

Chapter 5

BIOFILMS, NATURALLY OCCURRING COMMUNITIES OF IMMOBILIZED CELLS

ROBERT J. C. McLEAN,¹ DOUGLAS E. CALDWELL² AND
J. WILLIAM COSTERTON³

¹*Department of Biology, Southwest Texas State University,
San Marcos, USA*

²*Department of Applied Microbiology & Food Science,
University of Saskatchewan, Saskatoon, Canada*

³*Center for Biofilm Engineering, College of Engineering,
Montana State University, Bozeman, USA*

CONTENTS

5.1	Introduction	290
5.2	Colonization and Biofilm Formation.	291
5.2.1	Influence of Surface Properties on Colonization.	292
5.2.2	Bacterial Structures Involved in Adhesion	294
5.2.3	Strategies and Mechanisms of Biofilm Formation	298
5.3	Diversity of Biofilms in Nature	302
5.3.1	Natural Environments	302
5.3.2	Industrial Environments	303
5.3.3	Medical Importance	307
5.4	Biofilm Composition	310
5.5	Biofilm Ecology.	312
5.5.1	Role in Persistence and Nutrition	313
5.5.2	Resistance to Adverse Environmental Conditions	314
5.5.3	Relation of Biofilms to Other Bacterial Growth Strategies	316
5.6	Biofilm Physiology	318
5.7	Practical Aspects of Biofilm Formation	320
5.8	References	323

5.1 INTRODUCTION

The concept of using immobilized cells (addressed in other chapters of this book) has greatly enhanced the industrial scale production of a number of secondary metabolites by microorganisms. Generally the organisms are confined within a supporting matrix such as carrageenin or agar. In this environment, cellular growth is greatly reduced and the production of secondary metabolites thereby enhanced. Separation of microbial products from the immobilized culture is also facilitated due to the ease with which the relatively large clusters of cells can be removed.

Artificial cellular immobilization actually mimics a natural process. Microorganisms such as bacteria often incorporate into biofilm communities which adhere to a wide variety of surfaces. In lieu of an artificial supporting matrix, these organisms produce a variety of extracellular polysaccharide or polypeptide capsule polymers often referred to as the glycocalyx (Costerton *et al.*, 1981*b*, 1987). When growing on surfaces, biofilm or sessile bacteria are physiologically quite different from their planktonic counterparts (Marshall, 1985; Geesey & White, 1990; van Loosdrecht *et al.*, 1990). Yet virtually all knowledge of microbial physiology is based essentially on planktonic cells grown at maximum growth rates in rich liquid culture!

Biofilms are very widespread in nature. In a number of environments such as oligotrophic (nutrient poor) mountain streams, virtually all microbial activity occurs within biofilms, and the very few planktonic organisms present act as pioneers in that they enable bacteria to colonize new surfaces and form biofilms (Marshall, 1985, 1988; Geesey *et al.*, 1978). In an industrial context, biofilms are of great importance being responsible for such diverse activities as anaerobic corrosion in water pipelines and the removal of heavy metals and organic matter mineralization from waste water in trickling filters (Costerton *et al.*, 1987; McLean & Beveridge, 1990; MacLeod *et al.*, 1990).

In this chapter, we describe the formation, distribution, ecology, and physiology of these naturally occurring biofilm communities of immobilized cells. Whenever possible, we will present applications of biofilm physiology and ecology in relationship to industry. This represents a relatively new and important facet of microbiology in that the biofilm mode often represents the preferred growth strategy of bacteria in nature (Costerton *et al.*, 1987). It is our hope that we will generate an appreciation of this natural mode of bacterial growth and stimulate others to conduct investigations in this area since much basic and applied research remains to be done.

5.2 COLONIZATION AND BIOFILM FORMATION

In their natural environment, bacteria are subjected to a variety of stresses of a physical, chemical, and biological nature. Rather than being given a rich laboratory medium and culture conditions in which to grow, these organisms are often in oligotrophic environments, and must compete for any available nutrients. In addition, conditions such as temperature, oxygen concentration, Eh, water activity, chemical composition, presence of harmful chemicals, and predation often complicates matters even further (Marshall, 1985, 1988; Brock & Madigan, 1988).

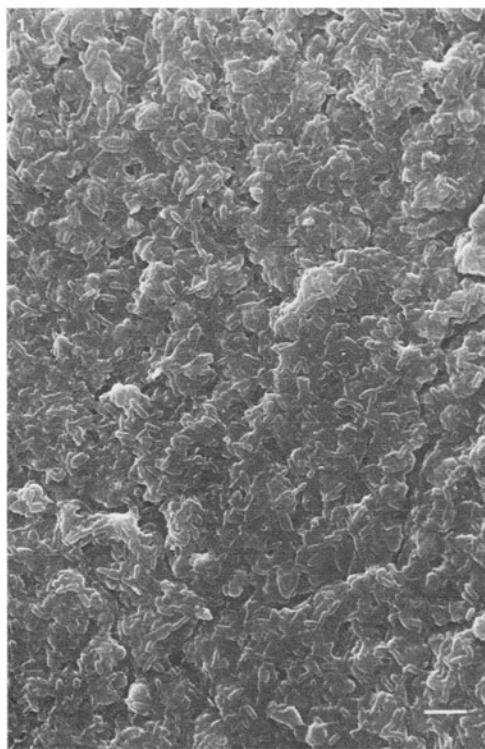


Fig. 5.1. Bacterial colonization and growth on a surface often results in the formation of a biofilm. Biofilm growth allows the component organisms to persist in spite of many adverse environmental conditions (liquid flow and accompanying shear forces, antibacterial agents, low nutrient concentrations, etc.), and can quickly change the chemical and physical properties of the underlying surface. In this case, *Pseudomonas aeruginosa* was allowed to colonize a metal pipe from a heat exchanger for 24 hours. In this short time span, the underlying surface was completely occluded by bacterial growth. Bar represents 5 μm .

Bacteria have adopted a seemingly simple strategy for dealing with these environmental stresses in that they colonize surfaces and form encapsulated communities of cells often referred to as biofilms (Fig. 5.1) (Costerton *et al.*, 1987). This mode of growth enables bacteria to demonstrate remarkable resistance to physical disruption (Reid *et al.*, 1990), predation (Caldwell, 1984, 1987; Lawrence & Caldwell, 1987), antibiotics (Nickel *et al.*, 1985a; Stiekler *et al.*, 1987; Brown *et al.*, 1988; Nichols *et al.*, 1988), disinfectants (Costerton, 1984a; LeChevallier *et al.*, 1988a,b; Millward & Wilson, 1989), and the immune response (Costerton *et al.*, 1983; Costerton, 1984b). In addition, adhesion allows these organisms ready access to insoluble nutrients such as cellulose (Cheng & Costerton, 1980b; Cheng *et al.*, 1981a), and the ability to persist in spite of liquid shear forces such as those present in the urinary tract (McLean *et al.*, 1988) and fast-flowing aquatic environments (Geesey *et al.*, 1978).

5.2.1 Influence of Surface Properties on Colonization

Attachment is the first prerequisite for biofilm formation. Here, an organism must encounter a suitable surface, attach to it, and eventually grow in order to produce a mature biofilm. During the initial attachment process, the chemical and physical characteristics of the surface, and the surrounding medium can greatly influence the relative ease with which a particular bacterium can adhere. In some cases, bacteria are attracted to and attach to a specific target site. This specific adhesive process is quite evident in the medical field, and is one of the major reasons for the localization of some pathogens to particular tissues (Svanborg Edén *et al.*, 1985; Beachey *et al.*, 1988). It has also been shown to be responsible for the specific binding of *Rhizobium* sp. to the roots of legumes during nitrogen fixation (Dazzo & Truehet, 1983). The specificity of bacterial adhesion can be shown experimentally by removing or chemically modifying the putative target molecule(s) or by using a surface analogue with the appropriate molecular target(s). Quite often blood cells are used for this purpose as sugars on their cell surfaces are chemically well defined. Specific bacterial attraction to the erythrocyte cell surface is shown by an agglutination or clumping of the blood cells (haemagglutination) and represents a strong indication that the adhesions expressed by the organism possess a lectin-like form of activity. If no suitable blood cell type exists, other cell types or carbohydrate-coated latex beads may be substituted in the agglutination test. The role of lectins in specific binding can be further verified by a variety of hapten-inhibition studies during which a variety of sugar compounds (mimicking potential target molecules)

are preabsorbed onto the bacterial cells in an effort to block adhesion (Dazzo & Truchet, 1983; Ofek & Perry, 1985; Svanborg Edén *et al.*, 1985; Beachey *et al.*, 1988). The lectins involved in specific bacterial adhesion are not always found on the bacterial cell. In the case of the *Rhizobium* legume symbiosis, lectins on the plant root hair cells recognize the carbohydrate capsule structures of the appropriate strain of *Rhizobium* (Dazzo & Truchet, 1983).

Non-specific adhesion of bacteria to surfaces is very common. As the name implies, this involves adhesion to a variety of surfaces, rather than a particular target site. Even so, a given organism may demonstrate a preference for a particular surface chemistry. The role of surface chemistry as it relates to bacterial adhesion has been an area of increasing study recently. Several investigations have shown that hydrophobic surfaces are more prone to colonization by dental and soil bacteria than are their hydrophilic counterparts (McEldowney & Fletcher, 1986; Pringle & Fletcher, 1986; Humphries *et al.*, 1987; van Loosdrecht *et al.*, 1987; Stenström, 1989). As might be expected, hydrophobic chemical interactions have been shown to play a major role in this type of bacterial colonization. In this case, adhesion can be diminished by the addition of detergents (Marshall *et al.*, 1989; Cowan *et al.*, 1987; Bryant *et al.*, 1984), or enhanced by the addition of cations (Fletcher, 1988) such as Na^+ , Ca^{2+} , La^{3+} , or Fe^{3+} .

Adhesion is not a simple hydrophobic interaction. In an examination of *Streptococcus sanguis* adhesion to hydroxyapatite, Cowan *et al.* (1987) found that while hydrophobic interactions were evident, electrostatic bonding was even more significant. In this case, the addition of the electrolyte, NH_4SO_4 , diminished binding. With the variety of surfaces which are colonized by bacteria, it is likely that the binding chemistry consists of a mixture of hydrophobic, electrostatic, hydrogen, van der Waals, and possibly even covalent bonds. The importance of each type of bond is influenced by the chemical environment of the surface and surrounding fluids, and the physiological status of the organisms.

