the cellulose fibers, whereas others are highly mobile cells (e.g., Treponema) that graze on "hot spots" of high butyrate concentration on the biofilm surfaces (32).

Because our speculations on the formation of digestive biofilms in the rumen predate modern perceptions of the role of cell-cell signaling in biofilm formation (16), all the mechanisms suggested to date have involved the attraction of cooperative species by nutrient advantage. The basic concept is that primary cellulose degraders attach to their preferred nutrient substrate and that secondary organisms are chemotactically attracted by butyrate and other volatile products of anaerobic cellulose digestion. However, as we emphasize in subsequent sections, nothing in the cellulolytic ecosystem precludes a possible role of cell-cell signaling in the development of these physiologically integrated biofilms. The notion that virtually all aspects of bacterial behavior are controlled by intercellular signals has a liberating effect on ecological speculations concerning the origin and organization of the complex physiologically integrated biofilms that actually dominate most ecosystems. We are no longer constrained by the necessity of explaining the recruitment of particular bacteria into particular ecological niches in terms of immediate

physiological advantage. We now understand molecular mechanisms, in both bacteria and archea, by which one prokaryotic cell can attract another prokaryote (or even a eukaryote) by means of a simple signal molecule. Furthermore, the cells that then find themselves in stable juxtaposition can manipulate each other's physiology, to mutual advantage, and produce efficient integrated physiological consortia. We can now adopt an approach similar to that of the embryologists who explain the developmental changes in multicellular eukaryotic organisms in terms of hormonal signals, sent and received, rather than in terms of immediate nutrient advantage to participating cells. Logically we can view the complex microbial communities that develop in a calf's rumen, initiated by bacterial genomes from its mother's microbial population, with the same awe we usually reserve for the development of its tissues and organs from its mother's fertilized egg.

Biofilms as Behaviorally Integrated Microbial Communities

The fruiting bodies formed by Myxobacteria comprise one of many examples of macroscopic biofilms that are produced by individual cells of many bacterial species in response to specific environmental cues, such as starvation and other adverse factors. In nutrient-sufficient conditions these same myxobacterial cells move through their environments, by a form of gliding motility that depends on the coordinated activity of retractable pili (27), in large swarms that react to nutrient gradients. This complex pattern of integrated behavior is facilitated by the production of surfactants and is controlled by cell-cell signals [acyl homoserine lactones (AHLs)] in a manner that is described in detail in a review by Kim et al. (28). When environmental conditions deteriorate, in terms of drying or of nutrient deprivation, swarms of myxobacteria form microcolonies in which individual cells pile up on each other and produce large amounts of extracellular matrix material to produce macroscopic fruiting bodies. The individual bacterial cells within these *de facto* biofilms differentiate to form cysts, and the biofilms themselves differentiate to form elaborate shapes, in which simple mushroom shapes may morph into complex structures resembling jester's caps or ornate crowns. The net result of this signal-controlled process of differentiation is that the cells of a particular species have formed a drying-resistant biofilm that is resistant to the environmental conditions that threatened its planktonic way of life in more halcyon times. Thus, in these mobile biofilms, control mechanisms based on cell-cell signaling may be integrated with cellular strategies for nutrient acquisition, and particular cells may burgeon when both processes bring them into a biofilm microniche, in which conditions are optimal (9).

Biofilms as Highly Structured Microbial Communities

Microbial biofilms are often sufficiently thick and extensive to be visible to the unaided eye, and they may contain millions of prokaryotic (and sometimes some eukaryotic) cells in arrangements that facilitate the stable juxtaposition of physiologically cooperative organisms. Cells of particular species are found consistently

