ANNUAL REVIEWS Further
Quick links to online content

Annu. Rev. Microbiol. 1995. 49:711–45
Copyright © 1995 by Annual Reviews Inc. All rights reserved

MICROBIAL BIOFILMS

J. William Costerton and Zbigniew Lewandowski
Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717

Douglas E. Caldwell and Darren R. Korber
Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A8, Canada

Hilary M. Lappin-Scott
Biological Sciences, University of Exeter, Hatherley Laboratories, Prince of Wales Road, Exeter, Devon, EX4 4PS, England

KEY WORDS: sessile bacterial communities, σ factor, phenotype change, mass transfer, consortia

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

CONTENTS

DEFINITION 712
INTRODUCTION 712
DISTRIBUTION AND UBIQUITY OF BIOFILMS 713
THE STRUCTURE OF BIOFILMS 716
As Revealed by Confocal Scanning Laser Microscopy and Fluorescent Probes 717
As Revealed by Microsensors 722
As Revealed by NMR and by Flow Studies 722
As Revealed by Electrochemical Measurements 726
As Revealed by Physicochemical Analyses 728
Summary of Our Current Concept of Biofilm Structure 733
PHENOTYPIC CHANGES IN BIOFILM BACTERIA 735
TELEOLOGICAL THOUGHTS ABOUT MICROBIAL BIOFILMS 736
MICROBIOLOGY AND REALITY 739

ABSTRACT

Direct observations have clearly shown that biofilm bacteria predominate, numerically and metabolically, in virtually all nutrient-sufficient ecosystems. Therefore, these sessile organisms predominate in most of the environmental,

0066-4227/95/1001-0711$05.00

industrial, and medical problems and processes of interest to microbiologists. If biofilm bacteria were simply planktonic cells that had adhered to a surface, this revelation would be unimportant, but they are demonstrably and profoundly different. We first noted that biofilm cells are at least 500 times more resistant to antibacterial agents. Now we have discovered that adhesion triggers the expression of a σ factor that derepresses a large number of genes so that biofilm cells are clearly phenotypically distinct from their planktonic counterparts. Each biofilm bacterium lives in a customized microniche in a complex microbial community that has primitive homeostasis, a primitive circulatory system, and metabolic cooperativity, and each of these sessile cells reacts to its special environment so that it differs fundamentally from a planktonic cell of the same species.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

DEFINITION

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media.

INTRODUCTION

The real significance of bacterial biofilms has gradually emerged since their first description (126), and the first recognition of their ubiquity (29). It has become increasingly clear that biofilms constitute a distinct growth phase of bacteria that is profoundly different from the planktonic growth phase studied so assiduously during the 15 decades following the discoveries of Louis Pasteur.

During the complex process of adhesion, bacterial cells alter their phenotypes in response to the proximity of a surface (49, 78). During the earliest stages of biofilm formation, sessile bacteria find themselves in a stable juxtaposition with cells of the same species and with those of other species, as single-species and mixed-species microcolonies are formed (82, 91). These cellular juxtapositions, and the exuberant exopolysaccharide matrix production within the developing biofilm, condition the microenvironment of each biofilm bacterium (115). Different biofilm bacteria respond to their specific microenvironmental conditions (59) with different growth patterns, and a structurally complex mature biofilm gradually develops. Physiological cooperativity is a major factor in shaping the structure and in establishing the eventual juxtapositions that make mature biofilms very efficient microbial communities adherent to surfaces (48).

Protein structure and sequential transcription dictate the elaborate structures of enzyme complexes. These complexes—because they are much more

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

efficient than random mixtures of floating enzyme molecules—are predominant in all biological systems. At a higher level of organization, bacteria within biofilms benefit from similar stable juxtaposition and similar physiological cooperativity; such bacteria therefore constitute a coordinated functional community that is much more efficient than mixed populations of floating planktonic organisms (31). In fact, biofilms resemble the tissues formed by eukar- yotic cells, in their physiological cooperativity and in the extent to which they are protected from variations in bulk phase conditions by a primitive homeostasis provided by the biofilm matrix. The analogy with eukaryotic organisms can even be extended to dissemination strategies, in which physiologically efficient, well-protected communities of cells constitute the most successful and most competitive expression of the genome, while less efficient planktonic cells are produced in order to disseminate and to colonize new locations.

As disciples of Koch and Pasteur, we have been taught to extrapolate from single-species laboratory cultures to predict bacterial behavior in actual environments. With modern tools we can now make direct observations of structure and of chemical function in living biofilms growing in specific ecosystems. This perception of functional biofilm communities, reinforced by novel methods for direct observation, will usher in a new golden age of understanding in virtually all fields of microbiology.

DISTRIBUTION AND UBIQUITY OF BIOFILMS

Bacteria constitute the most successful form of life on earth, in terms of total biomass and in terms of the variety and extent of habitats colonized. The pivotal reason for this success is phenotypic plasticity (16). The bacterial genotype is extensive, but it is the ability of this genotype to respond phenotypically to environmental stimuli, rather than the power of its genetic repertoire, that has produced the remarkable success of the bacteria. A general phenotypic strategy has recently become apparent in a majority of bacterial strains, as we have come to understand more of the modes of growth that these organisms can adopt in response to changing growth conditions.

The cells of such ubiquitous bacterial species as *Pseudomonas aeruginosa* respond to favorable nutrient conditions by adhering to available surfaces and by binary fission and exopolymer production to develop mature biofilms. These rod-shaped vegetative cells (0.8×1.2 μm) grow predominantly in this matrix-enclosed sessile mode of growth, in which they are protected from adverse environmental conditions and from biological and chemical antibacterial agents. This protection is sacrificed by the planktonic cells that are periodically shed from established biofilms so that new fresh habitats can be colonized with new biofilms. When nutrient conditions become unfavorable,

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

both sessile and planktonic cells (69) of this species are sharply reduced in size to form very small (± 0.3 μm), spherical ultramicrobacteria by a process that is now well documented as starvation survival (70). In this reversible process the DNA of the bacterial cell is stabilized while its metabolic capabilities are selectively jettisoned to produce dormant cells that can be fully resuscitated many years later. Reduced to its simplest form, this bacterial strategy clearly favors the formation of stationary, metabolically cooperative biofilms in favorable nutrient conditions and of planktonic cells or of dormant ultramicrobacteria when the interests of the species are best served by dissemination and/or by simple survival.

Prior to 1978, biofilms had been described in a certain number of aquatic systems (91, 92, 127), but the proportion of bacteria in a given ecosystem that grew in these adherent populations had not generally been determined. In 1978 Geesey et al (52) adapted a whole series of quantitative recovery methods to allow us to enumerate biofilm bacteria in a pristine mountain stream, and to compare their numbers and their activity with those of planktonic bacteria in the same aquatic system. When biofilm bacteria were clearly shown to predominate in numbers and in metabolic activity, these new methods of quantitative recovery were applied to a large number of aquatic systems in natural (34), industrial (12), and medical (33, 68) ecosystems. We can now make the general statement, based upon detailed analysis of hundreds of aquatic systems, that biofilm populations predominate in virtually all nutrient-sufficient aquatic systems independent of system geometry and of the type of ecosystem involved (78), and that these adherent populations have a very significant metabolic activity (47, 77).

This exhaustive quantitative analysis has now built a sufficient database to allow us to predict the extent of biofilm formation in a particular aquatic system, based on the following principles:

1. Metabolically active (vegetative) bacteria show a remarkable avidity for adhesion to surfaces, and this tendency is especially pronounced in wild-type cells in natural environments.
2. The extent of biofilm accretion on surfaces in any aquatic system is controlled by the amount of nutrient available for cell replication and for exopolysaccharide production.
3. In extremely oligotrophic environments, organic nutrients tend to associate with available surfaces, and to trigger local biofilm development, but bacteria generally do not adhere to surfaces in very nutrient-deficient ecosystems.

Using these principles, we can predict the extent to which biofilm will develop in particular water systems and compare these predictions with direct obser-

vations of natural systems within which many local factors may be almost equally important.

It is clear that virtually all body fluids provide sufficient organic nutrients for optimal bacterial growth, and we would therefore predict that most plastic and metal surfaces of medical devices would accrete bacterial biofilms when bacteria are present in these fluids. The development of the biofilms that are the actual etiological agents of infections associated with a wide variety of medical devices attests to the accuracy of this prediction and shows that this accretion process operates in spite of shear forces such as those generated on contact lenses by blinking and on heart valves by blood flow. Once established, the biofilm's natural resistance to natural surfactants (5), phagocytosis (65), and antibiotic therapy (4) allow it to remain as a continuing nidus of living bacteria long after all planktonic organisms have been killed by these host defense factors and by antibacterial agents. Natural endothelial surfaces are more resistant to bacterial adhesion and subsequent biofilm formation, because (*a*) antibacterial components of the tissue-surface milieu (e.g. tissue-associated antibodies) can kill planktonic bacteria as they approach the tissue surface and (*b*) cellular immunity (phagocytic activity) is fully functional on tissue surfaces. When tissue surfaces are covered by thick (>400 μm) biofilms of native (autochthonous) bacteria that condition the tissue-surface milieu by their physiological activity (e.g. lactobacilli in the vagina), the actual tissue surface is well protected from the adhesion of extraneous (allochthonous) organisms. Even though the intestine is equally replete with organic nutrients, allochthonous organisms require very special colonization mechanisms to adhere to the tissue surface, because this surface is covered by a >400 μm thick mucus layer (10) whose viscosity, movement, and autochthonous microbial population make colonization and biofilm development very difficult. The wholesale sloughing of colonized bladder endothelium and the remarkable antibacterial mechanisms of the eye are two additional examples of the many mechanisms that protect healthy tissues from bacterial adhesion, biofilm formation, and subsequent infection.

In natural and industrial aquatic systems that contain sufficient nutrients for bacterial growth and metabolism, we predict, and we note, rapid biofilm accretion on available surfaces (46, 77). This bacterial adhesion is especially rapid and specific if the surface in question is itself a nutrient, as in the case of cellulose and its analogs (32). The adhesion of the bacteria, and their subsequent formation of biofilms, is rapid and is remarkably unaffected by the physical or chemical nature of the surface concerned or by flow regimen in the bulk fluid. Biofilm formation leads to flow modification in linear systems (22) and to plugging in porous media; engineers are thoroughly familiar with the effects of these biofilms in such familiar configurations as heat-exchangers and sand filters. Indeed, sanitary engineers have for decades encouraged the

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

development of biofilm populations in trickling filters and in anaerobic digestors in order to decrease the organic content of treated wastewater by their metabolic activity. While sloughing events and reactions to toxic materials may cause variations in biofilm accretion and metabolic activity, these attached populations usually burgeon until they reach self-generated limitations of space or of sustained flow, and they can actually block conduits of considerable diameter.

In oligotrophic aquatic systems, in both natural and industrial ecosystems, the remarkable plasticity of bacteria is seen most clearly. First principles of surface chemistry predict that organic molecules will concentrate at certain areas of surfaces, and first principles of bacterial adhesion predict that bacteria will adhere to areas of the surface enriched by this process. Adherent bacteria will form biofilms to an extent dictated by nutrient availability in their particular microniche (61), but they may not adhere and they certainly will not form biofilms where nutrients are lacking (102). The ability of bacteria to form stable, essentially dormant ultramicrobacteria under very oligotrophic (starvation) conditions lends a remarkable plasticity to biofilms in these ecosystems. In extremely oligotrophic systems, the bulk fluid will certainly contain ultramicrobacteria (70) that may associate with surfaces but lack the metabolic capability for exopolysaccharide production to accomplish cellular adhesion and biofilm formation. If nutrients become available, then these very small (± 0.3 μm in diameter) ultramicrobacteria will resuscitate to form vegetative cells and even to form biofilms on available surfaces (88). When these nutrients are exhausted, the biofilm of resuscitated bacteria will persist, subsisting on its trapped nutrients, but newly produced cells at the periphery will experience starvation and will produce and shed ultramicrobacteria as their prime starvation-survival response (70). Thus, the overall strategy of bacteria in oligotrophic environments is to grow in biofilms on surfaces where nutrients are locally available and to persist in nutrient-deprived zones as floating, dormant ultramicrobacteria with the full capability of returning to the vegetative state when nutrients again become available.