The physical structure and location of the surface also plays a role in its ability to be colonized. Aquatic microorganisms tend to colonize surfaces more readily in streams than in a pond (Mills & Maubery, 1981). Presumably the higher water flow of the stream carried more organisms past these rocks and also created a stress factor in which these organisms were obliged to adhere in order to persist in this environment. Ferris *et al.* (1989b) established that colonization of rocks in a freshwater stream was inversely proportional to the hardness of the rock surface being colonized. They attributed this to an increased weathering of the softer

rocks which in turn produced a rougher surface more prone to colonization. Baker (1984) demonstrated that colonization of materials in a natural environment such as a stream was enhanced by the roughness of the particular surface. Hydrophobicity in this environment was not as important. Certainly liquid flow rates which are reflected in very high surface shear forces are quite important in determining adhesion. At very high shear forces, the organisms showed an increasing tendency to either not attach or to be swept away. Adherent microorganisms in regions of high shear forces such as the plaque organisms within the mouth must develop very tenacious means of attachment (Christersson *et al.*, 1988, 1989).

While there is ample evidence that physical and chemical properties of surfaces will greatly influence the rate of initial bacterial colonization, we have seen that over time virtually any surface will become colonized (Costerton *et al.*, 1987). There are a number of explanations for this. Certainly, after long periods (24–72 h) of colonization by planktonic bacteria, a monolayer will be formed even in the slowest adhesion systems and subsequent colonization will be influenced by the surface of this monolayer and not that of the original surface. If one attempts to ambush colonizing bacteria by incorporating an antibacterial agent onto a surface, the initial colonizers will be killed. However, should these dead cells remain attached to the surface, future colonizers will only experience and attach to this layer of dead bacteria and not encounter the original surface (Chang & Merritt, 1991).

5.2.2 Bacterial Structures Involved in Adhesion

Bacteria possess a wide variety of mechanisms that allow them to attach to surfaces and persist in various environments. These include the more familiar structures such as fimbriae (pili), adhesins and capsules, as well as the less familiar structures including various holdfast structures, stalks, prostheca, cell wall components and slimes. From a mechanistic viewpoint, the role of these various structures in the adhesion of bacteria to a variety of surfaces has been the subject of a large number of studies recently. Of equal or greater importance is the concept of overall microbial strategies during colonization. We now turn our attention to these two ideas.

Microbial colonization requires that the organism(s) encounter an appropriate surface. The target may be a source of potential nutrition such as a food particle in the digestive tract of a ruminant (Cheng & Costerton, 1980b; Cheng *et al.*, 1981a) or an inanimate object such as a polystyrene surface (McEldowney & Fletcher, 1986) which the organism

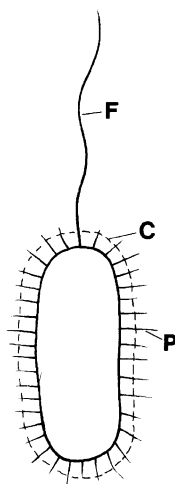


Fig. 5.2. Schematic representation of a bacterial cell showing the structures most responsible for initial surface adhesion. Motility usually arises from the propeller-like action of one or more flagella (F) (singular – flagellum). Adhesion generally occurs through the binding of pili (P) and/or the capsule (C) (also called the glycocalyx) to the surface. The capsule is a highly hydrated polysaccharide or polypeptide structure which can extend up to 10 μm from the cell surface. It represents the most significant structure in a mature biofilm.

would use as an anchor. Should some distance separate the organism from its particular target, then the ability to swim through a liquid to the target represents an obvious advantage. The most common bacterial structures involved in motility are flagella (singular – flagellum). These are proteinaceous whip-like structures, anchored in the cell membrane, which extend some distance from the cell surface (Fig. 5.2) (Beveridge, 1981). Motility arises when these structures are rotated by the cell. This overall process closely resembles the action of a propeller driving a boat (Larsen *et al.*, 1974).

In an elegant experiment, Korber *et al.* (1989) demonstrated that motility greatly enhanced the ability of *Pseudomonas fluorescens* to colonize glass surfaces in a laminar flow cell. In this experiment, motile and non-motile strains of *Ps. fluorescens* were added to a glass laminar flow cell and subjected to a flow velocity above ($120 \mu\text{m sec}^{-1}$) and below ($8 \mu\text{m sec}^{-1}$) the organism's maximum motility rate ($85 \mu\text{m sec}^{-1}$). In both cases, motility increased the ability of the organisms to adhere, enhanced dispersion over the surface, and increased the rate of colonization upstream, against the laminar flow. In other studies, positive chemotaxis, where the organisms are attracted to chemical(s) on a particular surface, has also been implicated in bacterial adhesion (reviewed in Finlay &

Falkow (1989)). Aside from playing a role in motility and chemotaxis, flagella themselves have also sometimes been directly implicated in adhesion (Marshall *et al.*, 1971; Belas & Colwell, 1982; Lawrence *et al.*, 1987). The long extension of flagella from the bacterial cell often enables these proteinaceous structures to come in contact with surfaces and to tether the cell to the surface.

The two major structures of bacteria most often associated with adhesion are fimbriae (also called pili), and the capsule (Duguid *et al.*, 1955; Fader & Davis, 1980; Costerton *et al.*, 1981*a,b*, 1987; Eighmy *et al.*, 1983; Doig *et al.*, 1987, 1988; Finlay & Falkow, 1989). Fimbriae are proteinaceous rods which extend out from the cell surface (Fig. 5.2). They are smaller structures than flagella having a typical dimension of 0.2–2 μm in length by 7 nm diameter (Eisenstein, 1987). The corresponding dimensions for flagella are 5–10 μm in length and 20 nm in diameter (MacNab, 1987). The fimbriae of *Escherichia coli* consist of repeating subunits of a single polypeptide of 17 kDa molecular weight. Biochemical analysis of this peptide subunit (reviewed in Eisenstein (1987)) shows it to be largely hydrophobic and to be acidic (pI, *ca.* 3.9). These two characteristics may help to explain the role it has in adhesion in that hydrophobicity and electrostatic bonding are major chemical components of this process (reviewed earlier). Many fimbriae of Gram negative organisms act as adhesins in that they bind very specifically to receptor molecules on the target surface often in a lectin-like manner. Indeed, pili and their associated adhesins have been shown to be a major virulence factor in many Gram negative pathogens such as *E. coli* in that these organisms bind specifically to tissues containing receptor molecules such as galactose and mannose containing residues in the urinary tract (Hagberg *et al.*, 1986; Eisenstein, 1987).

The other major structure of bacteria associated with adhesion is the capsule (Fig. 5.2). This is a highly hydrated extracellular polysaccharide or polypeptide polymer which may extend a considerable distance from the cell surface (Troy, 1973; Sutherland, 1977; Costerton *et al.*, 1981*b*). Capsules vary widely in their chemistry but quite often contain a number of components such as uronic acids, amino acids, phosphate, or sulphur groups, in addition to neutral sugars (Sutherland, 1977; Kenne & Lindberg, 1983). Most of these polymers are therefore anionic as evidenced by their staining with cationic ruthenium red and their ability to bind metals (McLean & Beveridge, 1990) (Fig. 5.3). As is the case with fimbriae, the anionic and to a lesser extent, hydrophobic character of these polymers suggests that electrostatic and hydrophobic bonding are involved in the attachment of these structures to surfaces. In addition

one would expect a role for hydrogen bonding in some instances due to the carbohydrate nature of these polymers.

In a study using freshwater bacterial isolates, Allison and Sutherland (1987) found that initial adhesion to surfaces occurred independently of capsule production. However, it was required for subsequent growth of colonized cells into mature biofilms. There are numerous instances where capsule and/or fimbriae production have been exclusively or predominantly implicated in adhesion (see for example Costerton *et al.* (1981a); Bryant *et al.* (1984); Costerton *et al.* (1987); Doig *et al.* (1987, 1988)).

A number of other cell surface components have been implicated in adhesion. These include Gram positive cell wall polymers such as lipoteichoic acids (Kenne & Lindberg, 1983). These polymers are thought

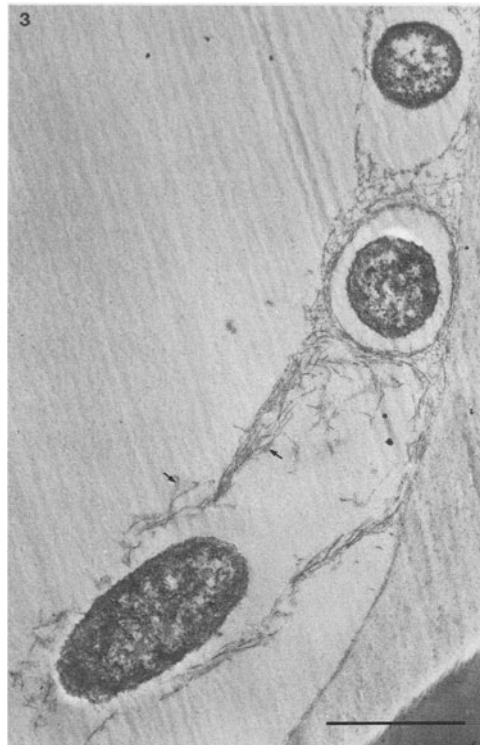


Fig. 5.3. Bacteria in an oligotrophic alpine stream adhere readily to available surfaces including experimental plastic disks. Dissolved minerals (iron, calcium, silicon, etc.) can precipitate onto bacterial structures such as the capsule. This results in the formation of minerals including iron hydroxides and silicates (arrows). Figure taken from Lappin-Scott & Costerton (1989) and reprinted with the permission of the copyright holder, Harwood Academic Publishers GmbH, Montreux, Switzerland. Bar represents 0.5 μm .

to act as adhesins in that they generally bind specifically to receptors on tissue surfaces (Op den Camp *et al.*, 1985; Aly & Levit, 1987). Other bacterial cell surface components such as outer membranes (Jacques *et al.*, 1988), regularly structured protein surface arrays, Streptococcal M proteins (Poirier *et al.*, 1989; Caparon *et al.*, 1991), etc. may be involved in adhesion in special instances. Other unusual stalked organisms such as *Caulobacter* sp. employ a holdfast mechanism for adhesion (Staley & Fuerst, 1989). With these exceptions, the capsule and fimbriae represent the major mechanisms of bacterial adhesion to surfaces.