in certain locations, near the colonized surface or at the apices of towers or mushrooms; the sessile cells that comprise single-species biofilms are located within the microcolonies in species-specific distribution patterns. In these single-species biofilms, the preponderance of the sessile cells may be found in the caps of mushrooms in a highly organized pattern with relatively regular cell-cell spacing, and the stalks of mushrooms formed by some species are virtually devoid of cells. We have up to this time explained these distribution patterns on the basis of nutrient advantage, as in cases where cellulolytic organisms occurred next to their substrate and fastidious aerobes occurred next to open water channels, but these explanations now seem to be unsatisfactory. They contain no provision for the movement of cells within the biofilm matrix or for the establishment of uniform cell-cell distance between sister cells, and it is difficult to see how some areas of the matrix are heavily colonized while others are devoid of cells. We hereby predict that a mechanism will be discovered whereby sessile cells within biofilms can move within the matrix, perhaps by the activity of pilus-like structures, attached to two or more cells, that can shorten or elongate to position the cells with some degree of precision. The high levels of lateral gene and plasmid transfer seen in biofilms may indicate that these or other structures may facilitate genetic exchange between neighboring cells, and the matrix may be structured in a manner that facilitates signaling between adjacent cells. Physiological cooperation between cells of the same or different species would be facilitated if the secondary structure of the EPS favored the preferential diffusion of particular molecules or even electrons. The random extrusion of EPS, with consequent cell displacement, is simply insufficient to account for the sophistication of the cell distribution patterns that we see in biofilms. Therefore we suggest that biofilms are surface-associated microbial communities, within which the position and spatial relationship(s) of each component cell are predetermined by means of a coordinated developmental cycle that is mediated (at least in part) by signal molecules and some type of positioning mechanism.

Biofilms as Self-Assembling Microbial Communities

The bacterial genome is expressed in many different phenotypes, as are the genomes of virtually all living things, and the proteomic data in the "Biofilm Formation as a Developmental Process" section of this review establishes the profound nature of the phenotypic changes that accompany the transition from planktonic to biofilm growth. Similar changes in gene expression occur when planktonic bacteria react to starvation (29). As a consequence of this phenotypic plasticity the genomes of thousands of bacterial species exist, in varying patterns of expression, in virtually all ecosystems in which environmental conditions allow bacterial growth or persistence. Recent examinations of oligotrophic environments, including the deep subsurface and the deep oceans (29), have revealed that approximately 1×10^5 bacterial cells are present in each milliliter of water and that most of these cells are in a dormant starved state. For this reason, the biosphere can be visualized as being

a continuum of fluids in which the genomes of thousands of species of bacteria exist in forms that range from virtually dormant to active in either the planktonic or the biofilm patterns of gene expression.

This ubiquity of the genomes of so many different species of bacteria provides the mechanistic basis for the self-assembly of microbial communities. In modern times, the ocean reservoir of bacterial genomes routinely serves as the genetic source for the development of self-assembled microbial communities that develop when black smokers suddenly deliver volcanic gasses (notably hydrogen and H_2S) into the marine environment. Dormant bacteria are resuscitated into a physiologically active state, and these planktonic cells undertake biofilm formation on available surfaces in a nutrient-rich environment. As H_2S is oxidized to form thiosulfate and other sulfur compounds, more species of sulfur-cycle organisms will be resuscitated and these organisms will be recruited into the developing black smoker biofilm community. A complex microbial biofilm community will develop in response to this new nutrient opportunity in a pattern that is remarkably similar in virtually all ocean regions, and the new availability of organic carbon compounds will recruit heterotrophic prokaryotes and macrophytes. Eventually, the anabolic processes of the heterotrophic organisms will balance the catabolic processes of the chemolithotrophs, and a stable climax community will have developed. Black smoker communities are representative of bacterial communities that may have developed in the primitive earth by the recruitment of hundreds of bacterial genomes and by the subsequent differentiation of these genomes to produce physiologically integrated communities. Because black smoker communities have thrived in the deep oceans for eons, the reservoir of prokaryotic genomes that float in the depths may be especially rich in potential members for the sessile communities that develop quickly in response to each new opportunity.

