Developmental biology of biofilms: implications for treatment and control

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Although of heterogeneous spatiotemporal and species compositions, all biofilms undergo certain common developmental events: organic molecules on the substratum can play a role in initial attachment, attached cells grow and additional cells attach from the bulk liquid. Biofilm growth is a four-dimensional (X, Y, Z and T) process similar to organ development.

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What is a biofilm? Although commonly thought of as bacterial, the vast majority of biofilms in nature include eukaryotic organisms as well as bacteria. Natural assemblages of algae, fungi and protists are found, together with bacteria, on substrata in most hydrated environments, even in those hydrated for only short periods of time. Pure prokaryotic biofilms are found exclusively as laboratory study systems or in the rare ecological regimes in which eukaryotes are excluded (some hot-spring sites and some deep subsurface sites). Only a small stretch of the imagination is required to consider the cell biologist's cultures as biofilms: confluent pure cultures of eukaryotic cells that develop on the wall of a flask. Generally, the substratum is thought to be inanimate: for example, a tooth, a rock, an artificial heart valve or the inner wall of a water pipe. However, microorganisms also attach to animate substrata: bacteria are found on the soft tissues of the oral cavity, in wound sites and on animal and plant epithelia. Furthermore, bacteria can aggregate with debris at air–water interfaces (e.g. the neuston of bodies of water): these are clearly biofilms although their attachment site is not easily recognized as a substratum. For the purposes of this review, the operating definition of a biofilm will be a collection of microorganisms (including cells in culture) and their associated extracellular products at an interface and generally attached to a biological (other cells or tissues, including matrix polymers) or abiological (mineral or synthetic) substratum.

It is likely that biofilms comprise the vast majority of the world's microbial biomass and it is also true that, until recently, microorganisms have usually been studied under conditions that do not reflect their preferred (biofilm) habitat. The exponential increase in publications on biofilm research reflects the relatively recent advent of the tools to perform biofilm research. An important concept in this work is that of the biofilm ecosystem. Many detrimental biofilms exist, including dental plaque, implant-associated infections, pathogens in drinking water distribution systems and corrosive biofilms in the oil industry. However, biofilms can also be beneficial to humans: wastewater treatment plants and activated-sludge processing facilities owe their effectiveness to biofilms. If the ecology of the biofilms exploited in these processes is disrupted by a change in environmental conditions, the species composition of the biofilm is affected and the facility becomes less efficient or may collapse[1].

With the possible exception of dental plaque, the bulk of microbiological research has been concerned primarily with the characteristics of microorganisms grown in liquid culture rather than with those of microorganisms grown on substrata. However, work on axenic strains in liquid culture can be profoundly misleading. For example, when grown under standard laboratory conditions in liquid culture, bacteria can be at least tenfold more susceptible to antibiotics than when growing in a biofilm[2]. The explanation for the antibiotic resistance of biofilms remains elusive, and the relevance of this example to patients with internal medical devices cannot be overestimated.

Biofilms are dynamic with respect to structure and to composition, but few studies have been concerned with the consequences of microbial growth as a biofilm or with the later stages of biofilm development. We therefore suggest that biofilms are best studied from a spatiotemporal/differential standpoint similar to that of developmental biology. Generalized patterns of biofilm development must be understood, and these patterns must be investigated under conditions that are equivalent to those experienced by that particular biofilm as it grows in nature. This review provides snapshots of spatial and compositional moments in the development of biofilms; it should be kept in mind that the developmental process is a continuum.

Initiation of biofilm formation

Cells attach to substrata, and a substratum to which no cell can attach has yet to be discovered. The lack of a comprehensive interpretation of cell surface data, such