5.2.3 Strategies and Mechanisms of Biofilm Formation

Biofilm formation can be essentially summarized into several distinct phases (Marshall *et al.*, 1971; Costerton *et al.*, 1987). Initially microorganisms encounter a target surface, bind reversibly, then irreversibly, and then grow into small clusters of cells (referred to as microcolonies). These microcolonies continue to grow and spread until a confluent biofilm forms over the surface. Once a biofilm reaches a certain stage of maturity, it begins to shed planktonic cells. These shed cells act as pioneers in that they are free to disperse and eventually colonize new surfaces.

If we examine colonization of a surface by an organism such as *Pseudomonas aeruginosa* only at specific time points, this behaviour appears to be the case (Fig. 5.4) (Costerton *et al.*, 1987). The true complexity of colonization strategy is only realized when microorganisms are subjected

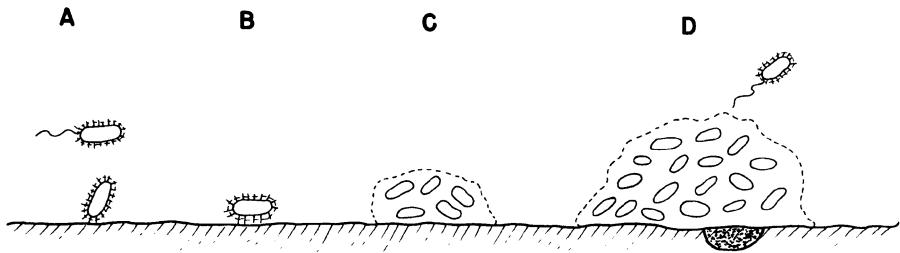


Fig. 5.4. Generalized schematic of bacterial biofilm formation. Planktonic cells in the fluid phase attach reversibly (A), then irreversibly (B) to a suitable surface (Marshall *et al.*, 1971) by means of pili and/or capsule. Further growth (C) results in the formation of an encapsulated microcolony. As the microcolony grows into a biofilm (D) some cells are released into the fluid phase and can then colonize new surfaces. In spite of the surrounding fluid characteristics (such as the presence of dissolved O_2), biofilms and microcolonies often generate a chemically distinct microenvironment which may lead to activities such as anaerobic corrosion of the surface (darkened region).

to continuous scrutiny. For practical purposes, this can only be accomplished by light microscopy coupled with computer-enhanced image analysis (Caldwell & Germida, 1985; Caldwell, 1985; Caldwell & Lawrence, 1988). Light microscopy, especially phase contrast or dark field microscopy, allows the visualization of individual, living, unstained microorganisms. Another related technique is the use of interference reflection microscopy (IRM) (Marshall *et al.*, 1989). IRM allows cell-surface separation distances from 0 to 100 nm to be estimated since the approach of bacterial cells to a glass surface induces interference in reflected light leading to an apparent darkening of the cell as the distance from the cell to the glass approaches zero (Marshall *et al.*, 1989). It allows the direct examination of unstained living material, yet allows an estimation of the distance between the adhering organisms and the surface to which they are attached (Fletcher, 1988; Godwin *et al.*, 1989; Marshall *et al.*, 1989). The addition of computer enhanced image analysis to these optical techniques allows microbial activity such as adhesion, detachment, growth, and spacing of individual cells and clumps of cells to be evaluated (Caldwell & Lawrence, 1988). Confocal scanning laser microscopy (CSLM) is an exciting new development in microscopy which employs a laser light source and highly refined optics. CSLM enables investigators to obtain optical thin sections through specimens with a resolution of $<1\ \mu\text{m}$ (Brackenhoff *et al.*, 1988). Through the use of appropriate fluorescent probes (Haugland, 1992) and computerized image analysis, CSLM allows one to construct three-dimensional maps of chemical microenvironments within biological materials such as cells and biofilms (Caldwell *et al.*, 1992). The major limitations of these techniques are that studies are limited to materials that readily transmit light, such as glass, and that once the adherent cells form a thick biofilm, a reduction in light transmission drastically diminishes resolution. In spite of these difficulties, we are now beginning to realize the tremendous diversity of colonization strategies that bacteria exhibit.

A wide variety of other techniques are available for measuring bacterial adhesion (summarized in Costerton *et al.* (1987)). The most basic technique used involves standard plate counts whereby bacteria are dislodged from a surface by scraping with a sterile scalpel blade or gentle sonication, agitated to break up cell clumps, and plated onto a suitable medium. During this, one assumes that all organisms can be separated and easily grown. As these assumptions are never completely met, microbial numbers are generally underestimated by this technique. Other techniques involve counting adherent bacteria directly using a viable stain (Haugland, 1992) coupled with epifluorescence microscopy (Ladd *et al.*,

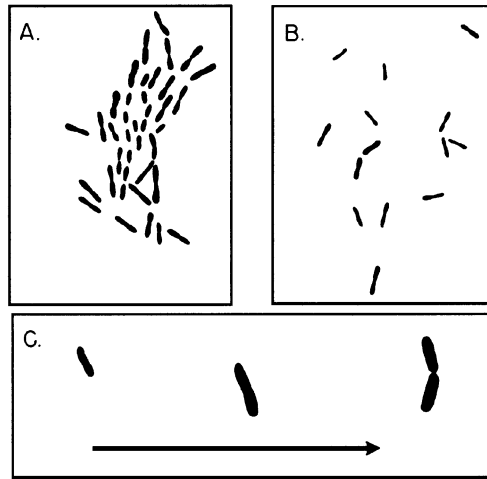


Fig. 5.5. Differences in colonization strategy can alter the appearance of microcolonies. Note the differences between packing (A) and shedding (B). Spreading of attached bacteria would result in a similar appearance to that of shedding (B). Rolling (C) involves loose attachment of an organism to a surface where it is moved along in the direction of flow (arrow). For details refer to text and also to references (Lawrence & Caldwell, 1987; McLean & Nickel, 1991). Figure reprinted from McLean & Nickel (1991) with permission of the copyright holder, Churchill Livingstone Medical Journals, Edinburgh, Scotland.

1985), measuring radiolabelled bacteria, measuring a biochemical component of the attaching organisms such as diaminopimelic acid (Beveridge, 1981), or measuring cellular metabolism using a radiolabelled substrate (Ladd *et al.*, 1987).

If bacteria adapted to colonization are allowed to colonize a glass laminar flow cell, several different types of cell movement strategies are seen (Fig. 5.5). Lawrence and Caldwell (1987) noted that once organisms from a freshwater stream colonized glass surfaces four distinct types of cell movement designated as 'packing', 'spreading', 'shedding', and 'rolling' were seen. Packing involved colonization by a mother cell. Upon growth and cell division, the daughter cells remained in tight proximity to the original colonization site thus forming a tightly packed microcolony. In the spreading manoeuvre, daughter cells remained attached, but moved some distance from each other producing a diffuse microcolony.

Colonies exhibiting shedding involved an attached mother cell which continuously shed daughter cells that then migrated to new locations. The fourth type of movement seen, rolling involved attached cells which did not firmly adhere, but rather rolled or tumbled along the surface in

the direction of the flow. Each form of colonization behaviour appeared to be associated with and be a unique characteristic of a specific species. In other studies, *Pseudomonas fluorescens* has been shown to colonize exclusively via a packing strategy (Caldwell & Lawrence, 1986; Lawrence *et al.*, 1987), and *Proteus mirabilis* via a shedding strategy (McLean *et al.*, 1991; McLean & Nickel, 1991). One can speculate that an organism such as *Ps. fluorescens* would be able to colonize and rapidly cover a given surface in a natural environment thus giving it a competitive advantage over other organisms. In the human urinary tract, colonization of bladder epithelial cells induces them to slough into the urine (Aronson *et al.*, 1988), so the shedding strategy of *Pr. mirabilis* whereby progeny continuously colonize other surfaces would aid in the persistence of this organism in this environment (McLean & Nickel, 1991).

Colonization of surfaces is often enhanced as their chemistry is altered and when a number of other organisms are present. This is most evident in the mouth. Here mineral surfaces (teeth) are continuously coated by a pellicle of salivary proteins, which can affect colonization (Rosan *et al.*, 1985; Pratt-Tepstra *et al.*, 1989). Primary colonizers of freshly cleaned teeth are predominantly *Actinomyces viscosus*, *Streptococcus sanguis* and related bacteria (Ellen *et al.*, 1988; Kolenbrander, 1988). Other organisms do not colonize teeth as readily, however they often specifically recognize and bind quite readily to the primary colonizers and are thus able to colonize these surfaces (Ofek & Perry, 1985; Mergenhagen *et al.*, 1987; Lilje-mark *et al.*, 1988). This process is now referred to as coaggregation since mixing two complementary strains of bacteria such as sucrose-grown cells of *Streptococcus mutans* and *A. viscosus* induces a clumping reaction (Gibbons & Nygaard, 1970; Kolenbrander, 1988). A number of studies (Kolenbrander, 1988) have shown this reaction to consist of a series of lectin-like reactions between accessible surface constituents in bacterial partners, and may be responsible in part for the spatial distribution of microorganisms within the oral environment. Other physiological features such as capsule synthesis by *Strep. mutans* are also involved in dental plaque colonization (Koga *et al.*, 1988). Synergistic relationships between bacterial species has also been reported during adhesion in other environments (Chang & Merritt, 1991), however, the importance of this is quite variable, and the mechanisms are at present unknown (McEldowney & Fletcher, 1987).

From the aforementioned studies, it is evident that bacteria possess very diverse mechanisms and strategies that enable them to colonize virtually any type of surface. If a particular surface contains an antibacterial compound it can still be colonized by killed microorganisms, or chemically

altered by the deposition of organic material of bacterial or other origin. Eventually incoming microorganisms will only encounter the chemically or bacterially modified surface, and be able to colonize. In this way the antibacterial nature will have been circumvented. From a practical viewpoint bacterial colonization and biofilm formation is ubiquitous – the problem is not so much inducing bacterial colonization, but how to prevent it. This is an area of active research, and will be addressed further.

5.3 DIVERSITY OF BIOFILMS IN NATURE

Surface adhesion and biofilm formation represent a fundamental strategy of bacterial growth in nature. Consequently it is not surprising to find that bacteria predominantly grow as biofilms in widely diverse environments so as to gain access to available nutrients, and protect themselves from predation and antimicrobial agents.

