

Biofilms in the New Millennium: musings from a peak in Xanadu

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INTRODUCTION

Most biological sciences had defined their subject organisms, grossly and at the microscopic level, long before the advent of molecular biology. For this reason, sciences like botany had characterized the structure, even at the ultrastructural level, of the cells and tissues that comprise all of the forms adopted by plants, including all vegetative and reproductive manifestations of thousands of organisms. Therefore, the detailed molecular mechanisms discovered by modern plant molecular biologists fit nicely into a framework of understanding of exactly where these mechanisms operate, in terms of the structure and function of the organism concerned. We visualize the process of transcription occurring on the cytoplasmic aspect of the endoplasmic reticulum, and we can follow the resultant peptides through their various fates, including excretion in vesicles via the Golgi apparatus. The intellectual synthesis within a field like botany is essentially very satisfying, because we could previously see the central genetic control of shape and function, and now we can understand the molecular mechanisms by which this control is exercised and we can manipulate plants using this knowledge.

The relatively new science of microbiology, which has done so much to eradicate epidemic diseases and to improve our lives, is presently reeling in confusion because it has not followed the same orderly development of concepts and techniques. At a very early stage in its development, microbiology embraced two *moda operandi* that served it well, but damaged it in the long term. First, following Robert Koch, we removed bacterial cells from the ecosystems in which they lived, and grew them in monospecies cultures in fluids or on agar. This decision served humanity well in the conquest of

many acute diseases, and we still use the same 150-year-old methods in such practical areas as diagnostic microbiology. Secondly, we set certain criteria (Koch's postulates) that licensed us to extrapolate from our *in vitro* single-species data to complex problems like the microbiology of the gut, in which many species operate in concert and we understand only what 'our' target organism is doing. The powerful armamentarium of classic microbiology was unleashed, generally, only when we were threatened by diseases. We developed the vaccines and antibiotics that have served us well, until recent times when the emergence of resistant strains has us profoundly worried, but we have crippled our science by three errors to which we subscribe:

- (1) We still study bacteria in monospecies cultures in rich fluid media, and thus we study only one of many phenotypes.
- (2) We still extrapolate from studies of the planktonic phenotype in monospecies culture in rich media to attempt to understand the behaviour of the same organism in the biofilm phenotype in a multispecies community living in a nutrient-limited ecosystem.
- (3) We have amassed huge amounts of molecular data, including several complete genetic sequences, but we still do not understand that the phenotypic expression of the virulence factors with which we are preoccupied is controlled by relationships with non-pathogenic bacteria, and with environmental factors that we rarely study.

Here amongst the mountains of Xanadu we see real bacteria growing in many different phenotypes, and forming integrated multispecies communities of remarkable complexity, and we weep over the errors and omissions of the past. What will our young colleagues find when they use confocal microscopes and species-specific probes to explore the wonderful and complex biofilm communities that we didn't even know existed? We have studied the same ecosystems for 150 years, and all we have to show for it are freezers full of monospecies cultures in broth, and reams of molecular data on the planktonic phenotypes that now cause only a minority of human infections in the developed world.

BIOFILM STRUCTURE

The confocal scanning laser microscope (CSLM), which allows the examination of living and fully hydrated biological materials, has revolutionized the study of bacteria actually growing in their native environments (Lawrence *et al.*, 1991). By subtracting out-of-focus planes from the image, the confocal microscope allows us to examine both the surface and the bulk fluid components of virtually any ecosystem, and thus to confirm the conclusion of earlier studies (Geesey *et al.*, 1977) that the vast majority of bacteria grow in slime-enclosed biofilms. These valuable but laborious confocal studies

have served to define the exact distribution of bacteria in a limited number of ecosystems, but they also provide a valid basis for the re-evaluation of the thousands of papers in which transmission and scanning electron microscopy have been used to study similar biofilms. The electron microscopy methods could only examine dehydrated specimens, because electrons cannot travel through water vapour. If we use our imaginations to 'rehydrate' the older images produced by electron microscopy, we can salvage information concerning the presence and number of cells on a surface, even if we cannot see the matrix material or define the complex cellular architecture of the sessile community. Both confocal and electron microscopic images will be used in this treatise, with this codicil in mind. In addition to its advantages in sophisticated optical analysis, and the fact that it can examine living biofilms and tissues, the confocal microscope can be used with a burgeoning array of physical and chemical probes. These probes, which range from oligonucleotide sequences to simple pH meters, now allow us to identify cells of various species and to measure physical and chemical parameters (e.g. dissolved oxygen) in living biofilms with a resolution of better than 5 μm . This is a wonderful time to be a microbiologist.

Bacterial adhesion

When a bacterial cell approaches a surface, which these cells do with notable avidity (Marshall, 1985), a very large number of behavioural and phenotypic changes take place. The cells adhere to the surface, in a reversible manner, and they use their pili (O'Toole & Kolter, 1998) and other surface appendages to move over the surface and to form aggregates and other formations of considerable variety. Darren Korber and his colleagues in Saskatoon have studied the mobile 'behaviours' of the recently adherent cells of several species of bacteria, and have noted that some roll and make 'windrows', while others form single-cell monolayers or discrete microcolonies. These recently adherent bacterial cells may compete for space on the surface, and Henk Busscher and his colleagues have correctly suggested that a whole array of surfactants and other bacterial weapons may come into play to determine which cells go on to form biofilms, and which interspecies alliances are formed. It would be intellectually facile to link these early arrangements of adherent cells to the complex towers and channels of mature biofilms, but, at this stage in this field, these connections simply have not been established with any certainty.