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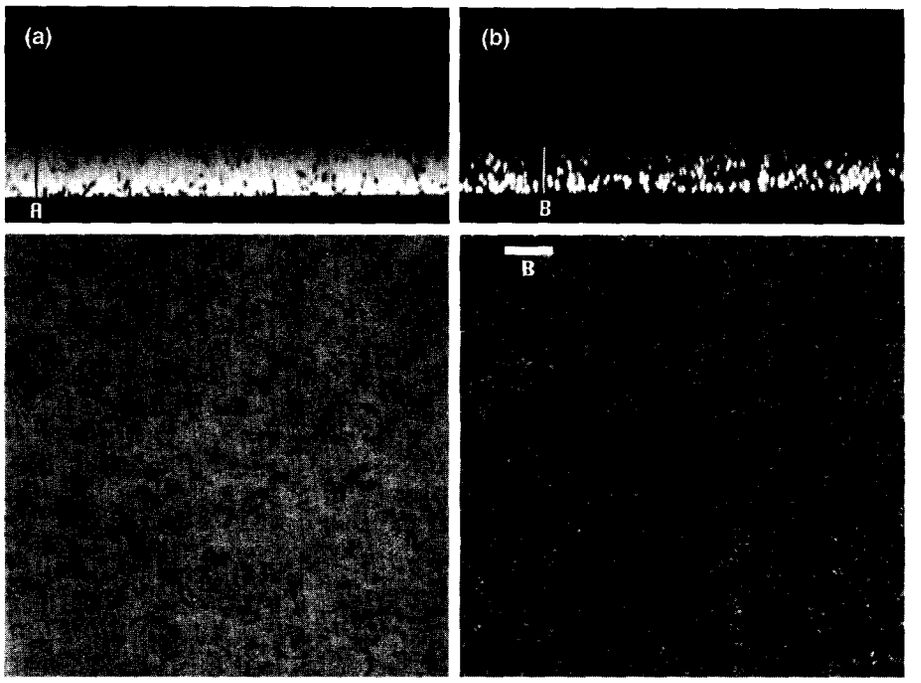

Fig. 1. Biofilm architecture is affected by exopolymer production. Confocal micrographs of pure-culture biofilms negatively stained with fluorescein. The upper panels in **(a)** and **(b)** represent XZ sections of the biofilms (cross sections, perpendicular to the substratum), whereas the lower panels represent XY sections (parallel to the substratum) at the middle of the biofilm biomass. **(a)** Fluorescein (present in the bulk liquid) is excluded by *Pseudomonas aeruginosa* AK1012 (serotype PA05) cells, thereby rendering them as dark 'holes' in the gray background fluorescence of the bulk dye. **(b)** In contrast, *Streptococcus gordonii* PK488 cells either actively concentrate the dye or have a significantly different transmembrane pH gradient from the *P. aeruginosa* cells and are thereby rendered brighter than the background fluorescein emission. The *P. aeruginosa* strain produces much exopolymer; cells are spaced far apart relative to spacing in the *S. gordonii* biofilm. Scale bars = 10 μm. Strains courtesy of Joe Lam, University of Guelph (AK1012) and Paul Kolenbrander, National Institute of Dental Research (PK488).

as hydrophobicity or charge, has resulted, at least as far as bacterial cells are concerned, in a lack of consensus on the importance of physicochemical factors to nonspecific attachment[3]. One perspective is that the distinction between specific (receptor-mediated) and nonspecific attachment is artificial[4]. However, it is clear that in nonspecific attachment a combination of all traits leads to an overall cell-surface milieu that sets the affinity for a substratum. If the substratum or the cell-surface composition is changed, the affinity can change[5,6]. Model organisms, such as strains with defined cell wall mutations[7] or known exopolymer composition, and model substrata for which surface physicochemical characteristics are known could be used to predict nonspecific attachment proclivity.

In the laboratory, the system under investigation is generally kept clean to prevent artifacts that might arise through contaminants, particularly organic molecules. However, in natural systems, clean substrata do not exist: they are rapidly (within minutes) covered with a thin film of organic contaminants, which has been referred to as the conditioning film or pellicle[8,9]. The composition of the pellicle may be influenced by the physicochemical properties of the substratum[10]. For example, it has been repeatedly demonstrated that the nature of the conditioning film in the mouth is critical to the selective recruitment of bacteria to substrata. Certain microorganisms recognize components of the conditioning film and bind to these components to the exclusion of other organisms[11]. A recognition site in the oral pellicle may result from conformational changes in a protein upon adsorption to the substratum[12]. Such specificity probably occurs in other environments as well, but our knowledge of conditioning films and biofilm community structure, for instance on ship hulls, is limited. For the relatively well-studied dental pellicle, individual components within the pellicle are known and their delivery to the substratum in micelle-like structures[10] is under study. However, although the kinetics of pellicle accumulation are just beginning to be understood[13], the spatial heterogeneity of pellicle components on the substratum is unknown.