5.3.1 Natural Environments

In natural ecosystems, the sessile biofilm population of heterotrophic bacteria was found to exceed the planktonic population by 3–4 orders of magnitude in oligotrophic alpine streams (Geesey *et al.*, 1978) (Fig. 5.3) and 200-fold in sewage effluent (Costerton *et al.*, 1987). The only natural community of microorganisms which are predominately freeswimming or planktonic are the photosynthetic, unicellular microorganisms in lakes (Caldwell & Tiedje, 1975*a,b*; Caldwell, 1977). By not stacking themselves as layered communities upon submerged surfaces, these organisms gain optimum access to light at the water surface (Caldwell, 1977). Significant numbers of particle-associated and planktonic organisms are also found in the open ocean and in deep terrestrial aquifers (Ghiorse & Wilson, 1988). In these environments, organisms are under advanced stages of starvation as readily metabolizable carbon is virtually non-existent. Consequently these organisms survive by drastically altering and slowing down their metabolism (Kjelleberg *et al.*, 1987), and many energy-requiring biosynthetic reactions, notably capsule production (essential for biofilm growth), are stopped. The capacity of these organisms to survive without metabolizable carbon is phenomenal with estimates ranging up to 1000–10000 years (W. C. Ghiorse, personal communication). Yet even here many organisms adhere readily to available surfaces (Bright & Fletcher, 1983; Eighmy *et al.*, 1983; Pringle & Fletcher, 1986; Kjelleberg *et al.*, 1987; Iriberry *et al.*, 1990).

One would expect that heterotrophic organisms would be the predominant benefactors from a strategy of biofilm formation in that by adhesion, they would gain access to insoluble carbon sources such as cellulose. It is therefore quite interesting that biofilm formation has been described in at least one instance involving an autotrophic organism (Kieft & Caldwell, 1984). One often sees other autotrophic organisms most notably algae readily adhering to surfaces.

5.3.2 Industrial Environments

The nutrient-rich aquatic environments and large surface areas common to many industrial processes stimulate the formation of biofilms. In many instances this has proved to be quite costly in that biofilm formation causes such problems as microbial plugging in oil injection wells (Shaw *et al.*, 1985; Cusack *et al.*, 1987; McKinley *et al.*, 1988; Lappin-Scott & Costerton, 1989), reverse osmosis membranes (Ridgway *et al.*, 1983; Payment, 1989), industrial filters (Costerton, 1984a; Camper *et al.*, 1985; Lytle *et al.*, 1989), heat exchangers (Costerton *et al.*, 1987) and water treatment plants (LeChevallier *et al.*, 1987, 1988a). Biofilm formation often represents a major nuisance in that filtration capacity, efficiency of oil injection wells, and heat exchange capability may be greatly diminished or even eliminated. When conditions are suitable, anaerobic microenvironments develop which allow the growth of sulphate-reducing bacteria and the initiation of associated anaerobic corrosion (Costerton, 1984a; Hamilton, 1985). In addition, biofilms in water and waste-water treatment plants usually represent reservoirs of potentially pathogenic bacteria such as *Klebsiella*, *Salmonella*, *Yersinia*, and other enteric pathogens (Camper *et al.*, 1985; LeChevallier *et al.*, 1987, 1988a). The industrial cost of biofilm fouling is astronomical in that millions of dollars are spent yearly on biocides, drilling of new injection wells, and equipment replacement.

Traditionally biocides have been somewhat indiscriminately applied in industrial pipelines, and the bacterial population has been monitored through the use of standard plate counting techniques of the fluid phase. While planktonic organisms are rapidly eradicated by this treatment, very little effect is seen against biofilms. The usual result is that anaerobic corrosion and/or microbial plugging continues in supposedly 'sterilized' industrial systems. This led to the development of the Robbins biofilm sampling device (Ruseska *et al.*, 1982). The Robbins device is a length of pipe (usually 4 feet long, made from brass). Its distinguishing feature is that sections of the pipe wall, mounted on studs, can be easily removed

and replaced. Biofilms which colonize the inside of pipes (including the Robbins device) can be easily sampled by removing the sampling plugs, dislodging the biofilm by scraping with a razor blade or scalpel, and plating on appropriate microbiological media. Incorporation of the Robbins device into industrial aquatic pipelines has allowed the continuous monitoring of microbial corrosion and the assessment of the efficacy of biocide treatment (Ruseska *et al.*, 1982; Costerton, 1984a). Other treatments which have been successfully employed to alleviate biofilm associated biofouling include the use of a slow freezing cycle to induce large ice crystals within the biofilms of heat exchangers (Costerton, 1990). Thawing of these crystals then leads to the complete removal of the biofilm. In oil formations, microbial plugging is most successfully controlled through the administration of sodium hypochlorite (bleach) to kill and dissolve organic biofilm matter, followed by acid to dissolve small particulate inorganic minerals or fines (Cusack *et al.*, 1987). The resistance of biofilms to antimicrobial agents is well-documented. Recently it has been shown that this resistance can be overcome if a biofilm is exposed to an antimicrobial agent in the presence of a small electrical field (Blenkinsopp *et al.*, 1992). This new discovery is highly significant, given the enormous expenditure in the industrial and medical fields in controlling biofilms.

There are many positive aspects of industrial biofilm formation. Waste-water treatment systems have made extensive use of biofilms in trickling filters, and other treatment protocols. In trickling filters, sewage is sprayed onto beds of loose gravel. Biofilms rapidly form on these surfaces, and due to the circulation of air through the gravel and the large surface area, they can aerobically degrade large amounts of organic matter (Hamilton, 1987). Biofilms under more anaerobic conditions have been used for methane production (Eighmy *et al.*, 1983; Eighmy & Bishop, 1984; LeChevallier *et al.*, 1987) and sulphate reduction (Nielsen, 1987). In addition to carbon mineralization, biofilms and their anionic bacterial cell wall and capsular components are also able to assist in the recovery of trace metals from mining and other industrial wastes (Olson, 1986; Ferris *et al.*, 1989a). The use of biofilms and bacterial surface components has tremendous potential for use in heavy metal pollution control and biologically catalyzed mineral formation (biomineralization) (Ferris *et al.*, 1986; Mann, 1988; Beveridge, 1989; McLean & Beveridge, 1990).

When an oil well is first drilled, gas pressure within the reservoir results in approximately 8–30% of the total oil being forced to the surface. In order to increase oil recovery when the gas pressure is depleted, water is pumped into the reservoir through injection wells and thus dis-

places the oil to the production wells. During water injection, 'fingering' occurs as the injection water will preferentially be driven through high permeability zones in the reservoir bypassing the residual oil in place (MacLeod *et al.*, 1988). The application of biofilm technology to this problem shows great promise in enhancing oil recovery from these reservoirs by enabling selective plugging of these high permeability zones. In a series of experiments, a strain of *Klebsiella pneumoniae* was isolated from the produced water in an oil well. Injection of the vegetative cells into model rock cores results in the formation of a thin biofilm 'skin' which forms predominantly in areas of high permeability. Penetration of these organisms into the rock cores is enhanced if the cells are first starved (and thereby inhibited in capsule production). The resulting ultramicrobacteria (UMB) (Fig. 5.6) can then be pumped into the formation where

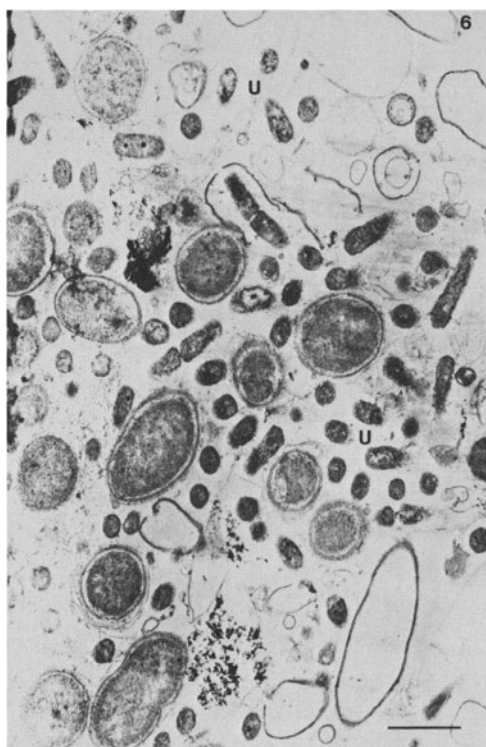


Fig. 5.6. Starvation of *Klebsiella* sp. isolated from produced water in oil fields results in the formation of ultramicrobacteria (U). Ultramicrobacteria do not produce capsule nor form biofilms under starvation conditions, yet upon nutrient resuscitation recover both of these capabilities (Lappin-Scott *et al.*, 1988a). Bar represents 1 μm .

they follow the injected water into regions of high permeability. After resuscitation with nutrients (Lappin-Scott *et al.*, 1988a), these starved cells rapidly recover and produce a relatively thick viscous biofilm (Lappin-Scott *et al.*, 1988b; MacLeod *et al.*, 1988) (Fig. 5.7) that plugs the high permeability water breakthrough zones and facilitates additional oil recovery.