THE STRUCTURE OF BIOFILMS

The advent of inexpensive high-speed computers and increasingly sophisticated software has transformed traditional microscopic observation into the discipline of analytical imaging. Of the many recent digital imaging devices now available, confocal scanning laser microscopy (CSLM) (108) has proven particularly well suited for the study of microbial biofilms. CSLM allows the nondestructive, in situ analysis of living, fully hydrated biofilms without the need for harsh chemical fixation or embedding techniques. This feature, coupled with the ability to digitally extract optical thin sections from specific

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

biofilm locations, free from out-of-focus optical interference, permits the direct examination of complex *xy* and *xz* spatial and chemical relationships that exist between bacteria, their extracellular products, and their environment (118). The episcopic nature of CSLM also enables the examination of biofilms cultivated on nontransparent surfaces (e.g. minerals, metals, gels, synthetics, etc) (23–25, 79), greatly expanding the types of analyses that may be performed. In conjunction with an expanding array of environmentally and chemically sensitive fluorescent molecular probes (2, 40, 60, 110, 114), CSLM may be used to provide detailed information on cell morphology, cellular metabolism, cell phylogeny, microenvironment chemistry, as well as the physical architecture and chemistry of the biofilm matrix and associated polymers.

Direct digital tools of examination, supported by indirect chemical and physical means such as microelectrode probes, have already led to the revision of our early rendering of biofilms, from one of a homogeneous distribution of cells in a uniform exopolysaccharide matrix (106, 116) to a model based upon significant variability and heterogeneity (34, 38, 73, 79). Earlier studies using both light and electron microscopy had sometimes suggested that microbial biofilms were heterogeneous in structure (8). Recent studies show living biofilms to consist of a variable distribution of cells and cellular aggregates, their extracellular polymers, and void spaces or water channels, which may or may not be continuous with the bulk liquid phase (67, 73, 74, 95, 111, 120). The spatially defined pattern of these elements has been termed biofilm "architecture" by Lawrence et al (79), and is often species-specific for pure-culture biofilms, as well as substrate-specific for certain microbial consortia (79, 120).

Notably, heterogeneous biofilm architecture does not appear to be the sole function, or the result, of mixtures of different organisms with variable growth habits inhabiting a similar niche. Pure-culture biofilms also accommodate many of the heterogeneous features found in mixed-cultured biofilms, suggesting a fundamental relationship between biofilm structure and in situ function. Recurrent patterns of various structural elements in both pure and mixed-species biofilms (72–75, 80) further support the premise that basic functional requisites underlie biofilm structure, and that structural diversity actually reflects the adaptation of unicellular organisms to a diverse range of physical, chemical, and communal circumstances on surfaces.

As Revealed by Confocal Scanning Laser Microscopy and Fluorescent Probes

CSLM analysis of microbial biofilms may be performed using a wide range of specific fluorescent probes and nonspecific fluorescent compounds. Nontoxic, nonspecific fluor compounds offer a number of clear benefits for architectural or structural investigations, however. For example, the exclusion by biofilm bacteria of fluorescent compounds added directly to the culture me-

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

dium (e.g. fluorescein) causes cells to appear negatively stained when viewed with CSLM (24). Bleaching, or fading, of the fluorescent signal following repeated laser scans is precluded using this method, as the fluor is continually replenished at the cell boundary. Furthermore, the nontoxic nature of fluorescein permits the temporal analysis of biofilm development or response without inhibiting normal cell function or growth (24).

Through use of CSLM and negative staining, qualitative differences in pure-culture biofilm architecture have been determined in terms of the amount of biofilm biomass present at different depths or *xy*-biofilm locations (71, 79, 80). Variability in the distribution of biomass of 24 h *P. fluorescens, P. aeruginosa,* and *Vibrio parahaemolyticus* biofilms has clearly demonstrated the tendency for biofilm bacteria to form aggregates at different horizontal and vertical sites (79), and that area of highest cell density varies among different species. For example, *P. aeruginosa* biofilms were characterized by a dense cell mass located near the biofilm base (27% biomass area at the attachment surface), whereas *V. parahaemolyticus* biofilms exhibited an inverted pyramid structure, with the most biofilm biomass located nearest the biofilm-liquid interface (16% biomass area at the ~3 μm section depth). Significantly, studies of this nature (71, 74, 79) have repeatedly confirmed that living biofilms are highly hydrated, with 50–90% of the total area at each sectioning depth consisting either of polymer and/or void space (liquid).

Recent CSLM examinations of *P. fluorescens* biofilms further detailed the spatial variability that exists within pure-culture biofilm systems. Depth measurements performed at defined *xy* locations revealed the depth of *P. fluorescens* biofilms after 24 h to be 42±19 μm. The variability in depth measurements was the consequence of adjacent regions where either channels or microcolonies predominated. After 72 h, the average depth of *P. fluorescens* biofilms remained constant (74), but the variability, which correlated with the continued growth of channels and the enlargement of cellular aggregates, increased to 42±28 μm. The actual depth measurements measured for *P. fluorescens* biofilms after 72 h varied from 0 to 90 μm. Similarly, Stewart et al (111) used a light microscopic procedure to characterize the variability in depth of *P. aeruginosa* biofilms. In this case, the mean biofilm depth was reported to be 33 μm, with a range of 13.3 to 60 μm. These data, which may be presented in the form of 3D topographic maps (73) or 2D distance-biofilm thickness profiles (111), have the potential for refining biofilm fluid friction coefficients and models of molecular diffusion. Figure 1 illustrates the clarity of these CSLM images, which can be manipulated to produce a sagittal (*xz*) or a horizontal (*xy*) section from the same data. The sagittal section (upper image) is produced at the top edge of the horizontal section (lower image), and this combination clearly shows the microcolonies and the water channels within a living 24 h *P. fluorescens* biofilm positively stained with aeridine orange.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

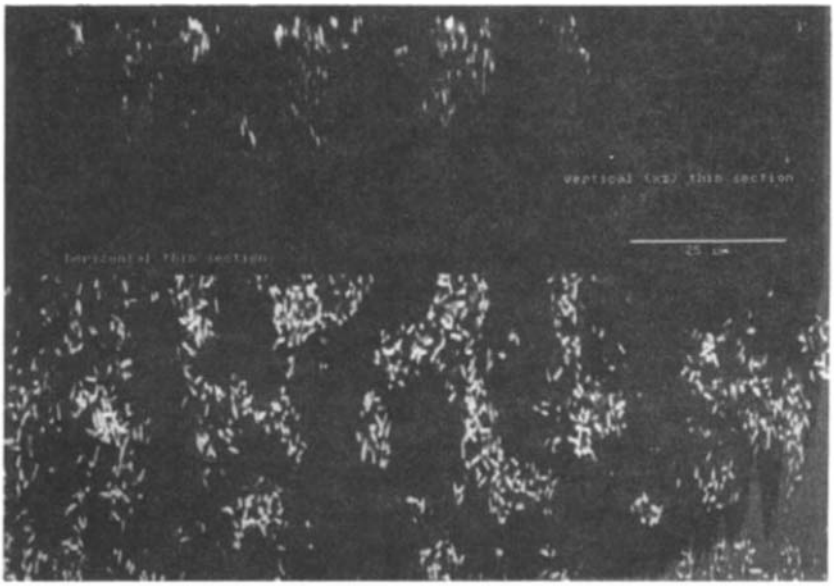


Figure 1 CSLM optical thin sections showing the bacterial microcolonies and the water channels within a living, positively stained 24h *P. fluorescens* biofilm. The upper section shows a sagittal (vertical) *xz* section, and the lower section shows the corresponding horizontal *xy* section, whose upper boundary indicates the location of the sagittal section. Bar = 25 μm.

Using high-magnification CSLM imaging, the volumetric significance of structures such as water channels is difficult to determine with certainty. Low-magnification CSLM analyses, performed over large regions of contiguous biofilm (areas ≥ 1 mm^2), lack the cell-level resolution afforded by high-numerical-aperture lenses but can resolve, and be used for quantification of, gross architectural features such as water channels and cell masses. Figure 2 shows a 1×10^6 μm^2 montage consisting of 12 low-magnification optical thin sections of a 48 h *P. fluorescens* biofilm grown under low-flow conditions (~0.1 cm s^{-1}). In this presentation, the quasi-regular array of channels and pores (occurring at 20–40 μm intervals) is clearly evident, and not a rare event. This very regular array of water channels is commonly seen in pure-culture biofilms formed under certain flow regimens, but mixed-species natural biofilms often display a much less regular architecture.

Detailed CSLM architectural studies of a diclofop methyl-degrading microbial consortium (120) linked the structure of biofilms with the nature of the carbon source. When aromatic hydrocarbons were provided as the sole carbon source, biofilm thickness increased, and spatial relationships—including coni-

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

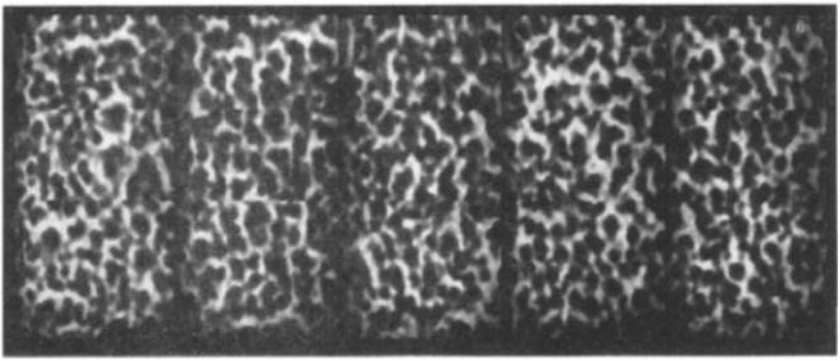


Figure 2 Montage of 12 low-magnification CSLM images of a 48 h *P. fluorescens* biofilm imaged using fluorescein and negative staining. Individual images were digitally positioned in *xy* alignment to create a montage ~ 1×10^6 μm^2 in area, clearly showing a spatial pattern of microcolonies (dark) and water channels (light).

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

cal bacterial microcolonies, grapelike clusters of cocci, and chemically defined intra- and intergeneric associations—developed after 14–21 days. When the same microbial consortium was grown on a more labile carbon source, biofilms were more homogeneous and less thick, and clear cellular associations were not evident. Furthermore, these architectural patterns could be controlled by alternating substrate. In these cases, biofilms initially grown on 300 $\mu g\ ml^{-1}$ trypticase soy broth (TSB) for 21 days regained the attributes of biofilms grown solely on diclofop methyl 2 days after switching to 14 $\mu g\ ml^{-1}$ diclofop methyl.

The difference between the architectures of pure and mixed-species biofilms may be rationalized in terms of nutrient limitation in the case of pure-culture biofilms, and niche exploitation in the case of mixed-species biofilms. The regular array of channels and aggregates seen in *P. fluorescens* biofilms would provide an alternate (lateral) route for the transport of nutrients and oxygen to the biofilm base without having to diffuse through the entire vertical length of the film. Trulear (113) previously showed that aerobic *P. aeruginosa* biofilms grew to ~30 to 40 μm in depth as monocultures, but increased in depth to ~130 μm when the culture was amended with anaerobic bacteria. This indirect evidence suggests that depletion of oxygen—not of nutrients—limited the vertical development of the *P. aeruginosa* biofilm.

In terms of penetration of nutrients, cellular wastes, or antimicrobial agents, a quantum model for molecular diffusion may apply (27). Biofilm channels, largely aqueous and under the influence of advective transport in addition to simple diffusion, would represent the primary level, and may consequently facilitate transport of molecules to the depths of the biofilm. Smaller pores or conduits, which permeate cellular aggregates, would represent the secondary

level, since (*a*) advective processes would be minimized and (*b*) microbial exopolysaccaride (EPS) may occupy the pore space. Pores are not necessarily connected with water channels, and may occur as voids or cavities within an aggregate of bacteria. At the tertiary level, cellular aggregates and associated EPS would hinder diffusion most significantly, as no advection would occur in these zones and molecular transport would be solely diffusion-driven.