Further developmental stages

Once cells have attached to a substratum, growth and division occur in three dimensions, and microcolonies are formed. In monoculture biofilms in the laboratory, this results in regular, reproducible 'architectures' that vary in a species-dependent manner[14]. Initial conceptual models of biofilm development generally showed tightly packed monolayers of cells that increased in thickness by ordered cell growth. Later, better recognition of spatiotemporal aspects led to the portrayal of the biofilm as a looser arrangement of cells containing distinct (generally pyramidal) structures. The application of confocal microscopy to biofilm research has resulted in a dramatic reassessment, and current models show microcolonial growth producing cellular islands (mushroom-shaped structures or columns) that are initially separated by extensive void regions but that coalesce, resulting in a loss of void space. The volume of void space, its relationship to the substratum, and the degree of liquid flow through the voids are currently areas of intense research[15,16]. Growth of biofilms is dependent on experimental conditions[17] (e.g. flow rate, nutrient content and temperature), and these factors should be taken into account when results are compared. One critical focus of biofilm physiology, which has only recently received much-warranted attention, is that of the role of exopolymer. This extracellular matrix that binds the biofilm together is responsible not only for aspects of biofilm architecture (Fig. 1) but is also a consequence and indicator of biofilm physiology[18,19].

Clear differences exist between the architecture and development of multispecies biofilms and those of pure-culture biofilms. Confocal microscopy of multispecies communities grown from natural inocula (saliva, river water, trickling filters) has shown that, at least initially, compartmentalization occurs; in other words, organisms tend to segregate into single-species microcolonies within the biofilm, despite conditions, such as low flow rate and low organic nutrient concentration, that might harbor cross-feeding[20–22] and the rapid establishment of mixed-species microcolonies. Presumed positional interdependence of these microcolonies has yet to be demonstrated. Likewise, heterogeneity of activity within a biofilm, which might be predicted on the basis of diffusion rates and nutrient depletion, is beginning to be explored[23]. Finally, most experimental protocols have, for the sake of simplicity and control, monitored biofilm development after a single inoculation. However, nature is not so simple. The oral cavity is an example of a milieu in which microorganisms are constantly present in the bulk liquid phase, and continuous selective recruitment takes place by coaggregation[24,25]. In the same way that certain organisms possess receptors for particular components of a conditioning film, some organisms possess receptors for ligands on other organisms. Through this type of selective attachment, it is possible that specific biofilm architectures might arise that are similar to stromatolites or microbial mats. This does not suggest that these structures must be layers that are clearly identifiable on the basis of cell morphology or pigmentation, but rather that structures based on the physiological functions of the cells could form. The four-dimensional (X,Y,Z and T) process of growth, division and continuous introduction of microorganisms can be seen as a type of differentiation during which the biofilm changes from a monolayer of cells (generally in the form of islands rather than confluent coverage) to a thicker structure with different cell types in locations determined by an interplay of architecture and environmental conditions (Fig. 2).

Mature biofilms, senescence and death

What constitutes a mature biofilm? In monoculture laboratory systems, it is usually defined simply by thickness/confluence, a factor that is in turn regulated by, for example, nutrient availability, flow rate and shear stress. In multispecies communities, maturity is difficult to define and can only develop if environmental conditions are constant and reinoculation is discounted[26]. The best known example of temporal development in attached microbial communities is that of the various types of dental plaque. The progression from a 'simple' community, consisting of predominantly actinomycetes and streptococci, to a complex association of at least ten genera composed of predominantly anaerobic and facultative microorganisms has been documented *in vivo* by classical bacteriological methods[27]. However, confocal microscopy and nucleic acid methods should yield a better understanding of community composition, architecture and evolution in this model system, as is being demonstrated in non-oral systems[22].

In addition to the temporal aspects of plaque development, specific bacterial populations are an etiological factor in periodontal disease. Strong evidence exists that specific and very different communities are associated with various levels of gum disease in humans[28,29]. The emphasis in periodontal disease research has shifted from identification of pathogenic species towards analysis of entire communities, mostly because the nucleic acid technology necessary to examine large numbers of samples in the necessary detail has only recently become available[30]. One of the most important questions raised is why clearly definable differences in community structure exist. At present, the relationship between environmental conditions and community structure is unclear. But what triggers the shifts, and are the environmental changes involved in a feedback loop that includes shifts in microflora?