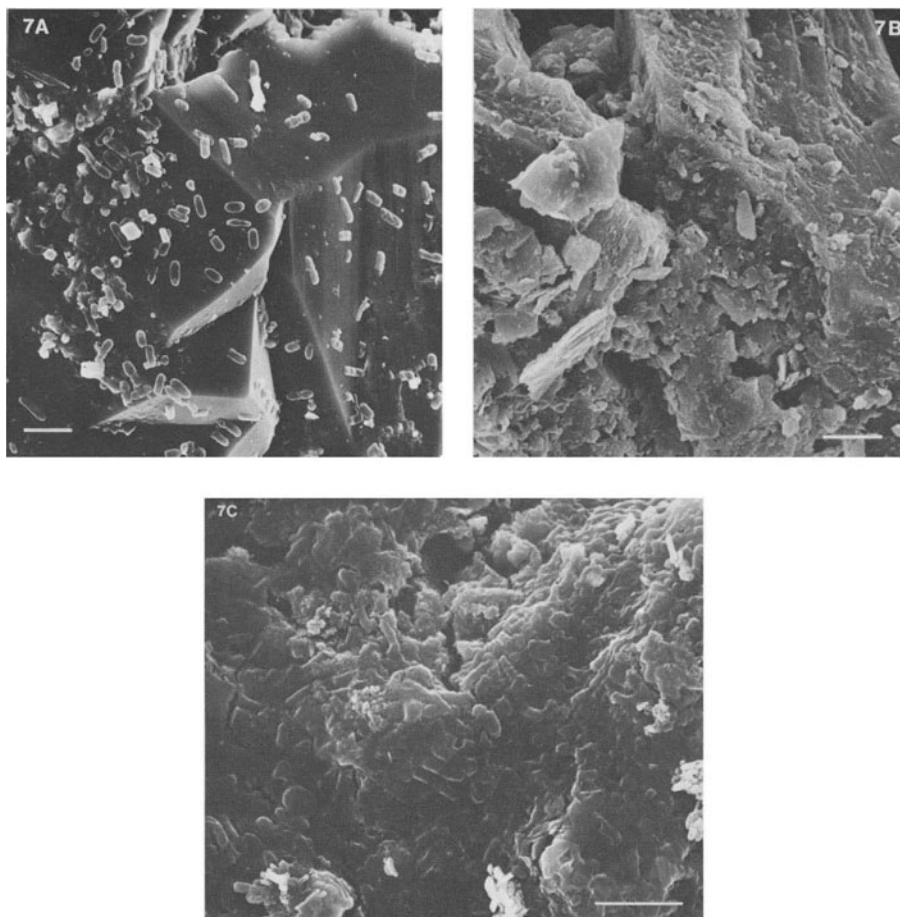


Fig. 5.7. Plugging of model sandstone rock cores using ultramicrobacteria (UMB). Introduction of UMB allows their penetration deep into the sandstone (7A). Exposure to nutrients results in rapid production of capsule (7B) and eventual biofilm formation (7C) which can then plug the high permeability zone of the oil-bearing rock formation. Bar represents 5 µm.

5.3.3 Medical Importance

Biofilm formation is an important bacterial strategy in the development of many chronic infections (see for detailed review Costerton (1984*b*); Gristina & Costerton (1985); Costerton *et al.* (1987); Jacques *et al.* (1987); Dart & Seal (1988)). Although this environment is obviously different from the industrial, aquatic and terrestrial habitats addressed above, organisms still face such basic stresses as predation (by leucocytes), competition with the host and other flora for nutrients, adverse chemical conditions (such as antibiotics and disinfectants) and nutrient limitation (notably iron). It is therefore not surprising that bacteria often employ the same basic strategy of biofilm growth in order to persist in

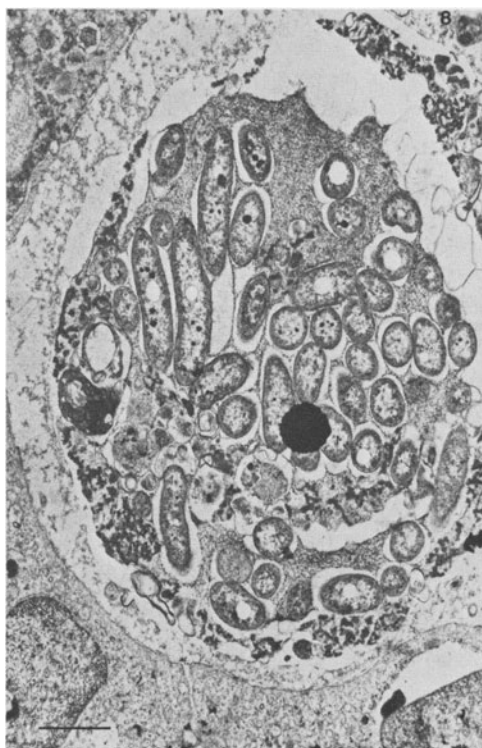


Fig. 5.8. Microcolony of *Pseudomonas aeruginosa* within the lung of a rat used in the study of cystic fibrosis. As a consequence of their microcolony mode of growth, these organisms can easily withstand the host immune system and large dosages of antibiotics. See Cochrane *et al.* (1988) for details. Bar represents 1 μm .

these pathogenic environments (Fig. 5.8). As in the case of the industrial environment, biofilm growth presents both its advantages and disadvantages.

Advances in modern medicine have resulted in the development of a wide variety of prosthetic devices and instrumentation which are implanted into various regions of the body. Although these developments have resulted in considerable reduction of morbidity and mortality, they do present an 'immunologically inert' surface that can be readily colonized. Biofilms which form on these surfaces act as a nidus of infection which may ultimately develop into a systemic, possibly life-threatening infection (for review see Jacques *et al.* (1987)). Probably the most prevalent nosocomial or hospital-acquired infections in North America are urinary tract infections associated with the use of urinary catheters. Using a modification of the Robbins device, Curtis Nickel and his colleagues have shown that the antibiotic resistance of catheter associated biofilm bacteria is considerably higher (up to 1000 fold) than their planktonic counterparts (Nickel *et al.*, 1985*b*). The cost of biofilm-associated infections is considerable, and has led to the development of active

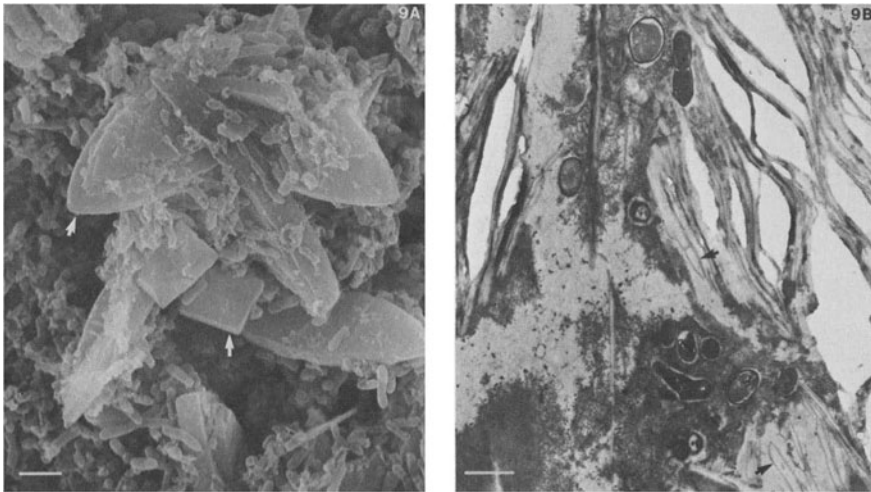


Fig. 5.9. Mineralization within biofilm-associated infections results in the formation of several types of medically important calculi. Two examples shown here are struvite ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$) calculi (9A) which form in the urinary tract from *Proteus mirabilis* infection, and calcium bilirubinate calculi (9B) that form in the gall-bladder from β -glucuronidase-producing *Escherichia coli* infection. Crystals are indicated by arrows. Bar represents 2.5 μm in 9A, 1 μm in 9B. Figure 5.9B was provided courtesy of Dr Joe Sung, University of Calgary.

research programs by a number of companies to develop polymers which are able to resist colonization. We have successfully used the modified Robbins device to examine the resistance of many experimental polymers to bacterial colonization and biofilm formation (Nickel and Costerton, unpublished observations; McLean and Nickel, unpublished observations) and no material has yet shown a total inherent resistance to this inexorable natural process.

Biom mineralization involving biofilms also has its parallel in the medical field in the formation of brown pigment gall stones (Leung *et al.*, 1989), dental calculi (Driessens *et al.*, 1985), and struvite ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$) urinary calculi (McLean *et al.*, 1988). In all three cases, mineralization is preceded by biofilm formation. The physiological activities of the bacteria within these biofilms alter the chemistry of the surrounding urine, saliva or bile resulting in the formation of precipitates. Formation of these crystals within the gel matrix of the biofilm ensures that they become cemented together and ultimately grow to become clinically significant calculi (Fig. 5.9). In the case of struvite calculi, we have shown that alkaline microenvironments created within *Pr. mirabilis* biofilms enable acid-labile struvite crystals to form in acidic conditions (Clapham *et al.*, 1990; McLean *et al.*, 1991).

The most striking beneficial aspect of biofilm formation in the medical and agricultural environments is perhaps the role of bacteria in digestive processes. In the absence of bacteria, animals are unable to digest complex carbohydrates such as cellulose. Symbiotic bacteria are intimately associated with all animals including man, and particularly in herbivores, play an integral role in the digestion of feed, most notably cellulose (Cheng & Costerton, 1980a). K.-J. Cheng and colleagues have shown that microcolonies and consortia enable digestive tract microorganisms to metabolize feed more efficiently in that the organisms gain access to their insoluble substrate and essential physiological interactions between component organisms are facilitated (Cheng & Costerton, 1980b; Cheng *et al.*, 1981a) (Fig. 5.10).

Examinations of human and animal tissues have also shown that autochthonous bacterial populations develop on the tissue surfaces of many organs (Cheng *et al.*, 1979, 1981b; Cheng & Costerton, 1980a; Costerton *et al.*, 1983; Jacques *et al.*, 1986) that are exposed to environmental sources of microorganisms (mouth, oropharynx, vagina, etc.). Natural biofilm populations often play a pivotal role in the resistance of these organs to invasion by pathogens (Chan *et al.*, 1985; Reid *et al.*, 1988) and in many instances, their maintenance in good order is assiduously cultivated (Cheng *et al.*, 1979; Reid *et al.*, 1988).

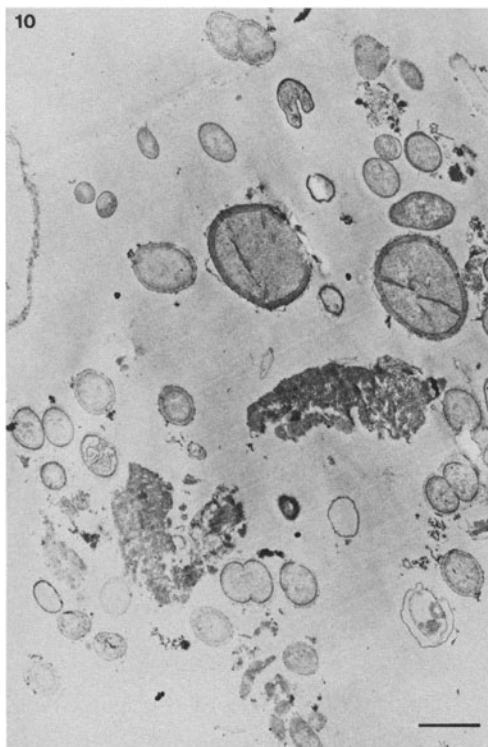


Fig. 5.10. Metabolic activities by natural populations of bacteria are often a result of cooperative (synergistic) metabolic interactions between several different organisms. In this micrograph are shown examples of the diverse microorganisms responsible for the digestion of cellulose and eventual production of volatile fatty acids in the digestive tract of cattle. During cellulose digestion, these mixed cultures adhere to feed particles and to each other, forming consortia in order to facilitate interbacterial metabolic reactions. See text and Cheng & Costerton (1980*b*), Cheng *et al.* (1981*a*) for details. Bar represents 1 μm . Micrograph was provided courtesy of Dr K.-J. Cheng, Agriculture Canada Research Station, Lethbridge, Alberta, Canada.