A number of fluorescent probes are suitable for the in situ analysis of biofilm population architecture. Thus, the role(s) that specific, or indicator, strains play during these processes may be examined at the phylogenetic, immunological, ultrastructural, or morphological level without the need to isolate the organism from other biofilm community members. This is a significant methodological advance, since many metabolically significant biofilm bacteria are not amenable to growth in pure culture, leading to an underestimation of the natural biofilm biodiversity. Furthermore, it is difficult to find a correlation between reproductive success of an isolate in pure culture and reproductive success of the same organism in a mixed-species biofilm. Fluorescently conjugated 16S rRNA probes are now routinely used for the phylogenetic analysis of microbiological systems. Such molecular probes, in conjunction with either epifluorescence microscopy or CSLM, have already been used to catalog (in some cases, to the subspecies level) the genetic biodiversity that exists within complex biofilm systems. Studies conducted to date include qualitative and quantitative examinations of the bovine intestinal microflora, sewage-degrading consortia, and soil communities (3, 58, 110). Immunological approaches have been used extensively for determinative epifluorescent microscopy applications (15, 62, 87), and have a similar potential for wide application in CSLM studies. Relatively few workers have utilized fluor-conjugated antibodies for determinative CSLM studies to date. One such study (64) used a polyclonal antibody specific for an *Acinetobacter* sp. to examine the distribution of these cells within a 24 h triculture biofilm also containing *P. fluorescens* and *Aeromonas hydrophila*. The *Pseudomonas* and *Aeromonas* spp. were identified based on their morphologies; thus the horizontal and vertical variability in biofilm biomass contributed by each species could readily be determined.

Broader parameters for the identification of bacteria may be established on the basis of cellular morphology, cellular ultrastructure, or cell metabolic capabilities. For example, commercially available fluorescent gram stains detect gram-positive or gram-negative bacteria through a simple, one-step staining procedure. Identification of methanogenic bacteria within mixed-species biofilms is facilitated by the autofluorescence of coenzyme F_{420} (involved in CO_2 reduction) following low-wavelength light excitation (43). *Methanothrix methanobrevibacter* and *Methanosarcina* spp. have been tentatively identified from sludge-digesting biofilms based on their 420-nm excitable fluorescence and unique cell morphologies.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

As Revealed by Microsensors

The structural heterogeneity that has been directly observed by CSLM, with and without the use of fluorescent probes, suggests very important ramifications regarding mass transfer within biofilms. Even before this structural heterogeneity was discovered, some workers (109) had hypothesized that there was convective flow through pores in the biofilm because the effective diffusion coefficient in aerobic biofilms was dependent on flow conditions and on biofilm structure. Once these pores were actually visualized using CSLM, we resolved to use microsensors (38) to measure the concentration of substrates directly in various regions of the biofilm during observation by direct microscopy. We used a dissolved oxygen (DO) microelectrode to study the distribution of oxygen in an aerobic biofilm within which water channels and cellular microcolonies could be clearly resolved by CSLM (Figure 3*A*, *C*). When this microelectrode was advanced from the bulk fluid through the bulk fluid-biofilm interface (at 100–200 μm) and into a dense, matrix-enclosed microcolony (Figure 3*A*), the data from the DO sensor showed that the DO values decreased at the interface and reached almost totally anoxic values in the center of the microcolony (Figure 3*B*) and at the colonized surface (0.0 μm). When the DO microsensor was moved to an adjacent position where it would advance from the bulk fluid through the bulk fluid-biofilm interface and into a much less dense, cell-free water channel (Figure 3*C*), significant levels of dissolved oxygen were seen at all levels (Figure 3*D*), including at the colonized surface (0.0 μm). These data clearly show that the structural heterogeneity of the biofilm predicates a corresponding heterogeneity in the distribution of an important physiological substrate: dissolved oxygen. The water channels appear to transport oxygen into the biofilm, but diffusion limitations and oxygen utilization produce very low oxygen levels at the centers of cellular microcolonies, and these direct observations of living biofilms may explain the existence, and even the physiological activity, of fastidious anaerobes within mixed biofilms in aerobic environments.

As Revealed by NMR and by Flow Studies

Another hypothesis that emanates from the conceptual model of biofilm structure presented in Figures 1–3 is the possibility of water movement within the biofilm. Intrabiofilm flow would profoundly affect mass transport within biofilms, and this concept would refute the fundamental assumption (116) of biofilm process modelling that mass transport within the biofilm is entirely due to molecular diffusion. This hypothesis has been examined by two direct methods: nuclear magnetic resonance imaging (NMRI) and particle image velocimetry (PIV).

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

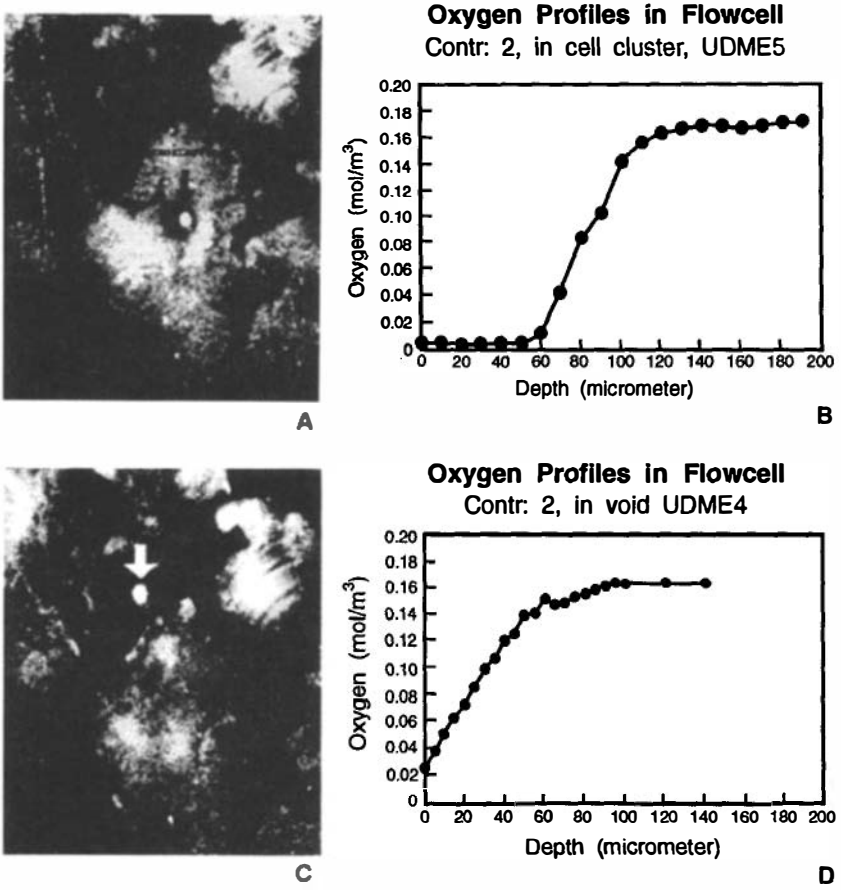


Figure 3 CSLM images (*A* and *C*) of a 24 h mixed-species biofilm showing the location (arrows) of a dissolved oxygen electrode within a microcolony (*A*) and within an adjacent water channel (*C*). *B* shows the oxygen profile as the probe is advanced from the bulk fluid into the microcolony (*A*) and eventually to the colonized surface (depth = 0 mm). *D* shows a corresponding oxygen profile as the probe is advanced into a water channel (*C*). These direct observations of a living biofilm clearly show that the microcolony is anaerobic, while the adjoining water channel contains oxygen throughout its depth.

NMRI provides a direct and non-invasive technique for the study of the chemical and physical properties of small samples and for spatial mapping, or imaging, of larger systems. Morris (97) provides detailed descriptions of current NMRI methods. Our most recent set of experiments (85, 86) used a reactor with a rectangular cross-section, and NMRI velocity measurements were performed at four different flow rates with and without biofilm in the reactor. The

flow profiles in spatial cross-sectional coordinates at 15 cm from the inlet indicated that in the reactor with biofilm, increased bulk flow velocity did not affect the character of the velocity profiles. On the other hand, in the reactor without biofilm, bulk flow velocities above 2.4 cm/s had a pronounced effect in these velocity profiles; an increase to 4.0 cm/s resulted in the formation of a jet, and an increase to 4.6 cm/s produced an even more dramatic jet. Such behavior can be explained in terms of the entry length required for development of the velocity profile. The entry length for laminar pipe and channel flows is proportional to the Reynolds number (Equation 1), where ν is the kinematic viscosity, A is the cross-sectional area, and P is the wetted perimeter and V is flow velocity. When the reactor walls are covered with a thick biofilm layer, the flow area does not change much, but the wetted perimeter may increase dramatically, since the biofilm has a very well-developed surface. This effect may stabilize the flow (i.e. decrease the Reynolds number), and consequently the coverage of the surface of a narrow conduit with a biofilm under certain circumstances may stabilize the flow near the surface.

$$Re = \frac{V4A}{\nu P} \qquad 1.$$

We also determined velocity profiles at the same location in identical flat-plate reactors in which thicker bacterial biofilms had been developed for either 3 or 4 days. Small non-uniformities (kinks) were apparent near the reactor walls, indicating that there is flow of the bulk solution in an area that is partially occupied by the biofilm. If flow above the biofilm surface is convective and if only molecular diffusion is operative below the biofilm interface (116), then the biofilm surface should behave as a rigid surface at which flow velocity reaches zero. Because this is clearly not the case, these data strongly suggest that there is convective flow of water within the biofilm layer, but the data are not unequivocal because the effective slice thickness in our NMR apparatus is 5 mm and the biofilm may not be uniform throughout this slice width.

We then turned to an almost equally non-invasive technique (PIV) that has yielded high-resolution data in a number of studies of flow velocity in complex systems. In our laboratory, a Bio-Rad MRC600 confocal scanning laser microscope (CSLM) was used in conjunction with an Olympus BH2 light microscope for simultaneous particle tracking and biofilm visualization. Neutral-density fluorescent latex spheres (Molecular Probes, Eugene Oregon, density 20°C = 1055 kg/m^2, Ex 580 nm/Em 605 nm, diameter = 0.282 μm) were added to the biofilm reactor to achieve a final concentration of 1×10^7 particles/ml, and velocity images were obtained by capturing images at various focal depths using a computer-controlled focus motor. Particles travelling across the field of view could readily be photographed at recorded intervals (Figure 4), so that their velocity could be calculated, and their position within the biofilm could also be

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

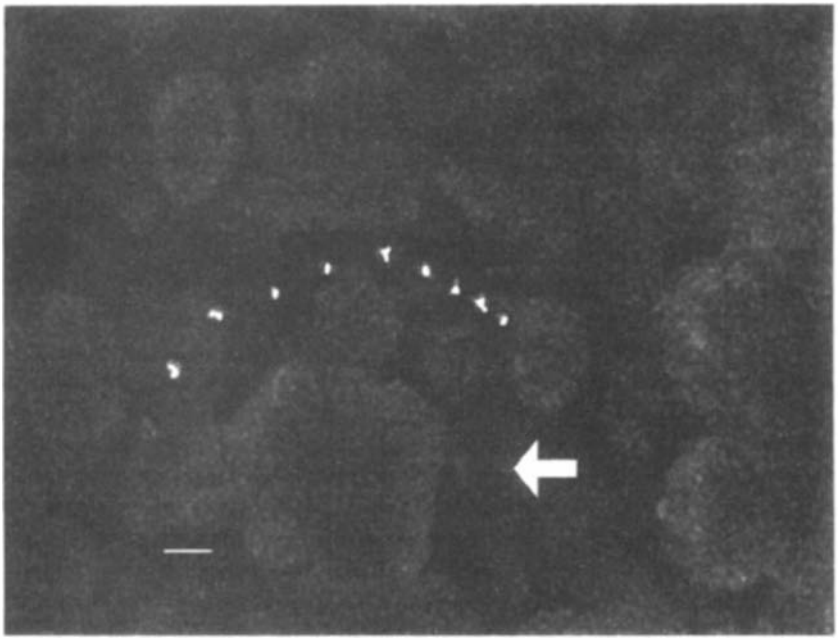


Figure 4 CSLM image showing the movement of a 0.3 μm fluorescent latex sphere, photographed at 2 s intervals, through a water channel within a 24 h *P. aeruginosa* biofilm. The movement of these spheres, which occurs in the same direction as the bulk fluid flow (arrow), is used to quantitate convective flow in the water channels within biofilms.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

continuously monitored (112). This direct method of monitoring particle movement within living biofilms produced unequivocal data to show that convective flow occurs in the water channels within bacterial biofilms.