The first indication that bacterial cells undergo a profound phenotypic change following their adhesion to a surface came from the imaginative use of confocal microscopy and a reporter construct in which an indicator enzyme gene (*lacZ*) was inserted downstream from the gene (*algC*) that encodes phosphomannomutase. Davies & Geesey (1995) used this reporter construct to show that cells of *Pseudomonas aeruginosa* up-regulate their *algC* genes within a few minutes of adhesion, as one might

expect because of the role of this enzyme in the production of the matrix material (alginate) that eventually cements the cells to the surface in an irreversible manner. Non-morphological studies (Yu, 1994) had previously shown that the *algD* gene, which also forms a part of the genetic sequence that controls the production of the alginate matrix of *P. aeruginosa* biofilms, is also up-regulated soon after cells of this species adhere to surfaces. Because these two genes are part of the alginate synthesis cascade, which is controlled by a sigma factor produced by *algU* (also known as *algT*), we know that the alginate synthesis system of this organism is up-regulated within minutes of adhesion. We must not extrapolate between species, but the observation that the cells of hundreds of different marine bacteria produce visible amounts of matrix material shortly after they adhere to surfaces suggests that this response to adhesion may be universal.

While the up-regulation of the matrix synthesis system following the adhesion of bacteria to a surface is expected, and the result of this up-regulation can be seen in the form of extruded exopolysaccharide, we were not prepared for the subsequent revelation that many other unrelated phenotypic changes are triggered by adhesion. Hongwei Yu has used PAGE gel techniques to separate the outer-membrane proteins (OMPs) from planktonic cells of *P. aeruginosa* grown in liquid media and in biofilms, and the OMPs of the planktonic phenotype are profoundly different from those of the biofilm phenotype (Fig. 1). Other laboratories have now used much more sophisticated techniques, including genetic library analysis, to examine the differences between the planktonic phenotype and the biofilm phenotype of *P. aeruginosa*, and it is clear that many more differences exist and that there is not a single biofilm phenotype. For example, it appears that the *rpoS* gene that controls senescence in *Pseudomonas* is up-regulated in biofilms, and it is of great interest to note that this gene is also up-regulated in cells of *P. aeruginosa* recovered directly from the lungs of cystic fibrosis patients (Foley *et al.*, 1999). The practical implications of a profound difference between the planktonic and the biofilm phenotype of a significant number of bacteria, if this proves to be the case, will take some time for full intellectual digestion, but the first logical reaction is that much work will have to be redone. Obviously the genotype of a bacterial species is constant, but phenotypes will differ in their response to environmental factors, and bacteria that produce a certain virulence factor when growing as planktonic cells in a monospecies culture may not even produce the same factor when growing in a biofilm. Cells growing in the biofilm phenotype may be resistant to antibiotics, not because the agents cannot penetrate the biofilm matrix (Stewart, 1996), but because the sessile phenotype has a profoundly different cell envelope structure, and perhaps even a different metabolic process that no longer constitutes a suitable target. We may have more success in the treatment of biofilm infections (Costerton *et al.*, 1999) when the antibiotics that we use have been selected