5.4 BIOFILM COMPOSITION

The predominant feature of any biofilm community is the large quantity of extracellular polysaccharide or polypeptide capsular material within which the organisms are encased (Costerton *et al.*, 1987). The quantity and composition of these polymers reflect the physiology and composition of the organisms within the community. Capsule synthesis and composition varies quite widely among bacteria and other organisms (Sutherland, 1977, 1985, 1988; Kenne & Lindberg, 1983; Whitfield,

1988). Various strains of a given bacterial species generally show a wide diversity in the quantity and composition of polymers produced. Recently, White and coworkers showed that analytical techniques such as infrared spectroscopy could be used to analyse biofilm polymers and in so doing help to determine the taxonomic composition of these bacterial communities (Nichols *et al.*, 1985).

We are only beginning to realize that the vast majority of bacteria in nature produce extracellular polymers. Early observations that some types of bacteria are non-encapsulated have been shown to be artifacts of the growth of these organisms in rich laboratory culture (Geesey *et al.*, 1978; Costerton *et al.*, 1981*b*, 1987; Chan *et al.*, 1984; Costerton, 1984*a*; Beynon *et al.*, 1992). Bacterial capsule production is a highly regulated physiological activity, especially in that it requires a considerable quantity of energy. If a particular organism is in the presence of a rich supply of nutrients such as a laboratory growth medium, capsule production will not be necessary for the survival of the organism. Switching off capsule production will therefore enable the metabolism of the cell to be more directed toward cell growth and division. The net result is that these non-encapsulated organisms are able to quickly overgrow their capsule-producing counterparts (Costerton *et al.*, 1987).

The prevalence of encapsulated bacteria in nature suggests that extracellular polymer production is vital to the survival of these organisms. As this often represents the outermost layer of the cell, it is with this region that bacteria first make contact with their environment. The anionic nature of many capsules is reflected in their affinity for cations such as metals (McLean & Beveridge, 1990; McLean *et al.*, 1990, 1992) and antibiotics such as tobramycin (Nichols *et al.*, 1988; Anwar *et al.*, 1989). In this aspect they serve as an ion exchange resin. One would also expect that anionic capsules might repel penetration by uncharged or anionic compounds. As a consequence, the bacterial cell surface will therefore only experience a greatly reduced concentration of a given toxic cationic compound. Conversely, should a cationic compound such as Mg^{2+} represent a nutrient, the capsule can act as a reservoir whereby Mg^{2+} is absorbed during periods of abundance and released to high affinity transport systems of the cell during periods of starvation.

Extracellular polymers represent the most common feature of biofilms. In its simplest form a biofilm will consist of a surface-adherent community of one or more species of prokaryotic and/or eucaryotic cells which is largely immobilized in an amorphous glycocalyx matrix. Three-dimensional examination of living biofilms by confocal scanning laser microscopy has shown that extracellular material can account for as

much as 73–98% of the biofilm volume. Bacterial cells generally grow as discrete microcolonies within the biofilm matrix (Lawrence *et al.*, 1991). At any time there will be a number of large and small molecules present in the matrix introduced from the resident population through cell metabolism or cell death and lysis, or introduced from the liquid phase. Interestingly many biofilms found in nature become encrusted with a wide variety of minerals that are not found in the liquid phase or within the organisms. These minerals may arise as a consequence of biofilm metabolism or non-biological chemical reactions of bound metals. Examples of metabolically induced biofilm mineralization are the production of struvite (Fig. 5.9A) in urinary tract biofilms (McLean *et al.*, 1988) and calcium bilirubinate in gall-bladder biofilms (Fig. 5.9B) (Leung *et al.*, 1989). Conversely, metabolic processes are not involved in the nucleation of several silicates and other types of minerals by bacteria in aquatic environments (reviewed in Beveridge (1989)). In a study of mine tailings ponds, Grant Ferris and Terry Beveridge showed that biofilm bacteria growing on surfaces suspended into these ponds could bind a wide variety of metals (Ferris *et al.*, 1989a). The net result of this metal binding was the formation of a number of minerals including iron ferrihydrite ($\text{Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}$) (an example of which is shown in Fig. 5.3). The most commonly seen example of metal binding by biofilms is the slimy rust coating that builds up along surfaces in a wide assortment of water containers such as toilet tanks. This is due to the sorption of dissolved iron from water by the bacterial biofilm and its oxidation into rust-coloured ferric hydroxide. The passive sorption of metals from solution by bacteria has implications far beyond the scope of microbiology. In a large number of studies Ferris, Beveridge and Fyfe reviewed in Beveridge (1989) have shown that various cellular constituents of bacteria and bacterial biofilms may in fact be largely responsible for the formation of several clays and minerals of geological importance. Future developments in this area may eventually lead to the use of bacteria and bacterial biofilms in the commercial reclamation of metals from mines and industrial wastes, and to the production of industrially important minerals (McLean & Beveridge, 1990).

5.5 BIOFILM ECOLOGY

For a particular species to survive, members must be able to obtain sufficient nutrition, grow, and reproduce. In addition, there must be mechanisms whereby at least some organisms can survive periods of stress

(including starvation, adverse environmental conditions and predation). The obvious price of a species' failure to meet these demands is extinction. Bacteria are no different in these basic requirements from other organisms. As a group bacteria have been enormously successful in that they represent the earliest and most ubiquitous organisms to have colonized the earth. Their range of habitats is truly impressive in that they survive and often grow in regions of extreme acidity, alkalinity, temperature, pressure, radiation, salinity, osmotic stress, and harmful chemicals (Alexander, 1971). Bacteria are also found in association with higher forms of life where they can exhibit a pathogenic, commensal, or symbiotic relationship with their host. In many of these environments, adequate nutrition is often not readily accessible and so microorganisms have developed elaborate strategies including adhesion to surfaces and growth as biofilms to circumvent these problems as well as to deal with the ecological stresses of competition and predation.

5.5.1 Role in Persistence and Nutrition

If we examine oligotrophic ecosystems such as fast-flowing streams, adherence to surfaces allows organisms to persist. Dissolved substances in aqueous environments tend to concentrate on surfaces – a process which is often called the interfacial effect (McLean & Beveridge, 1990). Because of this, adhesion to surfaces therefore places organisms in regions of higher nutrient concentrations (Hamilton, 1987).

Adhesion also permits microorganisms ready access to insoluble substrates such as cellulose, or to surfaces which exude nutrients. These two situations are quite commonly encountered within the gastrointestinal tract of animals and have been particularly well studied in the rumen and caecum of herbivores. The rumen represents the largest organ within the digestive tract of ruminant animals (cattle, sheep, goats, etc.) and is unique in that all of its digestive functions are performed by the bacteria and other microorganisms (Hungate, 1966; Cheng & Costerton, 1980*a*). Within this organ, cellulose-containing plant material is digested by a number of microorganisms (principally bacteria) into several simpler compounds such as acetate, propionate and butyrate which are then absorbed and used by the host animal. No one species of microorganism is capable of the complete digestion of cellulose. Instead several species of bacteria usually act as consortia in performing this task (Cheng & Costerton, 1980*a,b*; Cheng *et al.*, 1981*a*). Cellulolytic organisms such as *Ruminococcus albus*, *R. flavefaciens*, and *Bacteroides succinogenes* generally adhere to insoluble substrates such as crystalline cellulose (Hungate,

1966; Kudo *et al.*, 1987). Other bacteria which may utilize the metabolic end products of cellulose digestion are seen in close proximity to and often adherent to the initial layer of cellulose degrading organisms (Cheng & Costerton, 1980a; Cheng *et al.*, 1981a; Costerton *et al.*, 1983). The ecological advantages to these organisms in adhering to cellulose and to each other in microcolonies (Fig. 5.10) (Cheng *et al.*, 1981a) become quite apparent in terms of ready access to an insoluble substrate, and transfer of metabolic end products. In the rumen environment and probably in other environments the formation of microcolonies and biofilms will enhance synergistic metabolic activities among their component microorganisms. In addition the proximity of microorganisms within a biofilm community has been shown to greatly facilitate the exchange of genetic material between members (Bale *et al.*, 1988).