This direct measurement of flow using PIV can also be used in bulk fluids and in zones adjacent to surfaces in flowing systems (39), and we have used it to examine flow in clean systems and in conduits coated with biofilms. Flow velocities, as calculated by PIV, are reduced as bulk fluid approaches a clean surface. The same phenomenon is seen as bulk fluid approaches a biofilm-coated surface, except that flow does not reach zero at the biofilm surfaces and direct evidence of convective flow is seen within the biofilm itself (39). The measurement of velocity profiles in biofilm systems allows the determination of shear stress both at the canal wall and the biofilm surface, as well as the estimation of the thickness of the subviscous laminar boundary layer and of the thickness of the flow regime. These values in turn can be used to estimate mass, heat, and momentum transfer, which are important for under-

standing and predicting biofilm growth kinetics and (bio)fouling effects on various process operations.

It is clear that hydrodynamic factors influence biofilms in many ways, including their control of the transport of bacteria to the surface during the initial stages of colonization (44). After the biofilm has formed, these hydrodynamic factors—control of the transport of substrates to, and metabolites from, the biofilm and shear stress—are related to biofilm erosion and biofilm sloughing, and to the physical density of the sessile population formed in various flowing systems (72). Biofilm accumulation changes the hydrodynamics of flowing systems, especially as the thickness of the biofilm becomes comparable to that of the boundary layer (104). Biofilm accumulation is essentially an autocatalytic process in that it increases surface roughness (13) which, in turn, provides shelter from shear forces and increases both the surface area and convective mass transport near the surface. Exhaustive studies have shown that biofilm elements may extend outward through the boundary layer that characterizes flow at all surfaces (35), and increase eddy diffusion and external mass transfer (109) as well as erosion (105) due to increased shear forces. Lewandowski & Walser (84) demonstrated that biofilm thickness reaches a maximum within the transition zone between laminar and turbulent flow, at a colonized surface, and they postulated that this thickness is limited by transport of the substrate in the laminar zone and by erosion in the turbulent zone. These irregular extensions of the biofilm into the bulk fluid are regularly seen at the surfaces of mixed-species biofilms formed in natural ecosystems, where they must be presumed to induce significant levels of turbulent flow.

In summary, direct evidence from examinations of living biofilms has clearly shown that these sessile accretions of microbial cells may produce surface roughness that increases turbulence and mass transport at the colonized surface. Mass transport within the biofilm is further enhanced by convective flow of the bulk fluid through the water channels that anastomose throughout the biofilm. This convective flow within the water channels attains a significant rate, and follows the same direction as the flow of the bulk fluid, so that microcolonies within the biofilm are effectively bathed by the bulk fluid even at the deepest levels examined to date.

As Revealed by Electrochemical Measurements

The structural heterogeneity noted above in direct examinations of living mixed-species biofilms, by relatively non-invasive techniques, must necessarily result in a corresponding chemical heterogeneity vis-a-vis matrix composition and the concentration of substrate molecules (e.g. oxygen) within the biofilm. These differences in the structural chemistry of different areas of a mixed-species biofilm would be exacerbated and reinforced by the metabolic activity of microcolonies of specific organisms (e.g. organic acid producers),

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

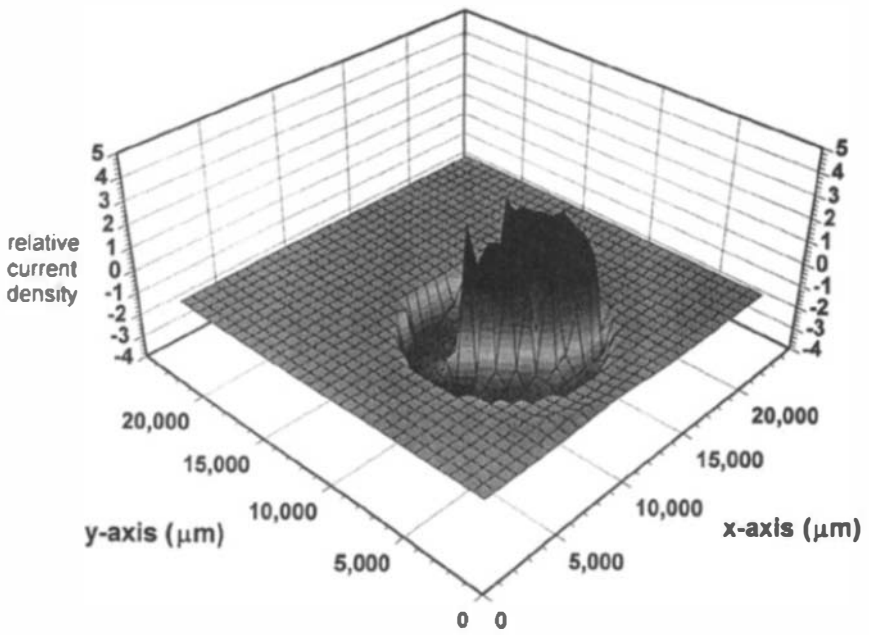


Figure 5 Plot of electrochemical data collected using a scanning vibrating electrode (SVE) when a drop of calcium alginate is placed on the surface of a mild steel coupon. The SVE data detect the development of a local anode (raised area) beneath the alginate deposit with a ringlike cathode (depressed area) on the adjoining area of the steel surface. This non-invasive electrochemical method can be used to detect anodes and cathodes on surfaces colonized by living biofilms.

and we must expect to find electrochemical differences between different loci in a mixed-species biofilm. These electrochemical differences between adjacent loci within mixed-species biofilms may constitute the mechanism of microbially influenced corrosion (MIC), and they must be considered to be a factor in the mass transport of charged ions and molecules within the biofilm. In the most extreme case, a microcolony of matrix-enclosed cells of an acidogenic species within a mixed-species biofilm would constitute a very difficult target for the diffusional attack of a cationic antimicrobial agent.

Because natural mixed-species biofilms are inherently very complex, we have chosen to define the factors operative in electrochemical heterogeneity using single-species biofilms and abiotic systems in which matrix polymers are applied to surfaces. Geesey et al (53) have shown that a measurable degree of electrochemical heterogeneity is established when two different areas of a copper surface are covered with two different acidic polysaccharides repre-

sentative of biofilm matrix material. More recently, Lewandowski's group used the scanning vibrating electrode (SVE) to map the electrochemistry of metal surfaces covered by single-species biofilms or by abiotic biofilm matrix analogs (polysaccharides). This SVE technique detects anodes and cathodes at the metal surface by detecting gradients of charged species as they stream from the surface into the bulk fluid. Initial studies have indicated that areas of metallic surfaces that are covered by real or by simulated biofilms are anodic, while uncovered areas are generally cathodic (F Roe, unpublished data). Figure 5 shows the electrochemical profile, as detected by the SVE, that results when an area of a mild steel coupon is covered with a drop of calcium alginate. The raised signal pattern indicates that a de facto anode has formed under the alginate deposit; the ringlike depressed trough surrounding this area suggests that a de facto cathode has developed on the immediately adjacent area of the uncoated metal surface. Several mechanisms have been suggested to produce electrochemical heterogeneity in these simple systems, including oxygen concentration differences and metal concentration differences, and we propose to validate these putative mechanisms before proceeding to the direct examination of living mixed-species biofilms using the SVE.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

As Revealed by Physicochemical Analyses

Microbial biofilms are characterized in part by the production of an extensive network of highly hydrated exopolysaccharides (31, 117). EPS production by biofilm bacteria serves many functions, including the facilitation of the initial attachment of bacteria to surfaces, the formation and maintenance of microcolony and biofilm structure, enhanced biofilm resistance to environmental stress and antimicrobial agents, protection of the biofilm from protozoan grazing, and biofilm nutrition (1, 20, 26, 93, 103). Biofilm EPS is also highly heterogeneous and has been demonstrated in situ to vary spatially, chemically, and physically (81, 121, 122, 123). In addition, chemically reactive EPS is generally the first biofilm structure to come in contact with potential substrates, predators, antimicrobial agents/antibiotics, and other bacteria, and thus is of considerable applied and ecological importance.

Biofilm bacteria also produce and maintain chemical microenvironments or microzones (23, 34, 38, 73, 83) within the biofilm. Spatial and temporal chemical variation within the biofilm (e.g. gradients of pH, oxygen, etc) permits the involvement of many fastidious bacteria with a limited range of metabolic capabilities. For example, chemical microzones allow the activity of anaerobic, corrosion-causing bacteria within aerobic biofilms, methanogenic syntrophy, reductive dehalogenation of pollutants, fermentative reactions, etc. The direct study of these features in living, fully hydrated biofilms is now possible using analytical CSLM and microprobe electrode methods. Examples

of how these tools may be used to elucidate some of the complex physical and chemical relationships present within biofilm systems are provided below.

Physical attributes of the biofilm-EPS matrix, such as effective diffusion coefficients (D_e, $cm^2 s^{-1}$), help to define the penetration of antimicrobial agents, the transport of nutrients and wastes, and partitioning that may exist between different biofilm regions. Barriers to diffusion imposed by the cell-polymer matrix may be examined using fluorescent compounds and CSLM. For example, fluorescence monitoring and fluorescence recovery after photobleaching (FRAP), using fluorescein and fluorescein-dextran derivatives, have been utilized to determine one-dimensional D_e values for pure-culture and mixed-species biofilm systems (75, 76, 81). Using size-fractionated fluors ranging in molecular weight from 300 to 2×10^6, it was shown that living biofilms and their polymers presented a molecular radius-dependent barrier to diffusion relative to the bulk liquid phase (from ~2 to 13% of D_{aq} values for water), and that D_e values also varied between regions of high and low cell/ polymer density. Regions where channels were directly connected with the bulk aqueous phase correlated with high diffusion coefficients, whereas adjacent areas with dense cell materials and polymers had relatively low diffusion coefficients. The depth of the biofilm material (ranging from ~30 to 100 μm) was not found to influence the magnitude of D_e for pure-culture *P. fluorescens* biofilm systems (76).

Fluorescent probes may also delineate interactions that occur between defined fluors and the microbial matrix. Early models of homogeneous biofilms depicted EPS as possessing a net negative charge. While on average this may hold true, regional variability in EPS charge distribution has been demonstrated for mixed-species biofilm model systems by comparing the binding patterns of polyanionically, cationically, and neutrally charged dextran-fluor conjugates. For example, both sewage- and herbicide-degrading mixed-species biofilms grown in continuous-flow slide culture exhibited considerable spatial variability in the in situ binding patterns of polyanionically and cationically charged fluorescent dextrans (75). In a detailed study involving a nine-member, diclofop methyl–degrading microbial consortium, Wolfaardt et al (122) demonstrated that sites that bound the fluorescent herbicide were predominantly cationic (determined using polyanionic dextrans) and hydrophobic (determined using Nile Red), even though immediately adjacent biofilm sites bound cationic dextrans.

Saccharide-specific fluorescent lectins can also partially characterize the chemical variability of bacteria and their "exopolymers." This approach has previously been utilized for examining polymer footprints left on surfaces by detached or dislodged bacteria (98), and for in situ characterization of exopolymers produced by bacteria present within a marine microcosm (23). Recently, Wolfaardt et al (123) applied a panel of fluorescent lectins (specific for various

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

isomers of mannose, galactose, glucose, fucose, *N*-acetyl-glucosamine, sialic acid, mannose, etc) to compare the EPS moieties produced by a diclofop-degrading consortium with the same organisms grown on labile substrate. During this study, considerable differences (in terms of the relative abundance and *xy-x* lectin-binding patterns of different lectins) were found between those biofilms grown on TSB and those grown on aromatic hydrocarbons. Notably, biofilms grown on a labile carbon source were considerably more uniform (both architecturally and chemically) than biofilms grown on more recalcitrant carbon sources.