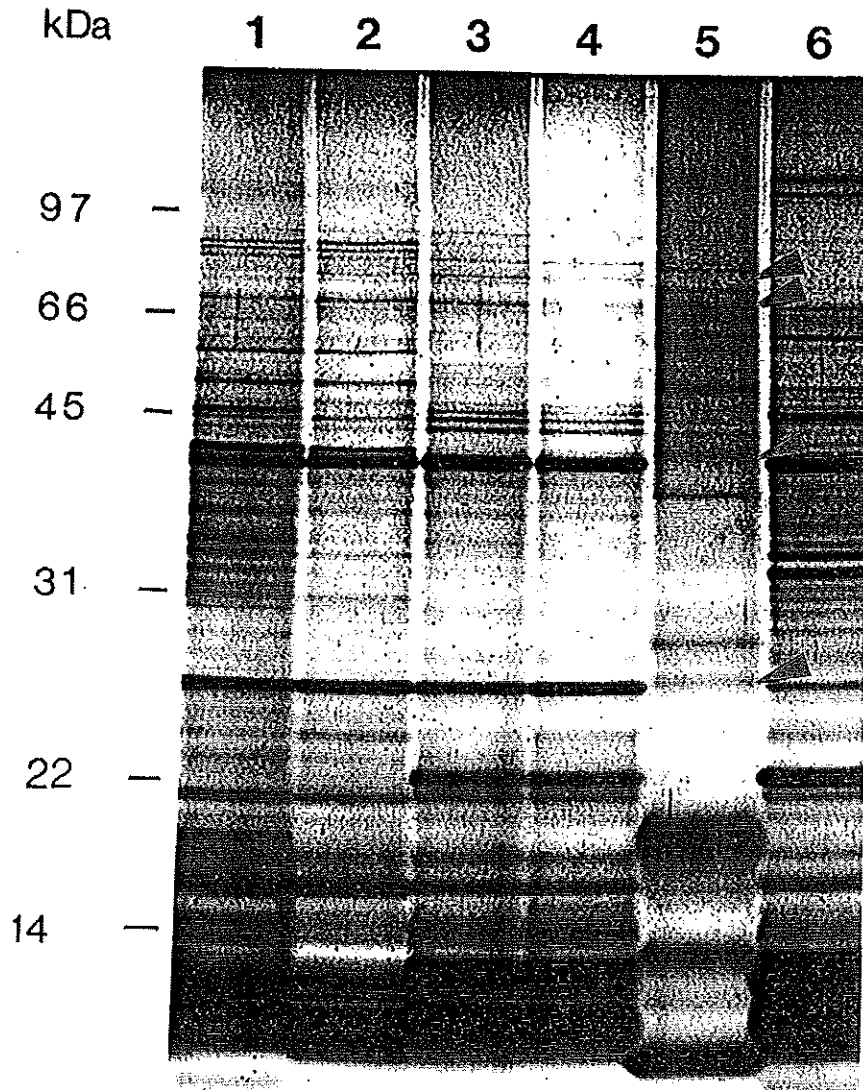


Fig. 1. PAGE gel of the outer-membrane proteins (OMPs) extracted from planktonic cells (lanes 1–4 and 6) and biofilm cells (lane 5) of *P. aeruginosa*. The planktonic OMPs seen in lane 6 are from cells grown in the same medium as the biofilm cells that yielded the OMPs seen in lane 5. Note the radical differences in the gene products of these two distinct phenotypes.

and evaluated for their ability to kill bacterial cells growing in the biofilm phenotype, and not planktonic cells growing in liquid media.

Biofilm formation

When bacterial cells have adhered to a surface, arranged themselves in species-specific patterns, assumed the biofilm phenotype, and up-regulated their matrix synthesis machinery, they have the potential to form a biofilm. Our direct examinations of living mature biofilms, by confocal microscopy, show that these sessile communities



Fig. 2. Confocal scanning laser micrograph, in the x-y-axis and just above the colonized surface, of a natural multispecies biofilm formed on a polished rock surface in the Bow River over a period of 10 days. Note the discrete microcolonies, many of which contain more than one morphotype (species) of bacteria, and the open water channels that separate these basic structural units of this natural biofilm. Bar, 10 μ m.

are very complex and they indicate that the newly adherent cell has a multitude of 'decisions' to make. Most of the natural biofilms examined to date are composed of discrete microcolonies, separated by open water channels (Fig. 2), and the sessile bacteria grow in the microcolonies where they comprise $\pm 15\%$ of the volume while the matrix material comprises $\pm 85\%$. Even in monospecies biofilms, the cells are not evenly distributed within the microcolonies, and some species tend to produce a majority of cells in the apical regions of these structural units of the biofilm. Many mature biofilms are composed of microcolonies in which cells of many different species are present (Fig. 2), and we know that many of these combinations are predicated on physiological co-operativity (Costerton *et al.*, 1995). As we begin a simple-minded analysis of the early stages of biofilm formation, it is relatively easy to conceive of the spatial association of cells of two or more metabolically co-operative species to form the initial stages of a microcolony. Individual cells would grow quickly where they experienced the metabolic advantage of an interspecies association, and the co-operative cells would proliferate most exuberantly where they were in closest proximity. However, a careful computer-assisted analysis of the

