MICROBIAL BIOFILMS

Edited by
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Microbial Biofilms

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CAMBRIDGE
UNIVERSITY PRESS

PUBLISHED BY THE PRESS SYNDICATE OF THE UNIVERSITY OF CAMBRIDGE
The Pitt Building, Trumpington Street, Cambridge, United Kingdom

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK
40 West 20th Street, New York NY 10011-4211, USA
477 Williamstown Road, Port Melbourne, VIC 3207, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain
Dock House, The Waterfront, Cape Town 8001, South Africa

http://www.cambridge.org

First published 1995
First paperback edition 2003

A catalogue record for this book is available from the British Library

Library of Congress cataloguing in publication data

Microbial biofilms/edited by Hilary M. Lappin-Scott and J. William Costerton.
p. cm. (Plant and microbial biotechnology research series; 5)
Includes bibliographical references and index.
ISBN 0 521 45412 3 hardback
1. Biofilms. I. Lappin-Scott, Hilary M. II. Costerton, J. W. III. Series.
QR100.8.B55M525 1995
576'.15 dc20 94-39377 CIP

ISBN 0 521 45412 3 hardback
ISBN 0 521 54212 X paperback

Formation of the microcolonies on surfaces is an important bacterial survival strategy. These biofilms occur on both inert and living systems, making them important to a wide range of scientific disciplines.

This book first provides an analysis of the chemical, ecological and physical processes involved in the development of biofilms and their interactions with surfaces.

The next section deals with biofilms on non-living surfaces. Biofilms have important engineering implications, such as in mining industries, the corrosion of pipelines and pure and waste water industries. They also have medical significance when associated with the mouth, urinary tract and urogenital tract. In addition, they form in plant root systems and in animals, for example in the ruminant digestive tract, and so are agriculturally important. The final section examines these interactions with living surfaces.

PLANT AND MICROBIAL BIOTECHNOLOGY RESEARCH SERIES: 5
Series Editor: James Lynch

Microbial Biofilms

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Series Preface
Plant and Microbial Biotechnology

The primary concept of this series of books is to produce volumes covering the integration of plant and microbial biology in modern biotechnological science. Illustrations abound: for example, the development of plant molecular biology has been heavily dependent on the use of microbial vectors, and the growth of plant cells in culture has largely dawn on microbial fermentation technology. In both of these cases the understanding of microbial processes is now benefitting from the enormous investments made in plant biotechnology. It is interesting to note that many educational institutions are also beginning to see things in this way and are integrating departments previously separated by artificial boundaries.

Many definitions have been proposed for biotechnology but the only one which has specifically defined *environmental biotechnology* is that of the European Federation of Biotechnology as *The specific application of biotechnology to the management of environmental problems, including waste treatment, pollution control and integration with non-biological technologies*. The study of microbial biofilms is clearly an excellent illustration of environmental biotechnology. The manipulation and control of biofilms is of great interest to industries, including agriculture, chemicals and healthcare.

One of the leaders in the study of biofilms has been Bill Costerton, especially in his early studies when he produced superb electron micrographs to demonstrate the fascinating microbial assemblages which developed in biofilms. However, he rapidly proceeded to demonstrate important physiological functions which occured in these interesting layers. In 1986, Hilary Lappin-Scott joined him to work partly in Cambridge and partly in Calgary on the biofilms associated with oil wells, so starting a long and productive association. Hilary went on to the University of Exeter in 1990 to create a research group on biofilms which is proving to have substantive inputs in a range of environmental and industrial fields. I had known Hilary since her days as a research student at the University of Warwick and found myself talking to her about biofilms on more than one occasion when we were both warming-up at the start of London marathons! I was delighted when Hilary said that she would be prepared to contribute a volume to the series with Bill Costerton. They have produced a textbook which covers not only the fundamentals of this important subject, but also provides a range of diverse applications.

Jim Lynch

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Introduction to Microbial Biofilms