Death is another concept that is currently difficult to define for biofilms. Death in a monoculture laboratory biofilm can be relatively easily defined as the cessation of cellular activity; however, the yardstick by which cellular activity is measured is important. For example, tetrazolium dyes indicate the degree of reducing activity within the cell, whereas propidium iodide indicates the integrity of the cell membrane. For mixed-species populations, death can probably be best assessed in terms of the loss of particular members of the community as a result of environmental change. However, it is unlikely that the species disappear completely from the community; rather, their numbers become too small to detect, and their function within the community (e.g. metabolism of a particular carbon source) is taken over by another species. If environmental conditions are altered, for example by the introduction of a new carbon source[26], a subset of cells previously identified as absent or inactive may reappear or become active again. In multispecies biofilms, the loss of key members of the community may result in a shift to a dramatically different population. Rearrangement of interspecies interactions could have profound effects on biofilm architecture if physical proximity is required for the interaction.

What are the consequences of death? If attachment points to the substratum deteriorating through, for example, enzymatic activity or loss of fimbriae, then sloughing of the biofilm can occur[31]. Complete loss of all cells is probably rare; the remains of the biofilm can act as a new substratum for other microorganisms.

Implications for control

The first opportunity for affecting biofilm development is at the substratum. Modification of the substratum to alter nonspecific attachment is an attractive, simple approach that can hinder biofilm initiation under controlled conditions[32]. However, as the diversity of relevant microorganisms increases, the likelihood of limiting biofilm growth by this approach decreases. As alterations in the composition of the conditioning film can affect attachment of specific microorganisms, modification of substrata to inhibit adsorption of specific classes of molecules is another way to control attachment. Alternatively, a substance can be applied that enhances attachment of particular microorganisms,

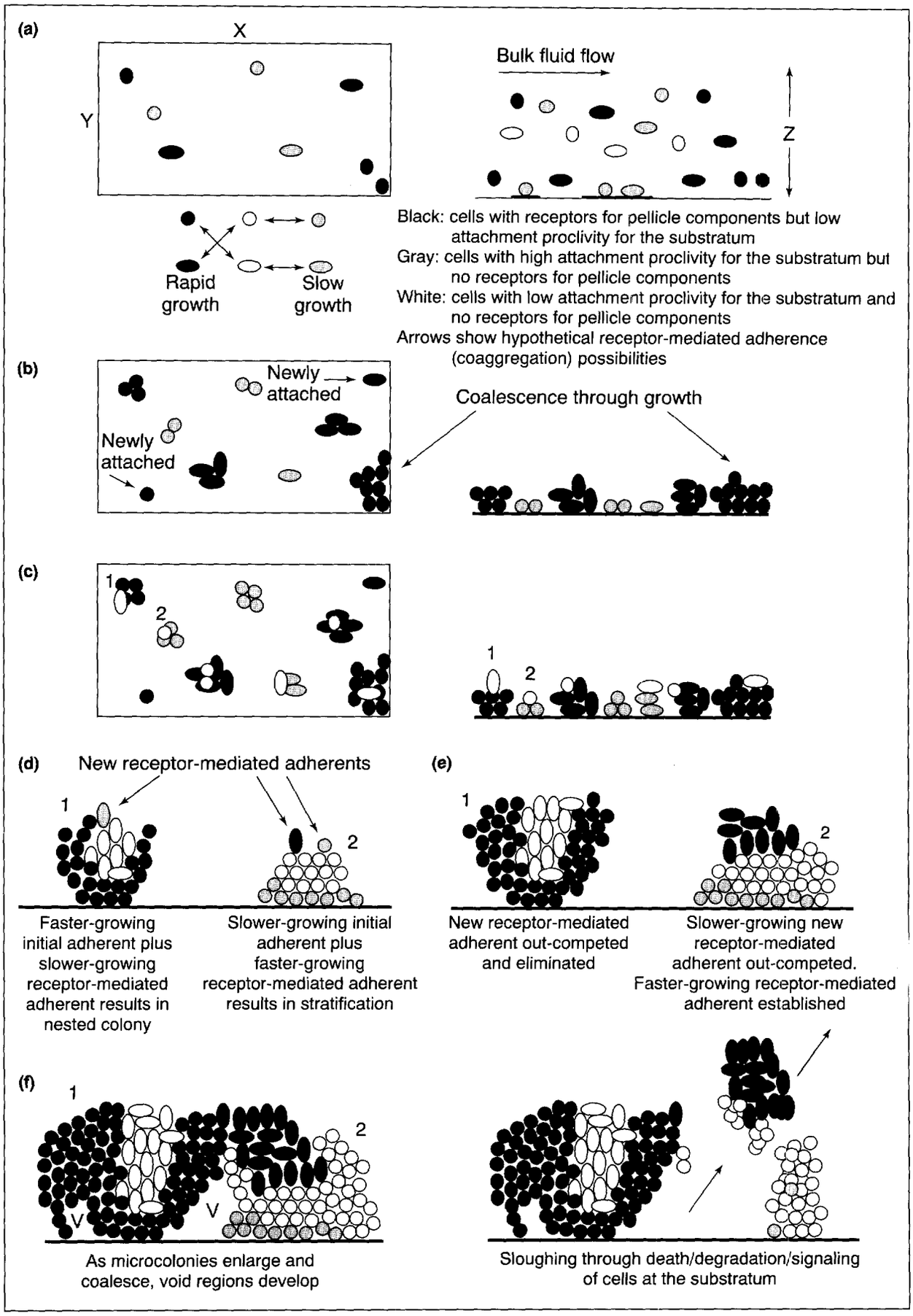
(a)
X
Y
Bulk fluid flow
Z
Rapid growth
Slow growth
Black: cells with receptors for pellicle components but low attachment proclivity for the substratum
Gray: cells with high attachment proclivity for the substratum but no receptors for pellicle components
White: cells with low attachment proclivity for the substratum and no receptors for pellicle components
Arrows show hypothetical receptor-mediated adherence (coaggregation) possibilities
(b)
Newly attached
Newly attached
Coalescence through growth
(c)
1
2
1
2
(d)
New receptor-mediated adherents
1
2
Faster-growing initial adherent plus slower-growing receptor-mediated adherent results in nested colony
Slower-growing initial adherent plus faster-growing receptor-mediated adherent results in stratification
(e)
1
2
New receptor-mediated adherent out-competed and eliminated
Slower-growing new receptor-mediated adherent out-competed. Faster-growing receptor-mediated adherent established
(f)
1
2
V
V
As microcolonies enlarge and coalesce, void regions develop
Sloughing through death/degradation/signaling of cells at the substratum