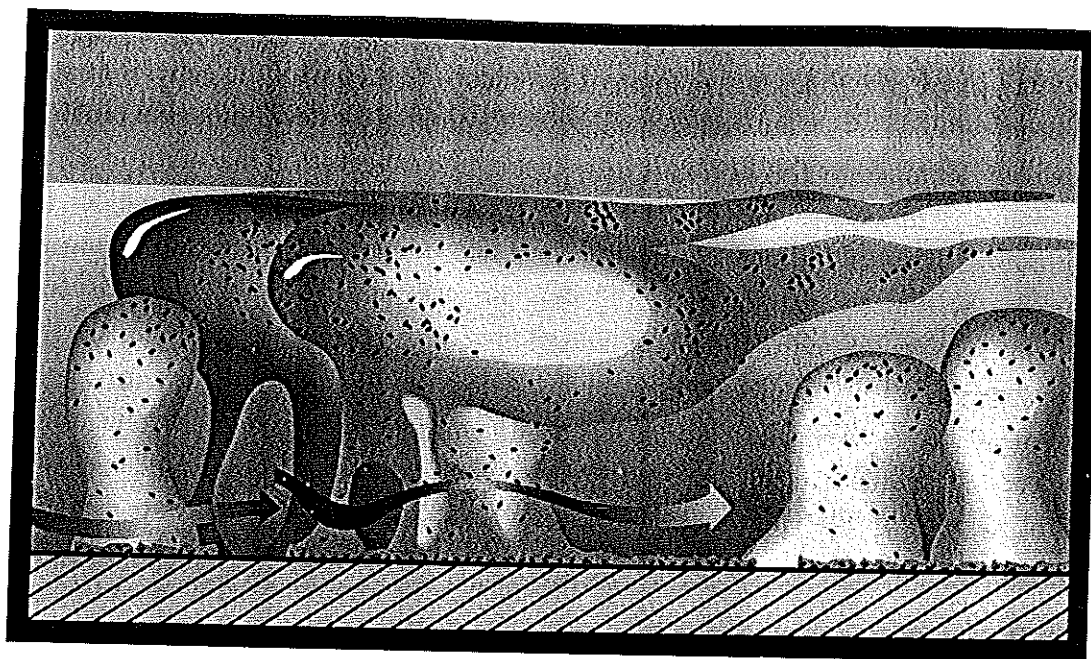


Fig. 3. Diagrammatic representation of morphological data from dozens of natural and *in vitro* biofilms, in the x-z-axis, showing the microcolonies and water channels that comprise these complex and highly structured communities. The sessile biofilm cells actually grow in matrix-enclosed microcolonies, of various shapes, and these microcolonies are often deformed by high shear forces to produce the streamers seen to project into the bulk fluid.

structure of monospecies and natural biofilms (Fig. 3) clearly shows that the microcolonies that comprise the biofilm are shaped like mushrooms, or stacks, and the water channels are open throughout the sessile community. This degree of structural complexity clearly precludes random growth, and dictates that we consider some sophisticated form of communication and control when considering the development of biofilms.

Biofilm structure

The first bacterial control signals were discovered in marine organisms that actually grow in biofilms in the light organs of higher animals (Fuqua *et al.*, 1994), but their manifold effects on cellular behaviour were studied using planktonic cells in monospecies fluid culture. For this reason, the actual ecological roles of these signal molecules may not have been realized, but a large and rapidly expanding literature now catalogues their manifold effects on planktonic cells (Parsek *et al.*, 1999). It became obvious that these acyl homoserine lactone (AHL) signal molecules, which control so many metabolic activities of planktonic Gram-negative cells, also control biofilm formation when mutants lacking the ability to synthesize these compounds were seen to be unable to produce structurally differentiated biofilms (Davies *et al.*, 1998). Other

signal minus mutants have been able to produce structured biofilms (Stoodley *et al.*, 1999a), but it still appears that some of the signals that control the general area of quorum sensing in bacteria are also involved in controlling the development of biofilms. Ancillary support for this notion is provided by the observation that natural signal analogues (furanones), which block the activity of specific AHL signals, also block the formation of natural biofilms in the marine environment. Fig. 4 is a simple-minded cartoon that suggests that the development of a structurally differentiated multispecies biofilm requires the control of cell proliferation and matrix production by signals that allow sessile cells to control the activities of neighbouring cells of the same, or of different, species. While the use of AHL signals in quorum sensing by planktonic cells is logical and well-documented, the transitory nature of the spatial associations between planktonic cells in most ecosystems make it unlikely that these cells communicate extensively in nature where quorums are rare. However, the sessile cells in the microcolonies that comprise multispecies biofilms (Fig. 4) are in stable juxtaposition with many cells, and I predict that the next few years will see the discovery of literally hundreds of signals that serve as the hormones and pheromones that regulate these complex communities. The small distances between adjacent cells in biofilms (6–8 μm) does not preclude the possibility that electrical signals may also operate within these communities.

BIOFILMS AS MULTICELLULAR COMMUNITIES

A successful community is invariably 'greater than the sum of its parts', and the history of microbiological research illustrates this point very well. In rumen microbiology, we could see that a biofilm community formed on the surfaces of cellulosic plant materials undergoing digestion, but the rates of cellulose digestion by isolated members of this community fell far short of the rates of digestion seen in the rumen of even the most inept bovine. Direct microscopic observation of material taken from the rumen showed that cells of two bacterial species tended to coexist on the feed (Fig. 5), and, when cells of these two species were mixed and used to colonize sterile cellulose (Kudo *et al.*, 1987), the rate of digestion approached that of the natural system. The mobile cells of the *Treponema* species scavenged butyrate from the biofilm formed by the primary cellulose degrader, and reversed the feedback inhibition that was inhibiting the whole process of cellulose digestion in the monospecies biofilm. It is obvious that the structured juxtaposition of metabolically co-operative cells is as beneficial to biofilm communities as it is to the tissues and organ systems of higher multicellular organisms. Doug Caldwell rightly insists (Caldwell & Costerton, 1996) that the characteristics of a bacterial species that make it an effective partner in a metabolically co-ordinated community are much more important to its evolution than any properties that allow it to succeed as a single planktonic cell in nature. It is perhaps serendipitous that the dental and industrial biofilms that were recognized long before the word was coined.