J. William Costerton and Hilary M. Lappin-Scott

In any scientific examination that addresses a subject as basic as the mode of growth of bacteria it is prudent to begin by considering the successful prokaryotic communities that clearly predated the development of the eukaryotic cell. During the millions of years in which bacteria constituted the only life form on Earth, we visualize an extremely oligotrophic aquatic environment in which specific ecosystems were impacted by many factors (e.g. heat, acid) hostile to their survival. It is the nature of aquatic systems to flow from one ecosystem to another and we can imagine a primitive stream connecting permissive and non-permissive bacterial habitats in the nascent Earth. Once bacterial cells had evolved, the planktonic (floating) mode of growth would deliver them from one habitat to another until they perished in the first non-permissive locus. The sessile mode of growth as attached bacteria would allow these primitive organisms to colonize a permissive habitat and persist therein. Biofilm formation would allow these sessile organisms to trap and retain scarce organic compounds and to develop a focused attack on complex or refractory nutrients whose processing required time and/or the cooperation of one or more bacterial species. Biofilm formation would also change the microenvironment at the colonized surface in a colonized habitat and render its inhabitants less susceptible to hostile chemical, physical, or even biological (e.g. bacteriophage) factors. Each colonized habitat would become a stable crucible of genetic adaption and physiological cooperativity that would flourish in its own location but would also shed its component organisms as planktonic cells so that, if they survived, they could establish a similar integrated biofilm community in any permissive habitat downstream.

Our image of aquatic systems in the nascent Earth militates against the survival of planktonic bacteria, and leads us to suggest that the sessile mode of growth and biofilm formation may have been the *sine qua non* of survival of newly evolved bacteria in this hostile environment. It is therefore germane to examine hostile oligotrophic environments on the modern planet to determine which mode of growth of prokaryotic cells is most successful. The ubiquity and predominance of bacterial biofilms was first noted in very oligotrophic high altitude alpine streams in Canada (Geesey *et al.* 1977) and subsequent detailed examinations of these systems clearly show that bacterial populations can only be maintained in their turbulent waters if these organisms live in biofilms adherent to available surfaces. In the equally hostile and oligotrophic Antarctic desert environment bacteria and algae can invade the exposed surfaces of rocks to produce complex biofilms or 'varnishes' whose matrices trap scarce rainwater and permit growth and primary production based on photosynthesis.

All modern bacteria are obviously descendant from the primitive forms that successfully colonized the planet Earth early in its biological history and their basic strategies of colonization and survival depend on patterns of phenotypic expression of their genetic material that made them successful in that primitive milieu. These patterns affect many modern processes because heat exchangers are fouled, pipelines are corroded, and medical devices are infected by

recalcitrant slimy bacteria, because bacteria have long ago evolved a set of basic strategies to colonize and persist and to survive in permissive habitats.

Laboratory cultures represent the planktonic mode of growth

The phenomenon of bacterial adhesion to surfaces is clearly visible in routine light microscopic examinations of natural populations and it was elegantly described (ZoBell 1943) long before its relationship to ubiquitous biofilm formation was recognized. Later, descriptions of this process emphasized its initial reversibility (Marshall *et al.* 1971) and its putative mechanisms (Fletcher & Loeb 1979) but what has emerged is a whole spectrum of adhesion phenomena, that range from the very specific pilus-mediated adhesion of bacteria to specific tissues to totally non-specific exopolysaccharide-mediated adhesion of natural wild bacteria to all surfaces within a stream (Geesey *et al.* 1977). What is perhaps most important in this ongoing area of research, which is mired in detail but driven by the search for colonization-resistant materials, is that genetic examinations of the best known adhesion mechanisms show that they are highly conserved during evolution. It has long been recognized that simple animal or natural ecosystem passage of a bacterial strain that has lost surface structures and adhesion capability during repeated subcultures as a planktonic single species culture restores these structures (pili, exopolysaccharides) and this capability. In some instances these surface structures and the adhesion capability can be restored by culture in menstrua that contain surfactants or antibiotics at concentrations that kill planktonic cells totally lacking in protective surface structures (Govan 1975) but allow the survival of glycocalyx enclosed wild type cells. These simple observations, some of which date back to the 1930s, probably should have alerted us to the fact that the planktonic single species laboratory culture exerts a powerful selective pressure on a bacterial genome that eventually produces a 'stripped down' cell lacking in protective and adhesive surface structures that simply cannot survive in natural environments where adhesion and protection are of paramount importance.

It is very sobering to realize, over a century after the development of the planktonic single species laboratory culture (Koch 1881), that the cells we have been studying so assiduously are phenotypically locked in a planktonic mode of growth. This is at the opposite end of a phenotypic spectrum from the sessile mode of growth clearly seen to predominate in most natural environments. The classic laboratory culture has been extremely useful for the exploration of the genome-driven activities of bacterial species, but bacteria are protean creatures whose survival depends on their phenotypic responses to environmental factors and we have generally studied cells locked by their test tube environment into the planktonic mode of growth. Decades of productive research have yielded dividends in the control of planktonic diseases and in modern genetic engineering but have been less successful in the control of biofilm diseases and industrial and environmental microbiology. Now that we realize that new culture methods, several of which are described by Caldwell in Chapter 3, can mimic the biofilm mode of growth that predominates in nature, and in many heretofore recalcitrant bacterial diseases, we can look forward to a new and equally exciting explosion of practical sequelae of modern microbiological biofilm research.