Fig. 2. (left) Four-dimensional development of a hypothetical mixed-species biofilm. Left-hand panels in parts **(a)**–**(c)** represent the XY plane of the biofilm (parallel to the substratum), whereas right-hand panels represent the XZ plane (cross section, perpendicular to the substratum). **(a)** Time zero – initial colonization. The pellicle is shown as light gray areas (height in Z exaggerated). **(b)** Growth in X, Y and Z – additional attachment. The bulk fluid and pellicle have been omitted from this point onwards. **(c)** Differentiation – co-aggregation results in mixed-species colonies. Continued growth and differentiation of numbered colonies (1,2) is depicted in parts **(d)**–**(f)**. **(d)** Development of structure within microcolonies. **(e)** Changes in species composition through competition. **(f)** Formation of channels and voids (V), followed by loss of cells from the substratum.

thereby reducing the efficiency of attachment of other undesirable microorganisms. These simplistic approaches do not work in complex natural situations (such as marine corrosion)[33], but they may succeed under the more controlled conditions to which catheters and other indwelling medical devices, and even dental appliances, are exposed. A technique that is more likely to succeed is targeting of specific gene products that are expressed when cells undergo changes from a planktonic to a sessile phenotype[34]. These critical studies with monoculture pathogen biofilms will define the regulation of phenotype switches and suggest strategies for control. Bioreporter technology (using green fluorescent protein, luciferase and LacZ) is ripe for application in biofilm studies[34,35].

During the growth/differentiation stage, expression of specific cell-surface moieties could be targeted to reduce the ability of microorganisms to attach to one another, or receptors could be blocked with very specific agents. These approaches would be expensive, but if biofilm-related infections could be reduced the gain would also be high. It is clear that microbial signaling of cell concentration (quorum sensing[36]), controls large banks of genes in many different organisms. As the role of quorum sensing in biofilm systems becomes better understood, it could also provide an opportunity for the control of attachment/detachment.