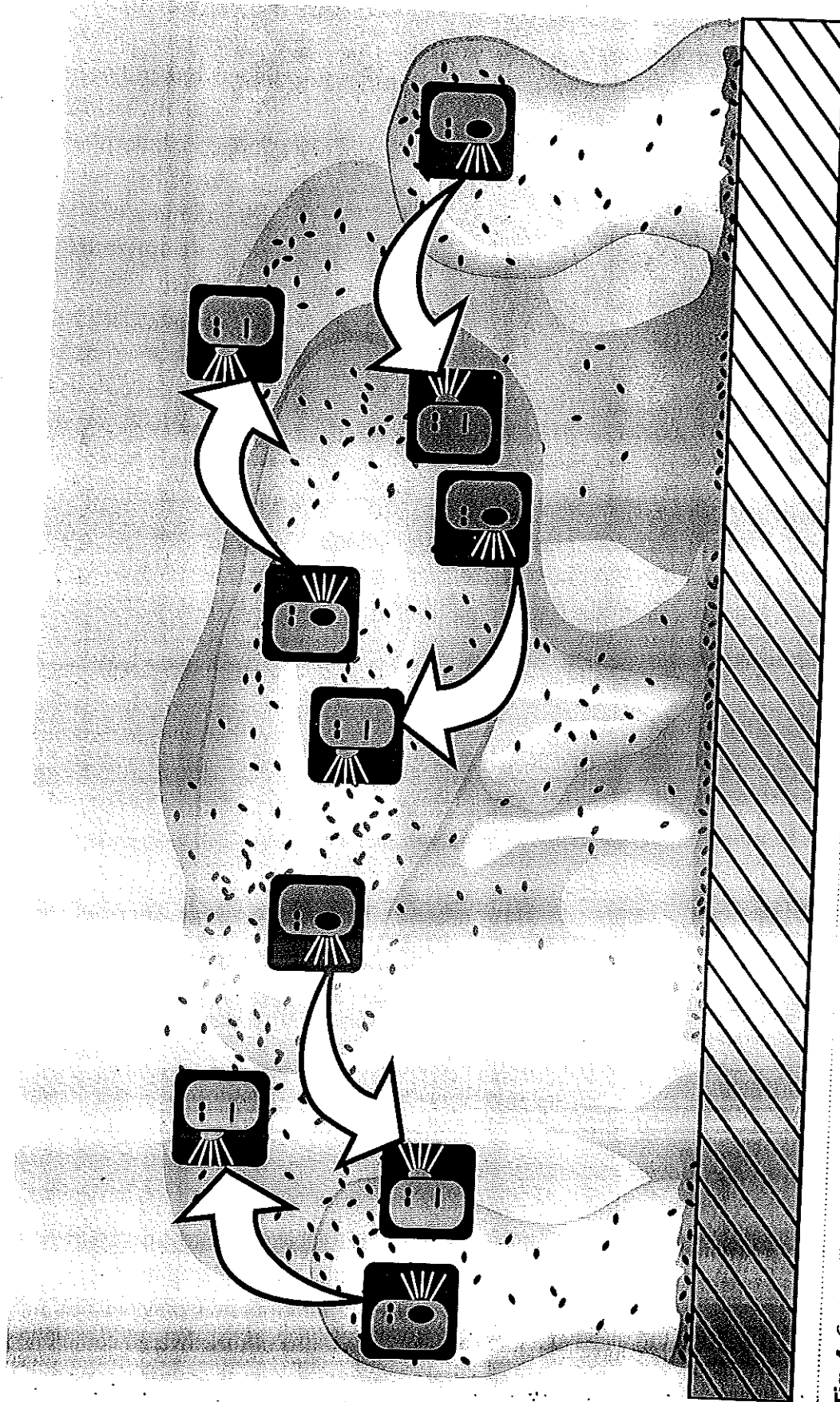


Fig. 4. Cartoon illustrating the point that interspecies and intraspecies signalling mechanisms must be operative in biofilms, in order for these sessile communities to form their elaborate structures and, especially, for the maintenance of their open water channels.

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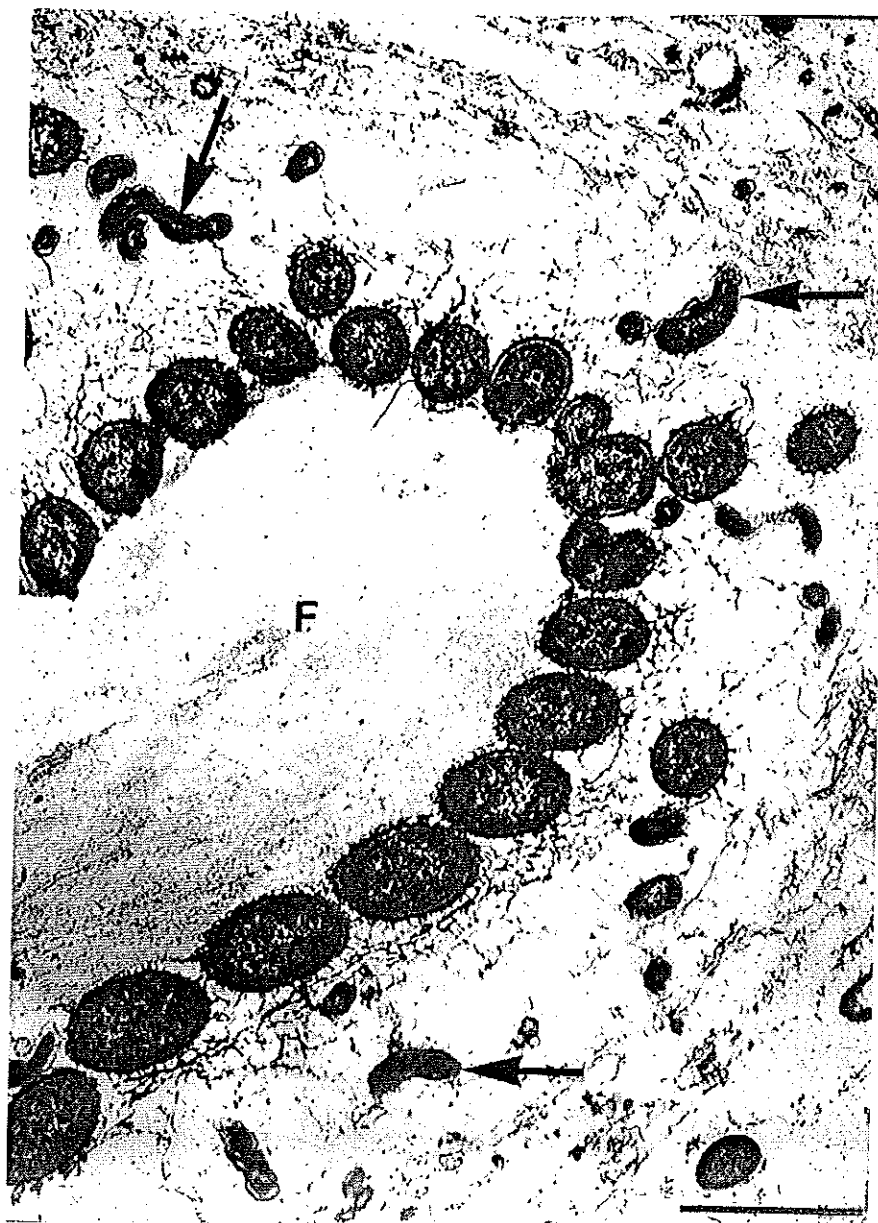