Phenotypic responses to adhesion

Modern research using reporter genes has clearly shown that the adhesion event triggers the expression of genes controlling the production of bacterial components (for example, the alginate of *Pseudomonas aeruginosa*) necessary for continued adhesion and biofilm formation. Reporter gene systems constructed by Chakrabarty and by Deretic have been used by Geesey's group (Davies *et al.* 1993) and by Costerton's group (Hoyle *et al.* 1993) to show that adhesion triggers the expression of the alg C and other genes that control the production of phosphomannomutase and of other enzymes in the alginate synthesis pathway.

Parallel work with Gram positive pathogens, notably *Staphylococcus epidermidis*, has shown that adhesion triggers the expression of enzymes which produce exopolysaccharides that are pivotal in continued adhesion and biofilm formation and in the aetiology of device related bacterial

infections (Costerton *et al.* 1987). These complex and focused reporter gene techniques have shown that adhesion triggers the rapid and specific phenotypic expression of several specific genes whose products are concerned with adhesion and biofilm formation. Parallel, general examinations, comparing the proteinaceous gene products made by sessile bacteria with those made by planktonic cells of the same species have shown (H. Yu and J. W. Costerton, unpublished observations) that adhesion changes the phenotypic expression of at least 30% of the proteins detectable in cell extracts by gel chromatography. Recent studies in Deretic's laboratory (Martin *et al.* 1993) indicate that a sigma factor similar to that involved in sporulation, and in the reversible rough–smooth lipopolysaccharide transformation in Gram negative bacteria, may be involved in the adhesion-mediated change between planktonic cells and sessile biofilm cells of the same bacterial species. If this fascinating hypothesis stands up under current intense scrutiny, the battery of phenotypic changes that occur as cells of bacterial species alternate between planktonic and sessile modes of growth will come to be regarded as a phase change mediated by a sigma factor that controls a whole cassette of genes related to adhesion and to biofilm formation. If biofilm bacteria do, in fact, constitute a different phase of phenotypic expression of the bacterial genome many of their observed characteristics, such as their almost complete resistance to antibiotics that are effective against planktonic cells of the same species (Nickel *et al.* 1985), will be partially explained.

We are presently studying the rate at which bacteria revert to the planktonic phase of phenotypic expression after they have become detached from established biofilms, by active shedding mechanisms or by simple fragmentation. These studies will provide insights into the nature of a phase change that may enable bacteria to control their cell surface components and to alternate between sessile and planktonic modes of growth to facilitate their colonization and survival within permissive habitats.

Biofilm structure

The confocal scanning laser (CSL) microscope has enabled us to examine living, fully hydrated, biofilms and the use of this microscope has provided structural information that is especially valuable because it is direct and consequently unequivocal (Lawrence *et al.* 1991). The examination of hundreds of biofilms formed by dozens of different pure cultures and by several natural bacterial populations has clearly shown that biofilm bacteria grow predominantly in microcolonies of similar morphotypes (Fig. 0.1) interspersed between water channels that contain few bacterial cells and appear to contain a more permeable matrix material. These clearly heterogeneous surface-associated bacterial populations can now be examined, living and fully functional, by CSL microscopy and by the use of non-intrusive chemical probes and of physical microprobes (5–10 μm tip diameter) that can be positioned at any location within the biofilm and visualized by the CSL microscope. The concerted use of these complementary analytical tools has produced a conceptual image of bacterial biofilms that is truly amazing in its complexity and sophistication.

The structural heterogeneity of biofilms

Soon after the initial adhesion of bacteria to a surface, in either a single species culture or a mixed natural population, certain adherent cells proliferate and elaborate exopolysaccharides until they produce a microcolony in which morphologically similar 'sister' cells are embedded in a thick polysaccharide matrix (Fig. 0.1). As the biofilm thickens and matures individual microcolonies may lose their associations with the colonized surface and, in multispecies populations, cells of several species may come together to produce functional consortia (Kudo *et al.* 1987a, b) that carry out complex physiologically cooperative processes such as methane production (MacLeod *et al.* 1990). The microcolony is the basic growth unit of the biofilm and we consider bacterial growth to be sessile in nature if these microcolonies are produced, even if their final geometric configuration differs from that depicted in Fig. 0.1. Specific data attest to the limited permeability of the thick matrices surrounding individual microcolonies, in that CSL microscopy has shown that fluorescein-conjugated dextrans and other permeability probes penetrate the water channels but fail to penetrate the microcolonies within biofilms. Very recent work by