Biofilm-EPS chemistry is significant in cases of industrial and medical biofilm control, because the penetration and reactivity of antimicrobial agents vary with the mesh size and chemical characteristics of the microbial EPS (81). Determined indirectly, transport of an antimicrobial agent to the base of a biofilm may be rapid (101); delivery of active molecules to target sites (e.g. the center of a cell-EPS aggregate) may be significantly hindered, however. Combined with the potential for bacterial EPS to act as an absorbent (99), this factor may contribute to the resistance of biofilm bacteria to a wide range of traditional microbial control strategies (33). Identification of unique zones of polymer chemistry using direct CSLM methods may thus be used to provide valuable insight into the mechanisms of resistance, particularly when in situ determinations of cellular viability are performed after inhibitory treatments.

Identification of chemical microenvironments associated with active bacterial cells and cell aggregates may also be accomplished using CSLM and chemically sensitive (e.g. pH-, Eh-, or ion-sensitive) molecular probes. Figure 6 shows an optical thin section of a living *Vibrio parahaemolyticus* biofilm probed with a nontoxic, pH-sensitive fluorescein derivative (5,6-carboxy-fluorescein). The fluorescent signal returned from regions of biofilm channels is much greater than that from areas associated with dense cell aggregates, indicating that the pH in these cell-dense regions is less than that associated with the bulk aqueous phase. Zones of reduced pH resulting from the activity of acid-producing bacteria have previously been inferred from similar studies using pH-sensitive compounds (23). To date, the majority of such studies have been conducted for eukaryotic systems in which the cytoplasmic chemistry remains relatively stable. Within biofilms, in which cell metabolism defines the chemical microenvironment, calibration of environmentally sensitive fluorescent indicators is extremely difficult. Simultaneous use of chemically sensitive fluors with microelectrodes (39, 83), or ratiometric pH indicators, which emit fluorescence in both a pH-dependent and -independent manner (60, 114), may prove helpful in this regard. Overall, there remains a clear need for the development of effective CSLM protocols suitable for routine quantitative analysis of biofilm chemistry.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

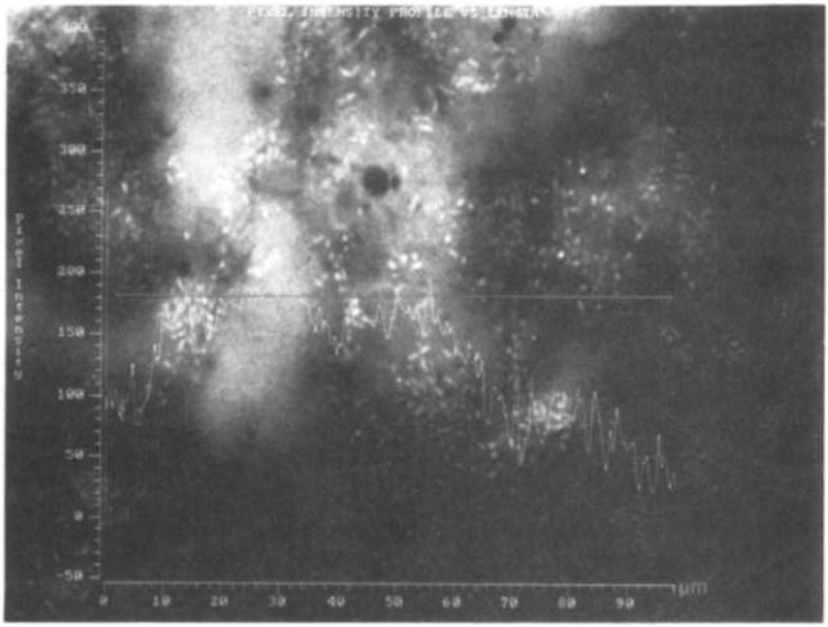


Figure 6 CSLM image of a horizontal (*xy*) section of a living *Vibrio parahaemolyticus* biofilm probed with a pH-sensitive fluorescein derivative: 5,6-carboxy-fluorescein. Areas of high pH return a stronger fluorescent signal, and the water channels are clearly more fluorescent than the bacterial microcolonies, indicating that these cellular aggregates are substantially more acidic. Pixel intensity is quantitated along a straight line that intersects both microcolonies and water channels.

The control of biofilm bacteria has been the focus of a vast amounts of applied and medical research (17, 18, 45, 54, 55, 100). The reason(s) why biofilm bacteria are less susceptible to treatments known to kill their planktonic counterparts remain unclear (18, 31, 100); thus the challenge remains to develop engineered strategies that take into account the features that make biofilms unique. One possibility is that a spatial distribution of cells of different physiological states exists within the biofilm. Such inherent variability would possibly function to maintain a pool of cells at different metabolic states (e.g. rapid or slow growth), which would be important during survival under adverse conditions (17, 54). Eng et al (45) recently demonstrated that increased rates of bacterial growth resulted in the increased efficacy of a range of antimicrobial agents. No antimicrobial agent tested by Eng and coworkers resulted in more than 3 orders of magnitude of killing against slowly growing *Staphylococcus aureus,* and only quinolones were effective against slowly growing or non-growing gram-negative bacteria. In contrast, rapidly growing cells experienced

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

almost complete killing. A wide range of fluorescent probes (based on cytoplasmic redox potential, electron transport chain activity, enzymatic activity, cell membrane potential, or membrane integrity) have been applied to provide information about the metabolic condition of individual bacteria (7, 107, 125). For example, formazan salts, propidium iodide, ethidium bromide, acridine orange, resazurin, rhodamine 123, and fluorescent substrate analogs have all been applied to various degrees in this regard (96). A wide range of newly developed probes, including RH795, Hoechst 33342, rhodamine 123, calcein AM, and carbocyanine derivatives (60), remain to be examined fully regarding their potential for ecological and applied biofilm research.

Owing to the wide range of different classes of physiological probes available, it is useful to apply the probe most likely to reflect the type of inactivation (or activation) that will occur. For example, during a study of the efficacy of fleroxacin (a fluoroquinolone DNA-gyrase inhibitor) against established (24 h) *P. fluorescens* biofilms, Korber et al (74) used acridine orange (AO) as an indicator of altered nucleic acid conformation. Fleroxacin inhibits the supercoiling of bacterial DNA, thereby altering nucleic acid conformation of affected cells and inhibiting cell replication (actively growing cells become elongated or pleomorphic). The fluorescence emission (36) of AO varies (either in the green or red wavelengths) with the conformation of DNA (the ratio of double- to single-stranded nucleic acids). Although this approach did not attempt to determine whether fleroxacin-affected cells were metabolically crippled or dead, it did identify cells that had undergone some nucleic acid conformational change as a result of fleroxacin activity. The specificity of this approach was verified morphologically by analyzing CSLM images; regions where AO fluorescence indicated cells had been affected by fleroxacin correlated ($P \leq 0.05$) with areas of cell elongation (fluoroquinolones inhibit cell division, and growing cells consequently elongate). This second morphological indicator of fleroxacin efficacy also suggested that a gradient of cell growth rates existed in *P. fluorescens* biofilms, as cells nearest the bulk liquid phase experienced the greatest elongation after 48 h exposure to 2 $\mu g\ ml^{-1}$ fleroxacin (average cell length was 8.1±2.4 μm at 15 μm section depth vs 1.7±0.6 μm at the biofilm base). Application of AO to detect the activity of fleroxacin proved much more appropriate than use of an indicator of redox potential, as initial tests revealed that cells affected by fleroxacin continued to be metabolically active for periods greater than 24 h.

CSLM studies using fluorescent dextrans with defined charge (either polyanionic, cationic, or neutral) have confirmed that the reactivity of mixed-species biofilm exopolysaccharide material with these probes is often variable (122). This is generally not the case for pure-culture biofilms, which are much more uniform in chemical and structural terms. The binding intensity of charged dextrans may be modified by specific treatments, however. For ex-

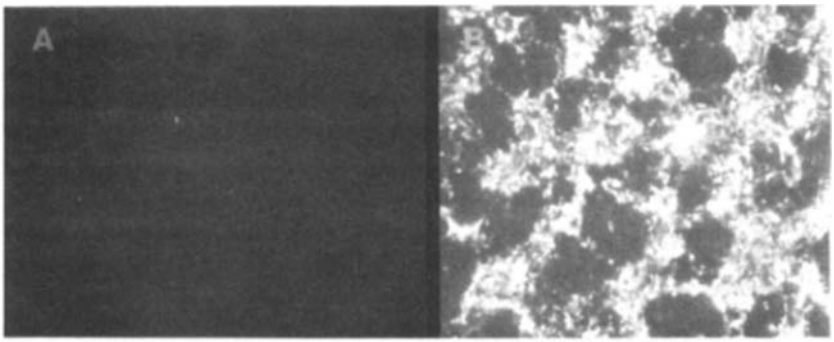


Figure 7 CSLM images of a 48 h *P. fluorescens* biofilm treated with 70 kDa polyanionic TRITC-dextran before (*A*) and after (*B*) the application of a DC electric field sufficient to produce the bioelectric effect (12). The native biofilm shows no detectable affinity for this polyanionic probe, but exposure to the DC electric field produces many cationic sites that react with the probe. These data suggest that DC fields may exert the bioelectric effect by causing profound changes in biofilm electrochemistry that facilitate penetration by antibacterial agents.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

ample, 70 kDa polyanionic tetramethylrhodamine isothiocyanate (TRITC)-dextran bound poorly with 48 h *P. fluorescens* biofilm polymer (Figure 7*a*), in agreement with reports that bacterial EPS is generally anionic in nature. Following application of a DC bioelectrical field (11) to an otherwise identical biofilm system, however, strong binding patterns were observed (Figure 7*b*). Such observations may prove valuable for explaining the mechanism by which mild electric current serves to enhance the efficacy of traditional biofilm-control strategies (11), and also explain why biofilms are more resistant to control treatments than are planktonic bacteria. One possibility is that exposure to DC current alters the electrical properties of the EPS by exposing cationically charged polymer sites or Mg^{2+}/Ca^{2+} ions, resulting in greater reactivity with the polyanionically charged dextran.

Summary of Our Current Concept of Biofilm Structure

Direct observations of living biofilms by optical and other non-invasive physical methods have revealed a novel and exciting complexity of structure (Figure 8). Although we have not yet established that this elaborate system of microcolonies and water channels is found in the same form in all biofilms, its occurrence within natural multispecies biofilms on surfaces in a typical river indicates that it may be present in this form in many common and important aquatic environments.

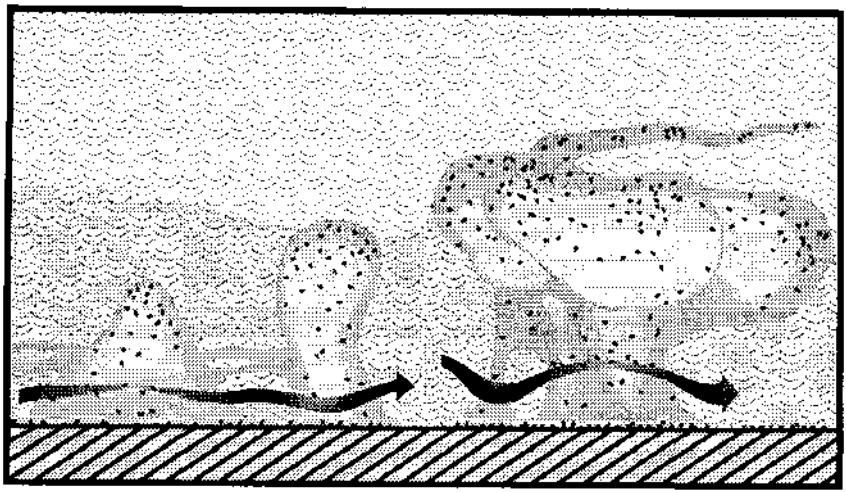


Figure 8 Conceptual model of the architecture of a single-species biofilm based on data collected by CSLM of living biofilms. Some microcolonies are simple conical structures, while others are mushroom shaped. Convective fluid flow is seen in the water channels between and even below the microcolonies within these biofilms.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

We find it useful to compare the de facto microenvironment of a bacterial cell in this complex biofilm to that of a planktonic cell of the same organism (34). Following adhesion to a surface, a bacterial cell undergoes a phenotypic change that alters many of its structural molecules (see the next section) and derepresses exopolysaccharide synthesis (37). Cell division coupled with exopolysaccharide synthesis would naturally lead to the development of microcolonies enclosed in dense slime and attached to the colonized surface. Some simple cone-shaped microcolonies are seen within developing biofilms. The persistence of water channels, however, even deep in the biofilm and at the colonized surface, together with the frequent development of mushroom-shaped microcolonies (Figure 8), argues powerfully for some form of cell-cell communication (66) similar to that which mediates the production of fruiting bodies by myxobacteria. Microcolonies would develop as the adherent bacteria that find themselves in the most favorable milieux divide and produce exopolysaccharide locally to provide a dense and coherent matrix to hold the microcolony together and to anchor it to the substratum or to other microcolonies. The microcolony is the basic unit of biofilm growth, just as the tissue is the basic unit of growth of more complex organisms, and each microcolony enjoys a measure of homeostasis in that its internal environment is conditioned by its matrix and by the metabolic activity of its component cells. In mixed

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

microcolonies (9), cells of two physiologically cooperative species may grow together as protected functional consortia. Obviously each microcolony must define its optimal size and must suppress cell division by quorum sensing (51) when a physiologically optimal size has been reached.