Fig. 5. Transmission electron micrograph of a stained section of a rumen specimen in which a cellulose fibre (F) is being digested by a monolayer of cells of *Fibrobacter fibrosolvens* within a fibrous biofilm. This biofilm has been invaded by mobile cells of a *Treponema* species (arrows), which accelerate this digestion by removing the products of primary digestion (butyrate) that would otherwise inhibit this process by feedback inhibition.

(Costerton *et al.*, 1978) are sufficiently thick as to be visible to the unaided eye. Dental plaque and the biofilms that form on the fixed film reactors used in sewage treatment have always been treated as integrated microbial communities by the practical folk who manage them, and they have usually been studied in terms of their overall community activities.

Biofilms as adaptive and ecologically functional communities

Biofilm communities assume a certain austere elegance when we consider their structure and function in terms of the evolutionary circumstances in which they evolved. It is certain that these assemblages of prokaryotic cells were the first multicellular communities to evolve on earth, and their basic organization certainly developed in response to the conditions operative in the primitive environment. Modern biofilms place their component bacteria in immediate juxtaposition to photosynthetic organisms and to other nutrient sources, including such autotrophic sources as oxidized minerals (e.g. elemental sulphur). Even when heterotrophic bacteria are not juxtaposed to sources of organic nutrients, the biofilm matrix has the property of trapping and concentrating organic nutrients (Costerton *et al.*, 1995), and biofilms are always the predominant form of bacterial growth in pristine mountain streams. The microcolony and channel structure of the biofilm, as illustrated in Fig. 3, represents a primitive circulatory system in terms of nutrient delivery and waste removal, and constitutes a very efficient way of supporting the growth of a large number of cells in a limited surface area. The short and stable diffusional distance between metabolically co-operative cells within mixed-species microcolonies represents a physiologically integrated structure, not dissimilar to that seen in the tissues of higher organisms, except that the co-operating elements do not all share the same genotype. In some ways, microbial biofilms presage the subsequent development of multicellular organisms whose cells are derived from the same genotype because these sessile communities were able to achieve a primitive circulation, and a measure of cellular specialization and metabolic integration, using cells of many different genotypes.

One of the most important properties of any successful community is its ability to respond to stress, and to survive in the face of adverse circumstances. In this matter, the biofilm mode of growth and the multigenomic nature of the mixed-species biofilm are both of pivotal importance. Biofilm communities are, by definition, stationary, and this stationary mode of growth reduces the hazards of a very hostile environment in which planktonic cells would be swept from one dangerous location to the next, until they perished. Cells growing in the biofilm mode of growth, in the protected biofilm phenotype, show a phenomenal resistance to chemical antagonists, including acids and antibiotics (Nickel *et al.*, 1985). They are also remarkably resistant to uptake by the amoebae that were probably amongst the first eukaryotes to challenge prokaryotic biofilms for dominance in the primitive earth. In addition to the survival values inherent in the biofilm mode of growth, and in the biofilm phenotype, the physiological and genetic heterogeneities that are characteristic of biofilms add another level of assurance of survival. Local areas within biofilms develop their own chemically distinct microenvironments, or microniches (Costerton *et al.*, 1994), and modern studies of the killing of biofilms with antibiotics and sterilants clearly show that sessile cells often