It may be useful to contrast the phenotypic plasticity and ubiquity of the bacterial genome with the same properties of higher plants and animals in order to examine their relative success in responding to transient nutrient availability. In the interests of homeostasis and physiological integration, plants and animals have amassed much larger genomes so that they can build large and complex multicellular organisms from a condensed and coordinated genetic base. Their genomes are usually kept isolated and protected, except during gamete release by some species, and many of the higher forms are dependent on seasonal sexual cycles for their reproduction. It is axiomatic that the standing crop of insects in a stream is roughly proportional to the availability of organic nutrients in the system, but a huge input of particulate organics would have no effect at all on the mayfly population if it did not coincide with a feeding larval stage of that insect. Similarly, the sudden availability of large amounts of soluble nutrients on the bank of the same stream would have no effect on the colonization of that environment by bullrushes, if the bullrush genome was not present or if rainfall was insufficient. In contrast, the sudden availability of either soluble or particulate nutrients would have a profound effect on the bacteria in both the stream and the soil, and both would produce complex biofilm communities to make maximum use of this largesse to

produce large microbial populations. Significantly, the phenotypic plasticity of the bacteria allows these organisms to process all the nutrients in stationary biofilm communities and then to disperse into both the stream and soil ecosystems as persistent genomes that are capable of responding to the next fortuitous nutrient event.

THE ROLE(S) OF SURFACES IN THE ORIGIN OF CELLS AND OF MULTICELLULAR COMMUNITIES

Our perception of prokaryotic cells has evolved from the notion of a membrane-enclosed bag of randomly seething protein and nucleic acid molecules to the concept of organized cytoplasmic elements enclosed within a highly organized membrane (and an even more structured cell wall). The concept of randomly floating or swimming bacterial cells has evolved into the thesis of this review, which is that most ecosystems operate on the basis of highly structured and coordinated communities of prokaryotic cells in which many members have specialized functions. The eukaryotic cell is also increasingly seen as a highly structured entity with a complex cytoskeleton that holds every organelle, and virtually every structural molecule, in a predetermined location that dictates its contribution to the coordinated function of the whole cell. Multicellular eukaryotic organisms are now known to coordinate the activities of their cells in patterns much more complex than those that were visualized when biologists first sat down to contemplate the origins of life. The role of random interactions of molecules or of cells has generally taken a beating in the past two decades, and it may be salutary to take a fresh look at the origins of life in light of these new concepts.

If an ordered structure lies at the root of our present concept of even the simplest cell, and of even the simplest multicellular community, it seems futile to look for this basic order in the planktonic state in which molecules and cells move about in a roiling mass in constant Brownian movement. In the nonliving world, high levels of order are found in crystals, and even the humble and ubiquitous clays display a highly structured series of alternating bands of different compositions and charges. Cairns-Smith (4) has speculated that the assembly of enzyme molecules might have been facilitated by the use of nonliving clay templates. This rudimentary replicating system may have later incorporated RNA, which eventually replaced the crystalline clay as the information template. Others have speculated that this association may have stabilized these pioneer nucleic acids against the entropy imposed by ionizing radiation. Our modern concept of prokaryotic cell membranes, with their complex arrays of protein molecules and their control over the replication of DNA, are barely recognizable from the early notion of a simple lipid bilayer, and logic seems to demand their original assembly on an ordered surface. These considerations would seem to suggest that the first organization of bacterial or archeal cells would have taken place at a surface: the surface acting as a locus for cells and absorbed nutrients to interact. The physical proximity of one cell to

another may have provided a selective pressure to develop complex interactions within these early communities.

The theme of this review is the notion that complex interactions within prokaryotic communities evolved in surface-associated biofilms. The basis for our speculation is the fact that the earliest fossil records of life occur in surface-associated microbial mats in ancient hyperthermal vents and hot springs environments (49a, 49b) and that biofilm mat formation is found in the most ancient linages of archaeal and bacterial lines, the Korarchaeota and Aquificales (20a, 27a, 49b). A stable structural juxtaposition seems absolutely necessary as cells developed the physiological cooperation and reciprocal signaling so implicit in the origin of bacterial biofilms. To the extent that it is literally mind boggling to think that any such interactive community could develop as individual prokaryotic cells wheel past each other in the endless and frantic Brownian dance that all of us have seen under our microscope lenses. The fact that physiological coordination and signal-based cooperation are both based on the orderly diffusion of organic molecules demands the stable juxtaposition of cells and makes biofilm development a *sine qua non* for the major step from single cells to increasingly coordinated communities. Our current observation that structured and physiologically integrated communities comprise the predominant forms of growth in both bacteria and archea imply convergent evolution in two kingdoms that have evolved both independently and successfully.