The discovery of convective flow within the water channels has revolutionized our concept of bacterial growth within biofilms. We must visualize the water channels as being very open, because they allow the passage of 0.3 μm latex beads (Figure 4), and they have been clearly shown to comprise an anastomosing network that carries bulk fluid throughout the biofilm. Because the convective flow in these water channels maintains the same direction as the bulk fluid flow, and because the demonstrated surface roughness of the biofilm must be expected to enhance mass transport by turbulence, the water channels actually represent a primitive circulatory system analogous to that of higher organisms. Because of this remarkable biofilm architecture, bacterial cells within a microcolony have a degree of homeostasis, optional spatial relationships with cooperative organisms, and an effective means of exchanging nutrients and metabolites with the bulk fluid phase.

PHENOTYPIC CHANGES IN BIOFILM BACTERIA

Direct microscopic observations have been particularly valuable in the use of reporter genes to elucidate the phenotypic changes that bacteria undergo when they adhere to surfaces (37). Reporter gene constructs have been produced by Chakrabarty and by Deretic, and these constructs have been used by Geesey's group (37) and by Costerton's group (63) to show that *algC* and *algD* are upregulated at the time of adhesion. This phenotypic change was intuitive because these genes control the production of phosphomannomutase, and of other enzymes in the alginate synthesis pathway, and alginate is necessary for post-adhesion activities such as biofilm formation. Davies et al (37) have developed a very useful direct visualization technique whereby cells of *P. aeruginosa* that are expressing the *algC* reporter system can be directly visualized by light microscopy, and we can now observe living cells in real time as they express the gene following adhesion and often cease to express it when surrounded by large amounts of alginate. Parallel work with gram-positive organisms, many of which are pathogens, has shown that adhesion triggers the expression of several enzymes that produce exopolysaccharides that are of pivotal importance in adhesion and biofilm formation. These functions are extremely important in the etiology of device-related infections (31).

Biochemical comparisons between biofilm cells and planktonic cells of the same species have shown that at least 30% of the cellular proteins that can be resolved by 2D electrophoresis are expressed to different extents by cells in these two growth phases (HW Yu & JW Costerton, unpublished observations).

Recent studies in Deretic's laboratory (41, 42, 94) indicate that this planktonic-biofilm transformation is controlled by a σ factor that is similar to that which controls sporulation in gram-positive bacteria and to that which controls the rough-smooth transformation in gram-negative organisms. If this fascinating hypothesis (42) stands up to current very intense scrutiny, its impact on the study of bacterial biofilms will be profound. If biofilm bacteria are the product of a σ factor–directed phenotypic change in a large cassette of genes, then they actually constitute a phenotypically distinct expression of the bacterial genome. Adhesion-induced phenotypic changes in proteins in the cell envelope, cell membrane, and cytoplasm may profoundly influence such characteristics as the enigmatic resistance of biofilm bacteria to antibacterial agents (101).

The reversal of this σ factor–directed change would produce cells with the planktonic phenotype, and the expression of alginate lyase (14) would assist in the detachment of these planktonic cells from the biofilm. We have consistently observed that microbial biofilms shed planktonic cells at a steady rate, and that this detachment is sometimes under physiological (6) and even diurnal control. We believe that the controlled shedding of planktonic cells from biofilms is a very important strategy in the bacterial struggle for survival and predominance in aquatic ecosystems.

TELEOLOGICAL THOUGHTS ABOUT MICROBIAL BIOFILMS

If we consider that bacteria thrive in a wide variety of environments throughout the Earth's crust and deep into its oceans, we must concede that they constitute a remarkably successful life form. As new methods of direct observation have been applied in many of these environments, we have seen that bacteria exist predominantly as dormant ultramicrobacteria in a wide variety of harsh oligotrophic systems, but that they actually grow predominantly in exopolysaccharide-enclosed biofilms in those ecosystems that permit growth and replication. Taken together, these data suggest that the planktonic mode of growth is favored for dissemination and for persistence in a dormant form, while the biofilm mode of growth is favored for growth. Because of their obvious success, it is germane to wonder why ultramicrobacteria are such optimal agents of the dissemination of the dormant genome while biofilms are such ideal communities for growth and for the phenotypic expression of metabolic activity.

Because bacteria obviously developed their very successful phenotypic strategy very early in their evolution, it is useful to consider the survival value of this strategy in the milieu of the primitive Earth. Bacterial cells would be attracted to the organic nutrients that concentrate naturally at surfaces in aquatic systems, and

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

the expolysaccharides that mediate their adhesion to surfaces would further concentrate dissolved organic molecules and cations out of the bulk fluid. Primary production by photosynthetic bacteria would be favored by cellular deployment at a surface, and heterotrophic bacteria would thrive because of their close juxtaposition to primary producers and because they can scavenge the biomass when these organisms die. Sustained juxtaposition to the cathodic surface of metallic ores is, in itself, sufficient to allow the growth of heterotrophic bacteria (21) in biofilms. In the primitive Earth we visualize physical (heat) and chemical (acid) threats to aquatic bacteria, and it is clear that the ability to remain essentially stationary in an optimal, or even in a permissive, local environment was one of the most valuable contributions of biofilm growth to bacterial survival. Biofilm bacteria are notably resistant to drying (103, 124), a characteristic that would also enhance bacterial survival as water levels fluctuated. Even in harsh environments in the modern Earth (e.g. hot springs), the growth of bacteria in biofilms allows certain species of bacteria to adapt to challenging conditions gradually as the whole population creeps along the surface towards the very boundary of nonpermissive conditions.

If bacteria can be spoken of as having a strategy, without lapsing into teleology, this set of reactions to environmental conditions is codified in patterns of gene expression triggered by specific environmental stimuli (41). We now know that attachment to a surface induces a de facto σ factor that triggers the expression of a large cassette of genes similar in scope to the sets of genes expressed in sporulation or in starvation survival. This heavily conserved and radically different biofilm phenotype is indicative of the fact that adhesion and biofilm development were positively selected early in the evolution of bacteria. If the elaborately controlled biofilm phenotype allowed bacteria to survive, concentrate nutrients, and grow profusely in the primitive Earth, then we can begin to understand the massive mineralogical monuments to bacterial success that still exist in the form of large ore bodies in which metals have been concentrated (50). The persistence of this positive selection for the biofilm phenotype in the modern Earth is evidenced by the predominance of this sessile mode of growth in virtually all permissive ecosystems.

The tendency of bacteria to grow in protected biofilms proved to be increasingly useful as other life forms evolved. Bacteria within biofilms are notably resistant to bacteriophages, to amoeboid predators, and to vortex-feeding protozoa, and these factors of modern ecosystems tend to reinforce the predominance of biofilms that we note in direct observations of natural systems. The fact that bacteria on plastic and metal surfaces within the human body in persistent device-related infections grow exclusively in the biofilm mode attests to the resistance of this defensive phenotype against host defense factors (antibodies, surfactants, phagocytes) and vigorous antibiotic chemotherapy (56).

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Although the aforementioned is remarkable, by far the highest expression of the biofilm phenotype is in the complex, metabolically cooperative, sessile populations that we have begun to study directly in nutrient-sufficient ecosystems. In the simplest case, the colonized surface may itself be a nutrient (e.g. cellulose), and primary colonizers then adhere and initiate the biodegradative process. In the process, these primary colonizers produce surface zones rich in their metabolic products (e.g. butyrate), and secondary and tertiary colonizers may respond to chemotactic cues and build a multilayered and highly functional biofilm community. In more complicated cases (119), many types of bacteria may be present at a surface, in response to a variety of adhesion cues, and each cell in these complex populations then responds to the ecological microniche within which it finds itself (34, 78). These responses may vary from virtual dormancy to wildly accelerated growth, much as the cells of mammalian tissues respond to local hormone and lectin concentrations during embryogenesis, and complex tissuelike biofilms may be formed. Within these tissuelike biofilms there are structural elements (the exopolysaccharide matrix) and a complex array of bacterial cells with specialized functions that are favored by their stable juxtaposition with bacterial cells of complementary metabolic capabilities (89, 121). Within these very complex tissuelike biofilms, the provision of nutritive substrates is important, but the removal of cellular end products is often a more important cooperative function (57). Specific adhesion mechanisms that would tend to initiate or promote physiological cooperation would be positively selected. The characteristic that enables bacteria to construct these elaborate, metabolically linked consortia is their ability to replicate in direct response to nutrient availability. Eukaryotic cells can respond to nutritional or endocrinological stimuli only during certain periods of development, but bacteria can always respond to favorable nutrient conditions very simply and very rapidly, and it is this programmed response that builds functional biofilm consortia.

Biological success can be determined by range or by biomass and, by either criterion, bacteria are eminently successful. Bacteria generally substitute phenotypic plasticity for complexity. In their simplest planktonic forms, bacteria can penetrate into a very wide variety of ecosystems and their range is truly phenomenal. In favorable environments their phenotypic elasticity allows bacteria to develop biofilms, and this mode of growth provides stable cell-to-cell juxtaposition and a measure of primitive homeostasis in that component cells are somewhat protected from toxic changes in the bulk fluid. Recent direct observations established that biofilms have a primitive circulatory system (39) and that cells with specialized metabolic capabilities can form tissuelike cooperative consortia (89). In essence, bacteria can be simple when it suits them to extend their distribution, but they also have the programmed capability of phenotypic change to the biofilm mode of growth, whose complexity and

efficiency rivals that of higher organisms and produces prodigious amounts of biomass.

MICROBIOLOGY AND REALITY

Recent advances in quantitative recovery and in the direct observation of microbial populations have led us to the unequivocal conclusion that the biofilm mode of growth is predominant in aquatic ecosystems. These new methods of direct observation, especially the confocal scanning laser microscope, allow us to examine living biofilms in real time, and it is difficult to imagine that they can be misleading. We believe, therefore, that we must now conclude that the biofilms that have assumed considerable importance in medical and industrial areas are actually predominant, numerically and functionally, in virtually all nutrient-sufficient aquatic ecosystems.

This predominance of biofilms was not evident during the several decades during which these same ecosystems were examined by traditional microbiological methods of sampling and culture. It is vitally important to examine the reasons for this very serious error, because the majority of students in microbiology are still taught to examine microbial ecosystems by extrapolation from planktonic samples and from monospecies laboratory cultures. Direct observations of aquatic ecosystems have shown that samples of the bulk fluid typically contain <0.1% of the bacteria in the ecosystem (52) and that swabbing of surfaces recovers a similarly insignificant proportion of the total bacterial population. Because aggregates of bacteria, like those routinely detached from biofilms, produce a single colony on the plates used for determination of colony-forming units (CFU), biofilm organisms have been radically underestimated. For this same reason, the simple rolling of colonized surfaces across agar plates (90) detaches large, slimy aggregates containing thousands of bacterial cells that produce only a single colony unless they are disrupted by special methods. Biofilm bacteria can be accurately quantitated if colonized surfaces are scrapped and if the resultant detached bacterial aggregates are dispersed, by mechanical shaking and/or ultrasonication (30), before dilution and plating by traditional CFU methods. In our experience, quantitative recovery methods must be developed for each ecosystem to be sampled, and success must be assessed by direct microscopic examinations, because some organisms may be difficult to detach and others may be difficult to grow. In many aquatic ecosystems, some of the biofilm population and some of the planktonic bacteria may be viable but nonculturable (28), and cells of many species (e.g. legionella) do not grow on commonly used laboratory media. The quantitative numerical analysis of the microbial ecosystem is not a trivial undertaking, and the best available methods must be constantly tested against the gold standard of direct microscopic examination.