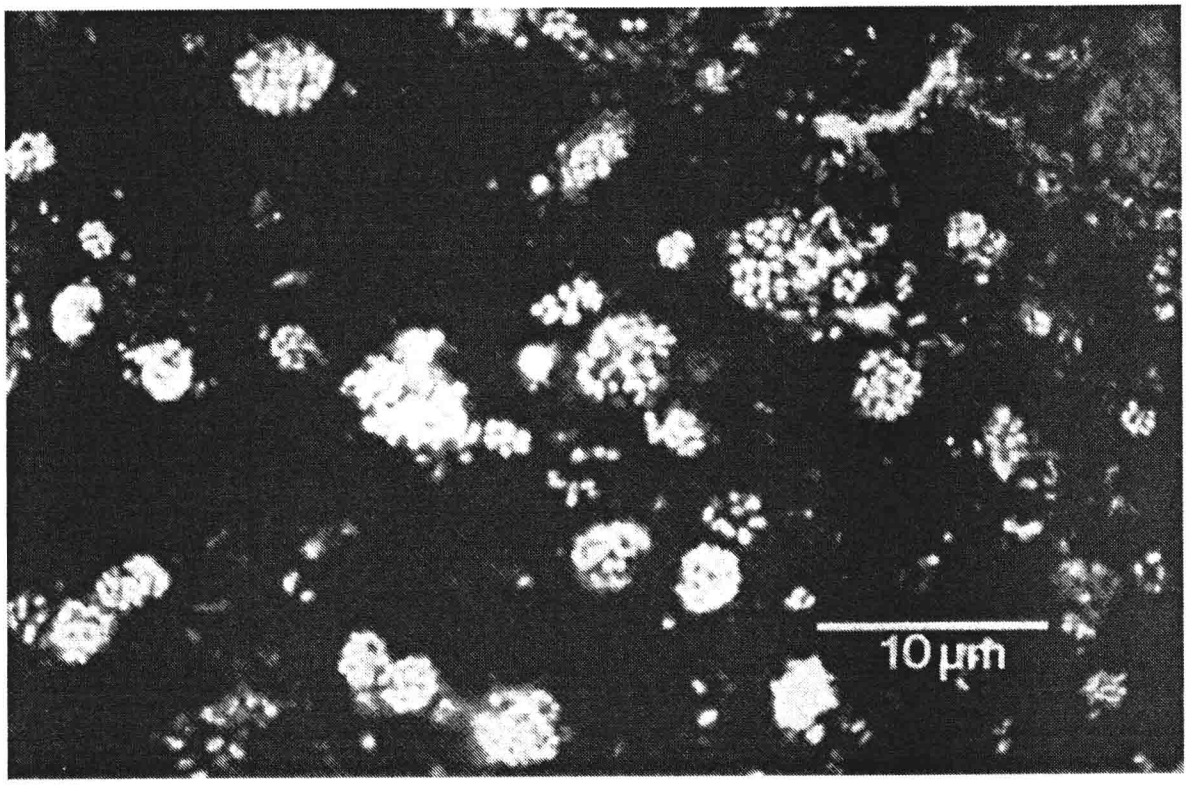

Fig. 0.1. Confocal scanning laser micrograph of an optical section of a mixed species natural biofilm parallel to a colonized surface. Note the occurrence of discrete bacterial microcolonies interspersed with broad and relatively unpopulated water channels within this living, fully hydrated, biofilm that developed in the Bow River, Alberta, Canada. (Photograph courtesy of Garth James.)

Dr Lewandowski's group at the Center for Biofilm Engineering has indicated that the water channels that lie between and sometimes below these microcolonies are actually sufficiently permeable to allow convective fluid flow, and the same group has obtained NMR data to confirm flow within these channels. These data, obtained in direct examinations of living biofilms, combine to present a concept of biofilm structure that is revolutionary in its complexity and sophistication. Biofilm bacteria clearly live in dense matrix-enclosed microcolonies, where they are exposed to a bathing flow of modified bulk fluid through the less dense water channels that anastomose throughout even the thickest and most mature biofilms. These morphological data suggest a biofilm within which bacteria live in specialized microniches that are served by a primitive circulatory system within a stationary matrix-protected population adherent to surfaces within a flowing system.