As the first bacterial and archeal cells arrived at an evolutionary state that allowed replication, perhaps through the intermediate step of functional replicating nanobacteria (59), their immediate need would be for a protective homeostatic environment. The primitive earth was undoubtedly hostile, in chemical and physical terms, and these pioneer organisms would only survive if they could avoid floating from a permissive niche into a hotspring or an acid pool. Morita has suggested (40) that the energy source that supported the first successful prokaryotes was hydrogen, and we can easily imagine a matrix-enclosed community of primitive prokaryotes attached to a surface adjacent to a hydrogen source. In this way, the first multicellular communities of both bacteria and archea would develop in the same protected nutrient-rich niches in which, over millions of years, their cell machinery had been assembled to produce the first replicating bacterial and archeal cells. Cells of cooperative species would be found in stable juxtaposition within biofilm communities, and bacteria could cooperate with archea in multispecies communities that would be the primitive equivalent of modern black smoker communities. In this thesis, biofilms are the predominant form of prokaryotic life because basic biological processes, including the first assembly of cells and the first assembly of multicellular communities, can draw their basic order and their stable juxtaposition from the inherent order of inanimate surfaces. Once formed, these cells and these communities are protected from antibacterial influences (first chemical and physical and then viral and eukaryotic) by their specialized biofilm phenotype and by their production of matrix materials that further condition their selected niche. The concept of highly structured and metabolically integrated bacterial communities

brings the prokaryotic domains much closer to the eukaryotic domain and provides a conceptual framework within which the transition of bacteria to mitochondria and of blue-green bacteria to chloroplasts may have occurred.

If we accept this notion that prokaryotic cells and prokaryotic communities developed preferentially on surfaces, we soon encounter the primary disadvantage of growth in biofilms, in that the conditioned niches become crowded with cells and access to nutrients is compromised. The first adaptation in response to this problem was probably the development of the structures inherent in the microcolony and water channel architecture of biofilms, which increase substrate access in sessile communities. The next adaptation in response to crowding in primitive biofilms may well have been the development of lateral surface–associated motility, which takes the form of twitching if it is mediated by type IV pili (45) and swarming if it is mediated by retractable pili (27). This twitching motility enables bacteria to colonize adjacent areas of the surface, and it is also involved in cellular rearrangements prior to biofilm formation during the colonization of newly available surfaces. Swarming motility involves a newly discovered mechanism that depends on retractable pili and can achieve amazing feats of coordinated cellular movement, so that its most efficient proponents (the Myxobacteria) can travel long distances and form elaborate structures. Some of the microbial biofilm communities that developed in these ways were macroscopic, and their early development is evident in the earliest fossil records as stromatolites.

We propose, for discussion, that the planktonic phenotype may have actually developed in the relative homeostatic environment afforded by the biofilm. The planktonic phenotype differs profoundly from the biofilm phentoype, and it involves the expression of a large number of genes that allow bacterial and archeal cells to detach themselves from biofilms and assume a floating or swimming mode of growth. Many planktonic cells are characterized by a heavy investment of genetic capability in detachment, in the formation of flagella, and in chemotactic mechanisms that allow them to find new and favorable niches for the development of stable protected biofilms. In the development of the Plant Kingdom primitive forms concentrated on growth and lateral spread in favorable niches, and higher forms only developed seeds when the available ecosystems became crowded and dispersal became a preferred strategy. In the same way, bacteria may have evolved their dispersal mechanisms late in their evolution, in response to the basic problem of crowding and to the opportunity of colonizing new favorable niches. We suggest that the evolution of the highly specialized planktonic phenotype has allowed certain species of bacteria and archea to colonize new environments, even at considerable distances from their original niches, and that this late-developing phenotype accounts for the phenomenal ecological success of these species.

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