survive in local 'pockets' in which the agent is less effective. An antibacterial agent must be active in a very wide range of pH values, and in an equally wide range of oxygen tensions, if it is to be able to kill all of the sessile cells even in a monospecies biofilm. If all of the microcolonies within a biofilm are not killed by an antibacterial agent, the community will recover to approximately its pre-challenge dimensions in only a few days. However, it is probably the multigenomic character of mixed-species biofilms that gives them their most effective properties in survival and in their ability to respond to changing environments. A biofilm community is usually composed of dozens of different species of bacteria, with different genomes that dictate both the metabolic potentials and the environmental susceptibilities of each individual sessile cell. In any given nutrient situation, or in any stage in the development of the biofilm, some sessile cells will be more metabolically active than others and we know that many of the cells in biofilms are often dormant or quiescent (Stewart, 1996). Each sessile cell has its own genetically determined metabolic potentials, and its own genetically determined susceptibilities to antibacterial agents, and this gives these microbial communities their remarkable resistance to adverse circumstances and their equally remarkable ability to respond to new opportunities. In any stress situation, one or two species are likely to survive, because of some property of their genome, and any new nutrient opportunity is bound to suit the genetically determined capabilities of some members of the community.

Biofilms as dynamic communities

Microbial biofilms are dynamic in both their mechanical properties and their community structure. Because of the limitations of pictures on a printed page, and of our imaginations, we imbue biofilms with a certain rigidity, even though we know that many macroscopic biofilms are relatively soft and very pliable. Recently, Paul Stoodley and some members of Zbigniew Lewandowski's group of engineers have explored the viscoelastic properties of biofilms, in the same terms that would be used to describe similar properties in any material, and biofilms have proven to be highly compliant materials (Stoodley *et al.*, 1999b). Microcolonies that grow symmetrically (Fig. 3) in low shear laminar flows become elongated when exposed to higher shear turbulent flows, where they form filamentous streamers that oscillate in the bulk fluid and exert a measurable drag on the fluid flow (Stoodley *et al.*, 1998). Also, microcolonies are not necessarily stationary at a given location on a colonized surface, as depicted in Fig. 3, but they move along the surface at a measurable 'creep' rate (Stoodley *et al.*, 1999c). Even more remarkably, biofilms in high shear environments (Reynolds number of >3200) can form well-defined wave structures (Stoodley *et al.*, 1999c) that travel downstream throughout the sessile community. Biofilms are not mechanically stable, and streamers detach into the bulk fluid when the tensile strength of their matrix is exceeded, while large pieces of biofilm are detached where the waves in biofilms 'break' on the 'shore' of the colonized area of a surface. Generally, biofilms that form at high

shear rates are stronger, and less liable to break and detach when stressed, but the mechanism(s) of this alteration in tensile strength is not understood.

The community structure of biofilms is as fully dynamic as their mechanical properties. Biofilms are formed by the recruitment of planktonic cells from the bulk phase of the ecosystem (Fig. 6), and these sessile communities shed similar planktonic cells into the same bulk phase throughout their life cycle. The detachment of planktonic cells from biofilm microcolonies is an active and natural process that, at least in biofilms formed by cells of *P. aeruginosa*, is mediated by a specific lyase enzyme (Boyd & Chakrabarty, 1994) and controlled by a specific AHL signal (D. Davies, personal communication). The detachment of planktonic cells from biofilm microcolonies is a spontaneous process that occurs frequently during the growth of a biofilm, and large areas of the sessile thermal mats that colonize hot springs are sometimes denuded by mass sloughing. It has even been suggested that there may be a measure of diurnal control of biofilm detachment in some aquatic ecosystems, so that planktonic cells of certain species are shed at certain times of the day. Detachment may involve the release of a few cells from a microcolony, but spectacular detachment events have often been recorded in time-lapse studies of biofilm development. In these cases, cells at the centre of a particular microcolony will start to move in a 'seething' manner, which indicates that they have dissolved the local matrix material and developed flagella, and the mobile cells will gradually come to occupy more and more of the bulk of the microcolony. At some point in this process, the mobile cells will breach the integrity of the matrix somewhere at the edge of the microcolony, and they will then swim away, as the remainder of the microcolony dissolves and releases the remaining sessile cells as transformed planktonic swarmer cells. Often a new microcolony forms in the space left by the wholesale detachment of all of the cells of the former microcolony, and the whole biofilm community looks little changed.

In many aquatic ecosystems, like deep groundwater and the abyssal areas of the oceans, there are few nutrients to support the growth of biofilms, and huge numbers of planktonic cells exist as dormant forms as a result of the well-documented starvation survival strategy (Kjelleberg, 1993). Because planktonic bacteria are swept into virtually all aquatic ecosystems, from rich terrestrial sources and from animal excretions, the bulk phases of these systems contain an almost infinite variety of bacterial species whose genomes are intact even if their cells are not metabolically active. These genomes are the building blocks of new biofilms, they are functionally ubiquitous, and even their dormant components are readily resuscitated when conditions become favourable. For these reasons, biofilms can self-assemble, in a matter of hours, wherever nutrients become available at an uncolonized surface. Cases in point are the 'black smokers' that spew H_2S wherever volcanic activity breaches the ocean floor, and virtually identical biofilms form at these widely distributed random