5.5.2 Resistance to Adverse Environmental Conditions

Biofilms offer many organisms an opportunity to dwell in what would otherwise be hostile environments. If we consider a typical single species biofilm of 10 or more layers of cells in thickness on an inert surface in an aerobic environment (Fig. 5.11A), any chemicals which reach the interior cells must first pass through the surface layer. In the case of nutrients, one would therefore anticipate that the surface microorganisms would demonstrate the fastest growth in that they would be exposed to the highest concentration of nutrients and their metabolic activity would largely deplete nutrients and oxygen available for the interior cells. Should the fluid phase contain any toxic materials (Fig. 5.11B), then we would expect the surface organisms to be inhibited or even killed, and growth or survival demonstrated by interior cells. Now if we consider a biofilm containing several different species on this same inert surface (Fig. 5.11C), the surface cells would be expected to again show the fastest growth, but in addition to depleting nutrients they would tend also to deplete oxygen. Should the biofilm be sufficiently thick, oxygen would be totally depleted allowing the growth of obligately anaerobic organisms within the interior. The growth of interior organisms whether anaerobic or not, would be enhanced if they require some metabolic end product(s) produced by the surface layer. If the surface on which the biofilm resides contains some form of nutrition such as cellulose (Fig. 5.11D), we could expect those organisms in intimate contact with the surface to utilize those nutrients and subsequent layers of other organisms to utilize preferentially their metabolic end products. These strategies nicely explain how anaerobic microorganisms can grow in otherwise

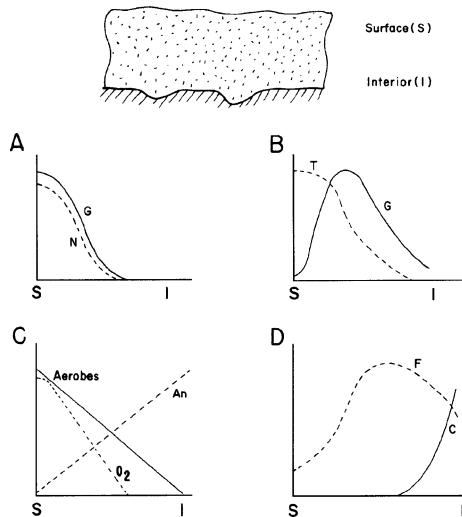


Fig. 5.11. Physiological profile within a model biofilm from the surface (S) into the interior (I). If the organisms are adherent onto an inert surface (example A), growth (G) will be most rapid near the surface since nutrients (N) will be rapidly metabolized by the surface layers of organisms. If a toxin (T) is present (example B), surface growth will be inhibited. Absorption or inactivation of the toxin by the first few layers of organisms allows the growth of underlying organisms. In example C, growth of aerobes is highest at the surface. Depletion of oxygen by these surface aerobes allows growth of anaerobes (An) in the interior. If adhesion is to an insoluble, metabolizable substrate such as cellulose (example D), cellulolytic activity (C) will be highest at the point of adhesion. Fermentation (F) of end-products of cellulolytic activity by other organisms will be highest in the immediate vicinity of the primary cellulose degraders.

hostile aerobic environments such as the skin (Costerton *et al.*, 1983, 1987), the mouth (Gibbons, 1989), and industrial water pipelines (Costerton, 1984a; Hamilton, 1987). Similar arguments can be used to explain the resistance of biofilms to other adverse environmental conditions such as antibiotics or heavy metals (Dean-Ross & Mills, 1989).

Many studies have shown that the biofilm mode of growth confers a considerable degree of protection to the component organisms against a wide variety of antimicrobial agents including antibiotics (Costerton, 1984a; Nickel *et al.*, 1985b; Ladd *et al.*, 1987; LeChevallier *et al.*, 1988a,b; Lytle *et al.*, 1989). In many cases this protection is quite significant in that biofilms can be up to several orders of magnitude more resistant than their planktonic counterparts. Several studies have been conducted to ascertain the mechanisms of this resistance. Anwar *et al.* (1989) showed that *Pseudomonas aeruginosa* biofilm resistance to the antibiotic tobramycin disappeared if the organisms were resuspended. In

addition older biofilms were most resistant. The authors concluded that the adhesion process was essential for antibiotic resistance. Although the capsule itself mediates some protection due to its binding of antibacterial compounds and reducing their penetration into the biofilm (Nichols *et al.*, 1988), the chief mechanism of this resistance is now considered to be related to reduced cellular metabolism (Ladd *et al.*, 1987; Brown *et al.*, 1988; Gilbert *et al.*, 1990). When compared to planktonic cells grown in conventional rich laboratory media, biofilm cells show a greatly reduced growth rate (expressed in terms of metabolic activity per cell or cell doublings per unit time). This is due to the energy required for biosynthetic production of capsular material as well as the necessity for diffusion of nutrients or other compounds (such as O₂) into the matrix. Since antibiotics largely act as inhibitors in many biosynthetic processes, the naturally lower growth rate within biofilms would reduce the effectiveness of these compounds. The surprising finding by Tim Ladd and Curtis Nickel (Ladd *et al.*, 1987) was that the metabolism of *Ps. aeruginosa* within biofilms (as expressed by ¹⁴C-glutamate uptake) is lowered even more upon exposure to the antibiotic tobramycin. This metabolic slowdown, which was not noted in planktonic cells, increases antibiotic resistance even further.

5.5.3 Relation of Biofilms to Other Bacterial Growth Strategies

As attested by their ubiquitous nature, biofilms represent an extremely successful ecological strategy for microorganisms in nature. However, they do not represent the only mode of growth available. As mentioned previously, introducing bacteria into rich laboratory media often induces them to forgo capsule production and biofilm growth and to grow as planktonic cells. These planktonic cells then quickly overgrow their sessile biofilm counterparts in that they are able to devote a greater fraction of their cellular metabolism to cell growth and reproduction (Costerton *et al.*, 1987). The other instance in which biofilm growth is rarely found is during conditions of long term starvation.

Under natural conditions, bacteria like other forms of life usually experience times of feast or famine. During periods of plenty, cellular physiology is directed towards growth and reproduction. Conversely during famine conditions, physiological strategy is geared mainly to survival. In higher forms of life such as animals and plants, dormancy often represents a common strategy. This is illustrated in seed production by annual plants and hibernation in many animals. In either case, these organisms survive periods of low nutrient availability during winter months in a dormant state and provide themselves with sufficient nutrients for their

basal metabolism from stored energy reserves such as starch, glycogen, and fat. Bacteria are certainly no different in their basic strategy from these other forms of life. Upon depletion of readily available nutrients, bacteria initially synthesize high affinity alternative transport systems and metabolic pathways to scavenge what nutrients are available. Eventually endogenous metabolism begins to predominate as the organisms utilize internal sources of nutrition such as non-essential proteins, and high energy compounds such as poly β hydroxybutyrate (Kjelleberg *et al.*, 1987; Matin *et al.*, 1989). Some bacteria notably members of the genera *Bacillus* and *Clostridium* produce endospores, while other microorganisms including yeasts, protozoa and fungi produce cysts or spores (Brock & Madigan, 1988).

There are several environments wherein bacteria encounter virtually no easily metabolizable carbon. These include the open ocean (Morita, 1982, 1988; Kjelleberg *et al.*, 1987; Roszak & Colwell, 1987; Matin *et al.*, 1989; Moyer & Morita, 1989) and porous rock aquifers deep below the terrestrial subsurface (Elliot *et al.*, 1984; Balkwill & Ghiorse, 1985; Balkwill *et al.*, 1989). The persistence and survival of some of these aquifer organisms is truly remarkable in that they may have been in their particular environments for several centuries or even millennia (W. C. Ghiorse, personal communication). Nevertheless the distinguishing feature of most of these organisms is that they are usually found as individual cells – not as biofilms. In the case of starved marine organisms, Morita and others have established that some organisms undergo a series of cell divisions without a net increase in overall population mass (Morita, 1982, 1988; Roszak & Colwell, 1987; Moyer & Morita, 1989). The net result of this process is the formation of spherical ultramicrobacteria, so named because they may only be 0.1–0.2 μm in diameter (in contrast to 0.5–2 μm size of many bacteria). In this case the strategy of these organisms is to make as many copies of their genome as possible so that when nutrients eventually become available, some representatives of the species will have survived. Other aspects of bacterial physiology during starvation stress are altered. A number of starvation-induced proteins are produced (Tormo *et al.*, 1990; Nyström *et al.*, 1992; Siegele & Kolter, 1992) and in at least one instance, starved microorganisms become much more resistant to heavy metals and ultraviolet radiation (Nyström *et al.*, 1992).

Very little experimental work to date has been done to study the effects of extreme and prolonged starvation upon biofilms. In a study with a marine *Pseudomonas* sp.; Wrangstadh *et al.* (1989) found that starvation induced the formation of capsular material which in turn promoted detachment from hydrophobic surfaces (Wrangstadh *et al.*, 1986, 1989).

Presumably these starved organisms went off in search of 'greener pastures' or at least more nutrition. Other investigators have found that starved microorganisms adhere more readily to surfaces (Marshall, 1988). This whole concept of starvation survival and its relevance to microbial and biofilm ecology and physiology represents an area of much needed future study.

5.6 BIOFILM PHYSIOLOGY

When compared to their planktonic counterparts, biofilm organisms would be expected to individually demonstrate slower growth (as expressed in terms of cell divisions and increased cell mass). This is due to the physiological requirements of capsule production, and possibly poor diffusion of soluble nutrients into and waste products out of the biofilm matrix (Rittmann *et al.*, 1986). This is more than offset by the many advantages of biofilms outlined above.

Biofilm physiology as an area of research is still basically in its infancy. The majority of basic physiological studies with bacteria have been performed using planktonic populations often under conditions that induce maximum growth. While this approach has yielded much in the fields of bacterial physiology and biochemistry, it must be realized that we are studying organisms under the stress of maximum growth. As most microorganisms in nature are starved or at least nutrient limited, it is possible that at least some of these discoveries will be shown to be largely irrelevant to the organisms as they exist in their natural environment (Brown & Williams, 1985). Most if not all the biochemical techniques that are available to us today require at the least ng to μg quantities of material and so we obtain values that reflect an average for a given population rather than individual cell values. In spite of these limitations, David White and his colleagues have adapted a number of sensitive biochemical tests to study the bacterial population characteristics of natural biofilm communities (White & Findlay, 1988). Using these and other techniques, we are able to detect specific chemical signatures of individual members within biofilm communities. Analysis of components such as membrane lipids, storage products, etc., unique to individual species and/or growth conditions allow us to estimate the overall physiological condition and constitution of biofilm communities. Other investigators, notably Ronald Atlas, Norman Pace, David Stahl, Betty Olson, and others are developing molecular techniques which enable specific gene sequences and viable organisms to be isolated, identified and differentiated from

natural populations *in situ* (Stahl *et al.*, 1988; Steffan *et al.*, 1989; Amann *et al.*, 1990a,b, 1992; Bej *et al.*, 1991a,b,c; Voordouw *et al.*, 1991; Tsai & Olson, 1992). Direct observations by light and electron microscopy greatly complement these other techniques in that they allow detailed examinations of individual cells (Costerton, 1979; Holt & Beveridge, 1982), and may often suggest appropriate future experimental strategies. Specific localization of enzymes within a population by either immunocytochemical or histochemical techniques can enhance morphological techniques in that it will illustrate the distribution of various metabolic functions among members of a biofilm microbial community (Costerton & Marks, 1977; McLean *et al.*, 1986).