The chemical and physical heterogeneity of biofilms

The planktonic mode of growth affords each individual bacterial cell an almost identical ecological niche, in that all cells communicate almost directly with the bulk fluid by simple diffusion. The simple immobilization of a bacterial cell within an anionic matrix introduces heterogeneity because these cells carry out many chemical functions, such as proton extrusion and oxygen consumption, and the matrix areas near the cells must, necessarily, differ from these further from the cells. If we then visualize different microcolonies, containing one or more physiological types of bacteria, within a biofilm, we must expect that the metabolic activities of these

clusters of cells would produce loci with sharply different chemical environments. If we consider the simple cases of acid generation and oxygen consumption, we can state that adjacent areas of the biofilm will be different at a given moment in time, depending on the extent to which acid generation or oxygen consumption exceed the diffusion of protons or of dissolved oxygen through the biofilm matrix.

Because of the development of the CSL microscope we can now introduce both chemical (Lawrence *et al.* 1991) and physical probes (Lewandowski *et al.* 1993) into living biofilms and record such parameters as pH and dissolved oxygen concentrations at particular loci. Chemical probes are difficult to calibrate and physical probes may be somewhat intrusive but both serve to show local differences very accurately. Early work with pH sensitive chemical probes clearly showed that some bacterial microcolonies in both pure culture and mixed natural biofilms operated at pH values significantly lower than the water channels (Lawrence *et al.* 1991) and that individual cells within microcolonies were surrounded by an acid zone that may be produced by proton extrusion. Recent work with dissolved oxygen microelectrodes has produced equally unequivocal data to indicate heterogeneity within biofilms. When the microelectrode (tip diameter 5–10 μm) is advanced from the bulk fluid through the biofilm interface and into a bacterial microcolony (Fig. 0.2a) the dissolved oxygen concentration is seen to decrease at the interface and to reach truly anaerobic levels within the microcolony (Fig. 0.2b). When the same microelectrode is traversed only 100 μm laterally and advanced from the bulk fluid through the biofilm interface and into a water channel (Fig. 0.2c) much higher levels of dissolved oxygen are recorded (Fig. 0.2d). These simple and direct measurements of pH and of dissolved oxygen concentration are made in living biofilms and they provide unequivocal evidence of the basic chemical heterogeneity of these structurally complex adherent populations.

If we grasp these basic concepts of the structural and chemical heterogeneity of biofilms and begin to apply them to natural biofilm populations that have been described and defined during the past two decades, a fascinating picture of the sessile mode of growth begins to emerge. Ultrastructural observations of cellulose digestion by some cellulolytic bacteria (Cheng *et al.* 1984) showed that these organisms adhere to this insoluble substrate and produce deep pits into which they and their progeny eventually penetrate. We can infer a local concentration of cellulolytic enzymes, within a biofilm, that mediate a focused attack on a surface that is typical of many instances of biodegradation. Acid generation by specific microcolonies within a biofilm could mediate local focused attack on surfaces ranging from dental enamel to stainless steel. In instances in which the concerted metabolic activities of several bacterial species are necessary to biodegrade a complex substrate (e.g. bitumen) cells of these species would form a microcolony within a biofilm and that microcolony would mediate a local attack on the substratum. One of the most important inherent characteristics of bacterial biofilms is their capability of focused and cooperative biodegradation, and this characteristic depends entirely on the sustained juxtaposition of cells with each other and with surfaces that is a feature of the biofilm mode of growth.

If we re-examine the structure and activity of bacterial consortia (MacLeod *et al.* 1990) in the light of these recent revelations of biofilm heterogeneity a similarly gratifying concept emerges. The biofilm mode of growth positions a wide variety of bacterial cells at a surface and the individual cells replicate to initiate microcolony formation at a rate that depends on how well their particular microniche suits their physiological requirements. If a particular cell is unable to replicate it may simply persist, entrapped in the biofilm, until suitable conditions develop. If a particular cell requires acetate it will replicate if this substrate is supplied by a neighbouring cell, and this type of metabolic cooperativity often produces structural consortia of considerable complexity and metabolic efficiency (MacLeod *et al.* 1990). The chemistry of a particular microniche within a biofilm depends on both the delivery of bulk fluid components through the water channels and the metabolic activity of neighbouring cells. The rapid asexual reproduction of bacteria enables them to react very quickly to favourable chemical changes within a specific microniche and their starvation survival strategies (Kjellberg *et al.* 1987) enable them to persist for very long periods of time in non-permissive conditions. Spatial juxtaposition within biofilms is essential to the development of