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Perhaps the most serious errors have arisen from extrapolation of data obtained from single-species laboratory cultures to explain bacterial behavior in real ecosystems. We now have unequivocal evidence that sessile biofilm bacterial are profoundly phenotypically different from their planktonic counterparts, but liquid cultures of planktonic cells are still routinely used to assess the antibiotic sensitivity of pathogens in device-related infections that have been shown to be caused by biofilm bacteria (19). Similarly, many microbial ecologists still rely on data from single-species planktonic cultures to explain the activity of multispecies biofilms in which the bacterial cells are sessile and are profoundly influenced by their interactions with neighboring organisms. We must begin to use modern methods of direct observation, from microscopy of living biofilms to NMR of whole reactor systems, if we are to understand processes within microbial ecosystems whose complexity is evident from simple, direct observation. We can readily correct the error implicit in our past and current reliance on planktonic sampling as well as our extrapolation from laboratory cultures, and perhaps we can reverse some of the damage to our collective credibility caused by this error. This damage includes the provision by medical laboratories of minimal inhibitory concentration (MIC) data, obtained from planktonic cultures, that erroneously predicted to clinicians that a given concentration of a certain antibiotic would be effective in killing bacteria in device-related infections known to be caused by biofilm bacteria. This damage also includes predictions of the capability of the microbial populations of aquatic ecosystems to degrade particular organic compounds based solely on the degradative capability of planktonic populations, when these planktonic populations have been unequivocally shown to constitute <0.1% of the total microbial community.

Mike Brown and his colleagues have pointed out (16) that the most remarkable characteristic of bacteria is their plasticity in response to changes in their environment. Recent developments have reinforced this contention. Much of what we know of bacteria is derived from their behavior in single-species cultures of planktonic cells in defined media, and this is only a single point on the vast spectrum of what bacteria can do in various environments. It is vitally important that we involve our students in the revision of our discipline, which has been singularly useful to humanity, so that they can lose no time in making microbiology more accurate and even more useful in the solution of modern practical problems.

ACKNOWLEDGMENTS

We are grateful for the sustained support of the Medical Research Council and the Natural Sciences and Engineering Research Council of Canada, and for the support of the Office of Naval Research and the National Science Foun-

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

dation (EEC-8907039) in the United States. Dr. JR Lawrence has provided excellent advice and generous collaboration throughout this research program.

Any *Annual Review* chapter, as well as any article cited in an *Annual Review* chapter, may be purchased from the Annual Reviews Preprints and Reprints service.
1-800-347-8007; 415-259-5017; email: arpr@class.org

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

Literature Cited

1. Allison DG, Sutherland IW. 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. *J. Gen Microbiol.* 133:1319–27
2. Amann RI, Kurmholz I, Stahl DA. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172:762–70
3. Amann RI, Stromley J, Devereux R, Key R, Stahl DA. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* 58:614–23
4. Anwar H, Strap JL, Costerton JW. 1992. Establishment of aging biofilms: a possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents Chemother.* 36:1347–51
5. Anwar H, Strap JL, Costerton JW. 1992. Susceptibility of biofilm cells of *Pseudomonas aeruginosa* to bactericidal actions of whole blood and serum. *FEMS Microbiol. Lett.* 92:235–42
6. Applegate DH, Bryers JD. 1991. Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes. *Biotechnol. Bioeng.* 37:17–25
7. Back JP, Kroll RG. 1991. The differential fluorescence of bacteria stained with acridine orange and the effects of heat. *J. Appl. Bacteriol.* 71:51–58
8. Bakke R, Olsson PQ. 1986. Biofilm thickness measurements by light microscopy. *J. Microbiol. Methods* 5:93–98
9. Banks MK, Bryers JD. 1991. Bacterial species dominance within a binary culture biofilm. *Appl. Environ. Microbiol.* 57:1874–79
10. Banwell JG, Howard R, Cooper D, Costerton JW. 1985. Intestinal microflora after feeding phytohemaglutinin (*Phaseolus vulgaris*) lectins to conventional rats. *Appl. Environ. Microbiol.* 50:68–80
11. Blenkinsopp SA, Khoury AE, Costerton JW. 1992. Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 58:3770–73
12. Boivin J, Costerton JW. 1991. Biofilms and biodeterioration. In *Biodeterioration and Biodegradation 8*, ed. HW Rossmore, pp. 53–62. London: Elsevier Appl. Sci.
13. Bouwer EJ. 1987. Theoretical investigation of particle deposition in biofilm systems. *Water Res.* 21:1489–98
14. Boyd A, Chakrabarty AM. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 60:2355–59
15. Brayton PR, Tamplin ML, Huq A, Colwell RR. 1987. Enumeration of *Vibrio cholerae* 01 in Bangladesh waters by fluorescent-antibody direct viable count. *Appl. Environ. Microbiol.* 53:2862–65
16. Brown MRW, Williams P. 1985. The influence of the environment on envelope properties affecting survival of bacteria in infections. *Annu. Rev. Microbiol.* 39:527–56
17. Brown MRW, Allison DG, Gilbert G. 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J. Antimicrob. Chemother.* 22: 777–80
18. Brown MRW, Collier PJ, Gilbert P. 1990. Influence of growth rate on susceptibility to antimicrobial agents: modifications of the cell envelope and batch and continuous culture studies. *Antimicrob. Agents Chemother.* 34: 1613–23
19. Brown MRW, Gilbert P, Costerton JW. 1991. Extrapolating to bacterial life outside the test tube. *J. Antimicrob. Chemother.* 27:565–67
20. Brown MRW, Gilbert P. 1993. Sensitivity of biofilms to antimicrobial agents. See Ref. 104a, pp. 87S-97S
21. Bryant RD, Laishley EJ. 1989. The role of hydrogenase in anaerobic biocorrosion. *Can. J. Microbiol.* 36:259–64
22. Bryers JD, Characklis WG. 1981. Early fouling biofilm formation in a turbulent

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

flow system: overall kinetics. *Water Res.* 15:483–91
23. Caldwell DE, Korber DR, Lawrence JR. 1992. Confocal laser microscopy and computer image analysis. In *Advances in Microbial Ecology,* ed. KC Marshall, 12:1–67. New York: Plenum
24. Caldwell DE, Korber DR, Lawrence JR. 1992. Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. *J. Microbiol. Methods* 15:249–61
25. Caldwell DE, Korber DR, Lawrence JR. 1993. Analysis of biofilm formation using 2-D versus 3-D digital imaging. See Ref. 104a, pp. 52S-66S
26. Caron DA. 1987 Grazing of attached bacteria by heterotrophic microflagellates. *Microb. Ecol.* 13:203–18
27. Characklis WG, Turakhia MH, Zelver N. 1990. Transfer and interfacial transport phenomena. In *Biofilms,* ed. WG Characklis, KC Marshall, pp. 265–340. New York: Wiley
27a. Characklis WG, Wilderer PA, eds. 1989. *Structure and Function of Biofilms.* New York: Wiley
28. Colwell RR. 1984. *Vibrios in the Environment.* New York: Wiley
29. Costerton JW, Geesey GG, Cheng K-J. 1978. How bacteria stick. *Sci. Am.* 238: 86–95
30. Costerton JW, Nickel JC, Ladd TI. 1986. Suitable methods for the comparative study of free-living and surface-associated bacterial populations. In *Bacteria in Nature,* ed. JS Poindexter, ED Ledbetter, 2:49–84. New York: Plenum
31. Costerton JW, Cheng K-J, Geesey GG, Ladd TI, Nickel NC, et al. 1987. Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.* 41:435–64
32. Costerton JW. 1992. The pivotal role of biofilms in the focused attack of bacteria on soluble substrates. *Int. Biodeter. Biodegrad.* 30:123–33
33. Costerton JW, Anwar H. 1994. *Pseudomonas aeruginosa*: the microbe and pathogen. In Pseudomonas aeruginosa *Infections and Treatment,* ed. A Baltch, P Smith, pp. 1–18. New York: Dekker
34. Costerton JW, Lewandowski Z, DeBeer D, Caldwell DE, Korber DR, James GA. 1994. Biofilms: the customized microniche. *J. Bacteriol.* 176:2137–42
35. Cunningham AB. 1989. Hydrodynamics and solute transport at the fluid–biofilm interface. See Ref. 27a, pp. 19–31
36. Daley RJ. 1970. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations and comparative accuracy. In *Native Aquatic Bacteria: Enumeration, Activity, and Ecology,* ed. JW Costerton, RR Colwell. pp. 29–45. Philadelphia, PA: Am. Soc. Test. Mat.
37. Davies DG, Chakrabarty AM, Geesey GG. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa. Appl. Environ. Microbiol.* 59:1181–86
38. DeBeer D, Stoodley P, Roe FL, Lewandowski Z. 1994. Effects of biofilm structures on oxygen distribution and mass transport. *Biotech. Bioeng.* 43:1131–38
39. DeBeer D, Stoodley P, Lewandowski Z. 1994. Liquid flow in heterogeneous biofilms. *Biotech. Bioeng.* 44:636–41
40. DeLong EF, Wickham GS, Pace NR. 1989. Phylogenetic stains: ribosomal RNA–based probes for the identification of single cells. *Science* 243:1360–63
41. Deretic V, Govan JRW, Konyecsni WM, Martin DW. 1990. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the muc loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. *Mol. Microbiol.* 4:189–96
42. Deretic V, Schurr MJ, Boucher JC, Martin DW. 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J. Bacteriol.* 176: 2773–80
43. Dolfing J, Mulder J-W. 1985. Comparison of methane production rate and coenzyme F_{420} content of methanogenic consortia in anaerobic granular sludge. *Appl. Environ. Microbiol.* 49:1142–45
44. Duddridge JE, Kent CA, Laws JF. 1982. Effect of surface shear stress on the attachment of *Pseudomonas fluorescens* to stainless steel under defined flow conditions. *Biotech. Bioeng.* 26:153–64
45. Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE. 1991. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob. Agents Chemother.* 35:1824–28
46. Fletcher M, Loeb GI. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Appl. Environ. Microbiol.* 37:67–72
47. Fletcher M. 1986. Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces. *Appl. Environ. Microbiol.* 52:672–76
48. Fletcher M. 1987. How do bacteria attach to solid surfaces? *Microbiol. Sci.* 4:133–36
49. Fletcher M. 1991. The physiological

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

activity of bacteria attached to solid surfaces. *Adv. Microb. Physiol.* 32:53–85