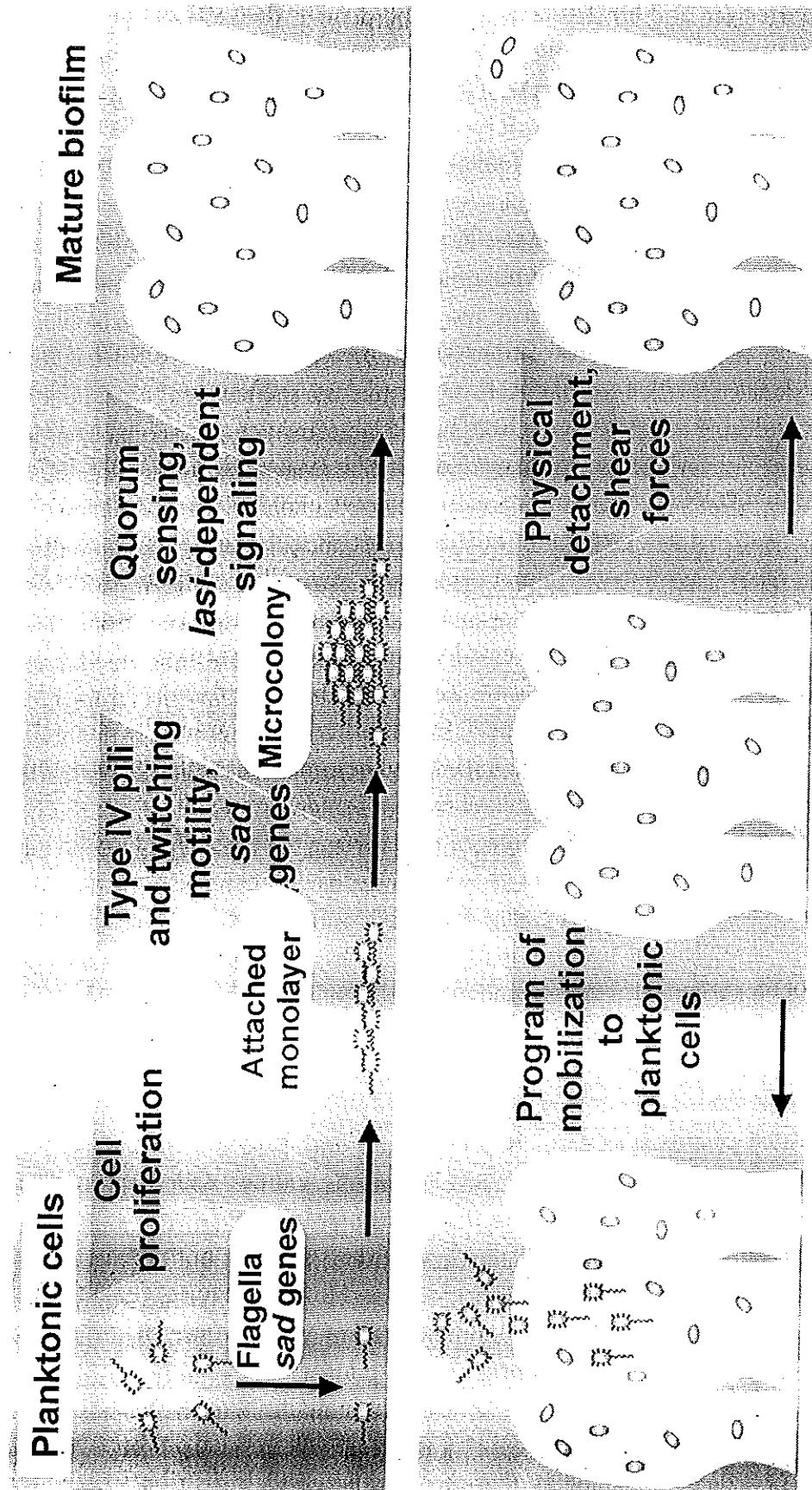


Fig. 6. Diagram showing the development of a biofilm following the attachment of planktonic bacteria to a surface, and the two main mechanisms of detachment of planktonic cells from the biofilm. Reprinted from *Science* May 21, 1999, p. 1321, with permission.

locations, from the reservoir of bacterial species in the bulk phase of the ocean and in response to the spontaneous presence of an available nutrient. The remarkable community dynamics of biofilms allow bacteria to react to colonization opportunities with amazing speed, and the detachment strategy of these sessile populations guarantees that planktonic cells of suitable species will always be readily available to colonize any suitable sites downstream from the 'mother' community. This dynamic interplay between biofilms and the planktonic cells in the bulk fluid of many ecosystems contrasts with the limited reactions of multicellular plants and animals, who usually must invoke sexual reproduction in response to similar opportunities and therefore cannot respond as quickly. This ability of microbial biofilms to self-assemble from a vast reservoir of planktonic cells of an almost infinite number of different genomes may be the most important single factor in the predominance of these remarkable multicellular communities on surfaces in even the most hostile of earth's environments.

VALETE

It should make us happy to discover new opportunities, and there should be no recriminations concerning opportunities missed. The present flood of exciting direct observations of biofilm structure and function leave us frantically searching for explanations, and rummaging through the molecular mechanisms discovered in planktonic cells to see if any of them fit these circumstances. Decades will probably pass before we understand the workings of different sessile communities sufficiently well to invoke the correct signals and enzymes to explain what we see by direct observations of living biofilms. However, we are catching up, and we are finally studying bacteria in the communities in which most of them actually live.

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