The realization that community composition is related to the extent of disease in periodontal patients suggests that a strategy for maintaining the community composition early in disease may reduce the pace of deterioration. It is possible that, through understanding of the interrelationship of community members, selective pressure could be exerted on the system to prevent transition to a community associated with more-severe disease or even to cause reversion to a community associated with less-severe disease. This pressure could be applied by treatment with simple molecules associated with biofilm community metabolism (e.g. sugars and organic acids) or even by changing the patient's diet.

Conclusions

Attempts to combat the initial stages of biofilm formation will be driven by the recognition that microbial attachment results from an interaction between the chemistry of the cell surface and the chemistry of the substratum and that the interaction is subject to modification by an organic thin-film (conditioning film). Cell–cell recognition events that influence the formation of mixed-species biofilms offer an opportunity for intervention in biofilm community composition, and a greater understanding of how microorganisms interact metabolically within the biofilm will result in intervention strategies based on community physiology.

Biofilm biology is a young field that requires much very basic, extremely cross-disciplinary and sometimes esoteric research. However, that research will set the stage for a true understanding of complex worldwide problems, such as microbially influenced corrosion and oral disease, and will contribute greatly to our understanding of the most significant microbial lifestyle on earth.

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Questions for future research

- How do cell-surface and substratum characteristics control non-specific attachment proclivity?
- Can specific attachment (coaggregation, pellicle recognition) be used to affect biofilm species composition and thereby modulate the effects of the biofilm on its environment?
- What are the consequences of attachment for a bacterial cell? What genes are activated and does this present an opportunity for intervention in biofilm accumulation?
- How is the composition of natural multispecies biofilms controlled through environmental cues? Are these communities homeostatic?
- Is it possible to visualize interspecies interactions within a biofilm at the level of the microcolony or the single cell?

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How rolling circle plasmids control their copy number

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Rolling circle DNA replication is inherently continuous and unregulated. This 'go-for-broke' strategy works well for lytic phages but is suicidal for plasmids that must coexist with their host. Plasmids have consequently evolved elaborate copy number control systems that operate at the transcriptional, translational and post-translational levels.

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Plasmids are double-stranded circular or linear extrachromosomal DNA molecules. A wide array of genes have been found on various prokaryotic plasmids, including genes that make the host resistant to external factors such as antibiotics, genes that alter host metabolism and genes that encode toxins[1]. Plasmids are readily transmitted between bacterial species and thus play an important role in prokaryotic evolution. Although plasmids replicate autonomously, they coexist stably with a host by limiting their replication to one round per plasmid per bacterial generation. Therefore, plasmids offer an opportunity to study the regulation of DNA replication.

Circular plasmids replicate primarily by either asymmetric rolling circle (RC) replication or theta replication. In RC replication of plasmids (as for RC replication of single-stranded phages), a plasmid-encoded replication (Rep) protein initiates replication by making a strand-specific nick at the plasmid double-stranded origin (DSO), which then serves as a primer for replication[2,3] (Fig. 1). Theta replication is initiated by RNA primers at the origin[4,5]. RC replication is the most common mode of replication for small (<10 kb), promiscuous, multicopy Gram-positive bacterial plasmids, and is also used by some Gram-negative bacterial plasmids[5,6].

Although we will focus on the mechanisms used to regulate RC replication of prokaryotic plasmids, this replication method is also used by viruses that infect a range of eukaryotes, including bovine papilloma virus (BPV), herpes simplex virus (HSV) and geminiviruses of plants. As many of these viruses coexist with the host cell, copy number control mechanisms must exist. The principles that apply to prokaryotic plasmid copy number regulation may also be relevant to the study of these eukaryotic replicons.

RC plasmids have a modular structure of sequence elements (cassettes or modules) that are frequently interchangeable[7]. Plasmids are classified genetically by incompatibility testing: two plasmids that share the same replication control machinery will not be able to coexist stably in a host[1]. The small RC plasmids have been classified on a molecular basis by comparing their main structural feature, the leading strand initiation and control region. These plasmids have been divided into five families, based on sequence homology within this region and Rep protein similarity: pT181, pMV158/pE194, pSN2, pC194/pUB110 and pTX14-3 (Ref. 5). All are maintained at ~10–50 copies per cell and lack a partitioning mechanism.

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