50. Ferris FG, Fyfe WS, Beveridge TJ. 1987. Bacteria as nucleation sites for authigenic minerals in a metal-contaminated lake sediment. *Chem. Geol.* 63: 225–32
51. Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the luxR-luxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176:269–75
52. Geesey GG, Mutch R, Costerton JW, Green RB. 1978. Sessile bacteria: an important component of the microbial population in small mountain streams. *Limnol. Oceanogr.* 23:1214–23
53. Geesey GG, Iwaoka T, Griffiths PR. 1987. Characterization of interfacial phenomena occurring during exposure of a thin copper film to an aqueous suspension of an acidic polysaccharide. *J. Colloid Interface Sci.* 120: 370–76
54. Gilbert P, Collier PJ, Brown MRW. 1990. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrob. Agents Chemother.* 34:1856–68
55. Gilbert P, Brown MRW. 1995. Mechanisms of the protection of bacterial biofilms from antimicrobial agents. See Ref. 78, In press
56. Gristina AG, Dobbins JJ, Giamara B, Lewis JC, DeVries WC. 1988. Biomaterial-centered sepsis and the total artificial heart: microbial adhesion versus tissue integration. *J. Am. Med. Assoc.* 259:870–77
57. Guiot SR, van den Berg L. 1985. Performance of an upflow anaerobic reactor combining a sludge blanket and a filter treating sugar waste. *Biotech. Bioeng.* 27:800–6
58. Hahn D, Amann RI, Ludwig W, Akkermans ADL, Schleifer K-H. 1992. Detection of micro-organisms in soil after in situ hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. *J. Gen. Microbiol.* 138: 879–87
59. Hamilton WA. 1987. Biofilms: microbial interactions and metabolic activities. In *Ecology of Microbial Communities, 41st Symp. Soc. Gen. Microbiol.*, ed. M Fletcher, TRG Gray, JG Jones. Cambridge: Cambridge Univ. Press
60. Haugland RP. 1992. Molecular probes. In *Handbook of Fluorescent Probes and Research Chemicals*, ed. KD Larison, pp. 1–421. Eugene, OR: Molecular Probes
61. Hermansson M, Marshall KC. 1985. Utilization of surface localized substrate by non-adhesive marine bacteria. *Microb. Ecol.* 11:91–105
62. Hoff KA. 1988. Rapid and simple method for double staining of bacteria with 4′,6-diamidino-2-phenylindole and fluorescein isothiocyanate-labeled antibodies. *Appl. Environ. Microbiol.* 54: 2949–52
63. Hoyle BD, Williams LJ, Costerton JW. 1993. Production of mucoid exopolysaccharide during development of *Pseudomonas aeruginosa* biofilms. *Infect. Immun.* 61:777–80
64. James GA, Caldwell DE, Costerton JW. 1993. Spatial relationships between bacterial species within biofilms. *Abstr. CSM/SIM Annu. Meet., Toronto, Canada*
65. Jensen ET, Kharazmi A, Lam K, Costerton JW. 1990. Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* biofilms. *Infect. Immun.* 58:2383–85
66. Kaiser D, Losick R. 1993. How and why bacteria talk to each other. *Cell* 73:873–85
67. Keevil CW, Walker JT. 1992. Nomarski DIC microscopy and image analysis of biofilms. *Binary* 4:93–95
68. Khoury AE, Lam K, Ellis BD, Costerton JW. 1992. Prevention and control of bacterial infections associated with medical devices. *ASAIO J.* 38:M174–78
69. Kjelleberg S, Humphrey BA, Marshall KC. 1982. The effect of interfaces on small, starved marine bacteria. *Appl. Environ. Microbiol.* 43:1166–72
70. Kjelleberg S. 1993. *Starvation in Bacteria*, pp. 1–277. New York: Plenum
71. Korber DR, Lawrence JR, Hendry MJ, Caldwell DE. 1992. Programs for determining statistically representative areas of microbial biofilms. *Binary* 4: 204–10
72. Korber DR, Hanson KG, Lawrence JR, Caldwell DE, Costerton JW. 1993. The effect of environmental laminar flow velocities on the architecture of *Pseudomonas fluorescens* biofilms. *Abstr. ASM Annu. Meet., 94th; Las Vegas, NV*
73. Korber DR, Lawrence JR, Hendry MJ, Caldwell DE. 1993. Analysis of spatial variability within mot$^+$ and mot$^-$ *Pseudomonas fluorescens* biofilms using representative elements. *Biofouling* 7: 339–58
74. Korber DR, James GA, Costerton JW. 1994. Evaluation of fleroxacin activity against established *Pseudomonas fluo-*

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

rescens biofilms. *Appl. Environ. Microbiol.* 60:1663–69
75. Korber DR, Caldwell DE, Costerton JW. 1994. Structural analysis of native and pure-culture biofilms using scanning confocal laser microscopy. *Natl. Assoc. of Corrosion Engineers (NACE) Canadian Region Western Conf., Calgary, Alberta*
76. Korber DR, Lawrence JR, Lappin-Scott HM, Costerton JW. 1995. The formation of microcolonies and functional consortia within biofilms. See Ref. 78. In press
77. Lappin-Scott HM, Costerton JW. 1989. Bacterial biofilms and surface fouling. *Biofouling* 1:323–42
78. Lappin-Scott HM, Costerton JW, eds. 1995. *Microbial Biofilms.* Cambridge: Cambridge Univ. Press. In press
79. Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. 1991. Optical sectioning of microbial biofilms. *J. Bacteriol.* 173:6558–67
80. Lawrence JR, Korber DR. 1994. Aspects of microbial surface colonization behavior. In *Trends in Microbial Ecology,* ed. R Guerrero, C Pedros-Alio, pp. 113–18. Barcelona: Span. Soc. Microbiol.
81. Lawrence JR, Wolfaardt GM, Korber DR. 1994. Monitoring diffusion in biofilm matrices using confocal laser microscopy. *Appl. Environ. Microbiol.* 60:1166–73
82. Lawrence JR, Korber DR, Caldwell DE. 1995. Surface colonization strategies of biofilm-forming bacteria. In *Adv. Microb. Ecol.* 14: In press
83. Lens PNL, DeBeer D, Cronenberg CCH, Houwen FP, Ottengraf SPP, Verstraete WH. 1993. Heterogeneous distribution of microbial activity in methanogenic aggregates: pH and glucose microprofiles. *Appl. Environ. Microbiol.* 59:3803–15
84. Lewandowski Z, Walser G. 1991. Influence of hydrodynamics on biofilm accumulation. In *Environ. Eng. Proc., EE Div/ASCE, Reno, NV, July 8–10,* pp. 619–24
85. Lewandowski Z, Altobelli SA, Fukushima E. 1993. NMR and microelectrode studies of hydrodynamics and kinetics in biofilms. *Biotech. Prog.* 9:40–45
86. Lewandowski Z, Altobelli S. 1994. Water flow in a narrow conduit covered with biofilm. *Proc. Int. Assoc. Water Quality,* pp. 19–21. Copenhagen, Denmark
87. Mackie RI, Kreced RC, Els JH, van Niekerk JP, Kirschner LM, Baccker AAW. 1989. Characterization of the microbial community colonizing the anal and vulvar pores of helminths from the hindgut of zebras. *Appl. Environ. Microbiol.* 55:1178–86
88. MacLeod FA, Lappin-Scott HM, Costerton JW. 1988. Plugging of a model rock system using starved bacteria. *Appl. Environ. Microbiol.* 54: 1365–72
89. MacLeod FA, Guiot SR, Costerton JW. 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Appl. Environ. Microbiol.* 56:1598–607
90. Maki DK, Weise CE, Sarafin HW. 1977. A semi-quantitative method of identifying intravenous catheter-related infection. *New Engl. J. Med.* 296:1305–9
90a. Marshall KC, ed. 1984. *Microbial Adhesion and Aggregation.* New York: Springer Verlag
91. Marshall KC. 1992. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. *Am. Soc. Microbiol. News* 58:202–7
92. Marshall KC, Stout R, Mitchell R. 1971. Mechanisms of the initial events in the sorption of marine bacteria to surfaces. *J. Gen. Microbiol.* 68:337–48
93. Marshall PA, Loeb GI, Cowan MM, Fletcher M. 1989. Response of microbial adhesives and biofilm matrix polymers to chemical treatments as determined by interference reflection microscopy and light section microscopy. *Appl. Environ. Microbiol.* 55: 2827–31
94. Martin DW, Schurr MJ, Yu H, Deretic V. 1994. Analysis of promoters controlled by the putative sigma factor *algU* regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to sigmaE and stress response. *J. Bacteriol.* 176:6688–96
95. McFeters GA, Bazin MJ, Bryers JD, Caldwell DE, Characklis WG, et al. 1984. Biofilm development and its consequences: group report. See Ref. 90a, pp. 109–24
96. McFeters GA, Yu FP, Pyle BH, Stewart PS. 1994. Physiological assessment of bacteria using fluorochromes. *J. Microbiol. Methods* In press
97. Morris PG. 1986. *Nuclear Magnetic Resonance Imaging in Medicine and Biology.* Oxford: Clarendon
98. Neu TR, Marshall KC. 1991. Microbial "footprints"—a new approach to adhesive polymers. *Biofouling* 3:101–12
99. Nichols WW, Dorrington SW, Slack MPE, Walmsley HL. 1988. Inhibition of tobramycin diffusion by binding to alginate. *Antimicrob. Agents Chemother.* 32:518–23

Annu. Rev. Microbiol. 1995.49:711-745. Downloaded from www.annualreviews.org
Access provided by Montana State University - Bozeman on 01/15/15. For personal use only.

100. Nichols WW. 1989. Susceptibility of biofilms to toxic compounds. See Ref. 27a, pp. 321–31
101. Nickel JC, Ruseska I, Wright JB, Costerton JW. 1985. Tobramycin resistance of cells of *Pseudomonas aeruginosa* growing as a biofilm on urinary catheter material. *Antimicrob. Agents Chemother.* 27:619–24
102. Novitsky JA, Morita RY. 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine *Vibrio*. *Appl. Environ. Microbiol.* 32:617–22
103. Ophir T, Gutnick DL. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl. Environ. Microbiol.* 60:740–45
104. Picologlou BF, Zelver N, Characklis WG. 1980. Biofilm growth and hydraulic performance. *J. Hydraul. Div. Am. Soc. Chem. Eng.* 106(HY5):733–46
104a. Quesnel LB, Gilbert P, Handley PS, eds. 1993. *Microbial Cell Envelopes: Interactions and Biofilms*. Oxford: Blackwell Sci. Publ.
105. Rittman BE. 1982. The effect of shear stress on biofilm loss rate. *Biotech Bioeng.* 24:501–6
106. Rittman BE, Manem JA. 1992. Development and experimental evaluation of a steady-state, multispecies biofilm model. *Biotech. Bioeng.* 39:914–22
107. Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58:1801–8
108. Shotton DM. 1989. Confocal scanning optical microscopy and its applications for biological specimens. *J. Cell Sci.* 94:175–206
109. Siegrist H, Gujer W. 1985. Mass transfer mechanisms in a heterotrophic biofilm. *Water Res.* 19(11):1369–78
110. Stahl DA, Flesher B, Mansfeld HR, Montgomery L. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* 54:1079–84
111. Stewart PS, Peyton BM, Drury WJ, Murga R. 1993. Quantitative observations of heterogeneities in *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 59(1):327–29
112. Stoodley P, DeBeer D, Lewandowski Z. 1994. Liquid flow in biofilm systems. *Appl. Environ. Microbiol.* 60:2711–16
113. Trulear MG. 1980. *Dynamics of biofilm processes in an annular reactor*. M.S. thesis. Rice Univ., Houston, TX
114. Tsien RY, Waggoner A. 1990. Fluorophores for confocal microscopy: photophysics and photochemistry. In *Handbook of Confocal Microscopy*, ed. JB Pawley, pp. 169–78. New York: Plenum
115. van Loosdrecht MCW, Lyklema J, Norde W, Zehnder AJB. 1990. Influence of interfaces on microbial activity. *Microbiol. Rev.* 54:73–87
116. Wanner O, Gujer W. 1986. A multispecies biofilm model. *Biotech. Bioeng.* 28:314–28
117. White DC. 1984. Chemical characterization of films. See Ref. 90a, pp. 159–76
118. Wilson T. 1990. *Confocal Microscopy*. London: Academic
119. Wimpenny JWT, Kinniment S. 1995. Biochemical reactions and the establishment of gradients within biofilms. See Ref. 78, In press
120. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE. 1994. Multicellular organization in a degradative biofilm community. *Appl. Environ. Microbiol.* 60:434–46
121. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE. 1994. The role of interactions, sessile growth and nutrient amendment on the degradative efficiency of a bacterial consortium. *Can. J. Microbiol.* 40:331–40
122. Wolfaardt GM, Lawrence JR, Headley JV, Robarts RD, Caldwell DE. 1994. Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. *Microb. Ecol.* 27:279–91
123. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE. 1993. In situ visualization of exopolymer chemistry and selective binding of a chlorinated herbicide in microbial biofilms. *Abstr. CSM/SIM Annu. Meet.*, Toronto
124. Wright JB, Ruseska I, Athar MA, Corbett S, Costerton JW. 1989. *Legionella pneumophila* grows adherent to surfaces in vitro and in situ. *Infect. Control* 10: 408–15
125. Yu FP, McFeters GA. 1994. Physiological response of bacteria in biofilms to disinfection. *Appl. Environ. Microbiol.* 60:2462–66
126. Zobell CE, Anderson DQ. 1936. Observations on the multiplication of bacteria in different volumes of stored seawater and the influence of oxygen tension and solid surfaces. *Biol. Bull. Woods Hole* 71:324–42
127. Zobell CE. 1943. The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* 46:39–56