Bright and Fletcher compared the metabolic activity of adherent and free living bacteria using a marine *Pseudomonas* sp. (Bright & Fletcher, 1983). In these experiments, the organisms were allowed to attach to a variety of surfaces, and their metabolic activity was evaluated by uptake of radiolabelled amino acids as shown by microautoradiography, and electron transport activity measured by staining with a tetrazolium salt. Activity by adherent organisms was compared to that of detached organisms, and to free living organisms. The authors found that as the hydrophobicity of the surface increased, there was an increase in the proportion of adherent cells showing metabolic activity (^3H -leucine uptake) and electron transport system activity but overall this relationship of activity between adherent and non-adherent cells depended upon the substratum to which they were attached. Their only firm conclusion was that metabolic activity appeared to promote firm attachment, and that cells that detached from the surface substrata often assimilated fewer amino acids. Overall they found no general effect of surfaces on the activity of attached bacteria, and that the relative activity of attached versus free-living bacteria depended upon the amino acid used, the concentration of the amino acid, and the properties of the particular surface involved. Several studies have shown the adhesion process itself to have some influence on microbial physiological activity (Pedersen *et al.*, 1986; Geesey & White, 1990). Other investigators have found that metabolic activities of the dental microorganisms, *Streptococcus mutans*, and *Veillonella* sp. (as expressed in DNA and acid production) were enhanced when these organisms were growing as a mixed biofilm or plaque (Noorda *et al.*, 1988).

Cells within the biofilm matrix would not be expected to have equal access to substrates due to problems of diffusion and possibly competition. If a particular substrate is necessary for growth, one would expect bacterial cells to possess several mechanisms by which the substrate in question might be assimilated. In order to economize biosynthetic

requirements, individual cells normally only express one particular system at any given time. However due to the aforementioned problems of diffusion and competition within a biofilm, both low and high affinity transport mechanisms for a given substrate might be expected within this microbial community. This has been shown in the concurrent expression of both low and high affinity aspartate transport systems within wastewater biofilms (Eighmy & Bishop, 1984). Indeed physical disruption of a biofilm has been shown to augment its overall collective metabolism in that the access of all cells to nutrients is enhanced (Murray *et al.*, 1987).

Metabolic activities unique to biofilms have been described in the literature. Many animals are dependent upon microbial fermentation in their digestive processes. In ruminants, fermentation occurs predominantly in the rumen and caecum (Hungate, 1966). Cheng and coworkers have repeatedly observed that microbial digestion of cellulose within the rumen occurs as a result of colonization of this insoluble substrate by consortia of mixed microbial biofilm populations (Cheng & Costerton, 1980b; Cheng *et al.*, 1981a). As described previously, metabolic waste products (such as volatile fatty acids and H_2) of the primary cellulose digesting organisms represent substrates for other organisms (such as methanogens) (Wolin & Miller, 1983). Biofilms, in this instance, increase the efficiency of cellulose digestion in that synergistic microbial interactions are greatly enhanced. The other example of metabolism unique to biofilms is the anaerobic corrosion processes within pipelines. Under appropriate environmental conditions, consortia of bacteria colonize pipes and through their metabolic processes actually generate an anaerobic reducing microenvironment in the interior of the biofilm. Quite frequently these anaerobic microenvironments possess a sufficiently low Eh to support the growth of sulphate reducing bacteria. Due to localized differences in metabolism, bacteria in one region may scavenge electrons from the metal itself, thereby converting the metal to soluble cations. In another region, bacteria may transfer electrons to an electron acceptor such as sulphate. The resulting sulphide ions then are able to react with the metal to form a metal sulphide. The net consequence is the generation of a biological electrochemical cell and corrosion of the metal (reviewed in Hamilton (1985); Costerton *et al.* (1987); Hamilton (1987)).

5.7 PRACTICAL ASPECTS OF BIOFILM FORMATION

The ubiquitous nature of bacterial growth as biofilms ensures that they will influence many aspects of industry. Detrimental aspects of biofilms

include their ability to induce microbial fouling and corrosion in industrial pipelines and other aquatic systems. In addition, the biofilm mode of growth is very instrumental in the ability of bacteria to persist. Consequently they can act as a reservoir for potentially harmful or pathogenic bacteria in many environments such as waste-water treatment systems, and infections. These topics have been addressed previously.

Many industrial processes employ the fermentation or biosynthetic capabilities of microbes. In a relatively simple process, ethanol is produced by the anaerobic fermentation of glucose by a monoculture of *Saccharomyces cerevisiae* or *Zymomonas* sp. These organisms are provided with appropriate levels of substrate (often a saccharified grain mash), and as a result of their growth and primary anaerobic metabolism, produce ethanol. In more complicated microbial biosyntheses, the desired product (such as an antibiotic) may only occur at the cessation of cell growth as a result of secondary metabolism. The efficiency of these biosynthetic processes is often enhanced by the use of immobilized cells (addressed elsewhere in this book) in that the immobilization process induces the formation of secondary metabolites. In either of these examples, the microbes in question are provided with appropriate levels of substrate, and other growth requirements, and the fermentation end-products removed through centrifugation, filtration, distillation, or other procedures. More difficult problems arise when poor substrate quality and quantity, and adverse environmental conditions are present.

Many industrial and agricultural wastes contain high concentrations of organic matter. In anaerobic digesters, these substances are converted to methane through microbial fermentation (MacLeod *et al.*, 1990). No single species of organism is responsible for all aspects of anaerobic digestion. Rather these fermentation processes follow several metabolic pathways with one or more species being responsible for and deriving benefit from particular reactions. During microbial cometabolism, each organism adapts to its particular niche, and relies upon its neighbours to remove inhibitory metabolic waste products. Such cell-cell interactions are enhanced when these organisms coexist within biofilms (Eighmy *et al.*, 1983; Carey *et al.*, 1984; Eighmy & Bishop, 1984; Camper *et al.*, 1985; Nielsen, 1987). As substrate levels in waste-water are rarely constant, and small or significant levels of harmful compounds may be present, biofilm growth becomes very advantageous to the organisms in terms of survival.

Recent technological developments in molecular biology have enabled us to get a very detailed understanding about many physiological processes of bacteria. Through selection, amplification, appropriate regulation, and

expression of particular genes or gene sequences we are able to develop 'super organisms' which in the laboratory are able to perform all aspects of a desired metabolic function, produce huge yields of product and/or consume huge quantities of substrate. Often, introduction of foreign genes, or alteration of naturally occurring genes, disrupt overall cell biology in that the loss of some other essential cell function can result. The outcome of these metabolic imbalances may not become apparent until these engineered organisms are moved into less ideal environments outside the laboratory and are exhibited when laboratory scale processes fail miserably at the pilot plant or field testing stage.

Biofilm growth offers several possible strategies for the development of new industrial microbial processes. Aspects particularly evident in wastewater treatment are that interspecies metabolic processes are enhanced and persistence during times of nutrient depletion and/or the presence of harmful compounds is enhanced. Rather than develop one super organism to perform all aspects of a desired process, it might be prudent to employ more naturally efficient metabolic processes involving several types of organisms growing as consortia within biofilms.

Another potential of biofilm technology would be preliminary testing of genetically engineered organisms as to their suitability and survival capabilities in the environment. Biofilms containing one or more species of microorganism are relatively easily produced in the laboratory (Costerton, 1984a; Costerton *et al.*, 1987). This being the case, one could conceivably produce biofilms containing the test organism and any number of other organisms. Due to the close proximity of the individual members within biofilms, cell-cell interactions are maximized and such processes as microbial synergy or competition, cometabolism (such as interspecies hydrogen transfer), and even intercellular genetic transfer would be enhanced. The potential for biofilm technology is therefore quite significant.

ACKNOWLEDGEMENTS

Work in the authors' laboratories has been funded through grants from the Kidney Foundation of Canada (RJCM), Alberta Heritage Foundation for Medical Research (RJCM and JWC), Medical Research Council of Canada (JWC), Natural Sciences and Engineering Council of Canada (RJCM, DEC, and JWC), Alberta Oil Sands and Technology Research Authority (JWC), Alberta Farming for the Future Program (RJCM and JWC), Office of Naval Research (DEC), numerous industrial contracts

(JWC), and the Ontario Ministry of Health Career Scientist Program (RJCM). J. W. Costerton was the holder of a Natural Sciences and Engineering Research Council of Canada (NSERC) Industrial Research Chair in Biofilm Microbiology and is now the Director of the Center for Biofilm Engineering, Montana State University, Bozeman, MT.

These basic concepts and developments of biofilm technology would not have been possible without the enthusiasm and help of a large number of our colleagues and collaborators through the years.

We dedicate this chapter to Kevin C. Marshall, a gentleman and a scholar, and a pioneer in biofilm microbiology, on the occasion of his retirement.

5.8 REFERENCES

- Alexander, M. (1971). Biochemical ecology of microorganisms. *Annual Review of Microbiology*, **25**, 361–92.
- Allison, D. G. & Sutherland I. W. (1987). The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology*, **133**, 1319–28.
- Aly, R. & Levit, S. (1987). Adherence of *Staphylococcus aureus* to squamous epithelium: role of fibronectin and teichoic acid. *Reviews of Infectious Diseases*, **9**, S341–50.
- Amann, R. I., Krumholz, L. & Stahl, D. A. (1990a). Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, **172**, 762–70.
- Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990b). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, **56**, 1919–25.
- Amann, R. I., Stromley, J., Devereux, R., Key, R. & Stahl, D. A. (1992). Molecular and microscopic identification of sulfate-reducing bacteria in multi-species biofilms. *Applied and Environmental Microbiology*, **58**, 614–23.
- Anwar, H., Van Biesen, T., Dasgupta, M., Lam, K. & Costerton, J. W. (1989). Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system. *Antimicrobial Agents and Chemotherapy*, **33**, 1824–6.
- Aronson, M., Medalia, O., Amichay, D. & Nativ, O. (1988). Endotoxin-induced shedding of viable uroepithelial cells is an antimicrobial defense mechanism. *Infection and Immunity*, **56**, 1615–17.
- Baker, J. H. (1984). Factors affecting the bacterial colonization of various surfaces in a river. *Canadian Journal of Microbiology*, **30**, 511–15.
- Bale, M. J., Day, M. J. & Fry, J. C. (1988). Novel method for studying plasmid transfer in undisturbed river epilithon. *Applied and Environmental Microbiology*, **54**, 2756–8.

- Balkwill, D. L., Fredrickson, J. K. & Thomas, J. M. (1989). Vertical and horizontal variations in the physiological diversity of the aerobic chemo-heterotrophic bacterial microflora in deep southeast coastal plain subsurface sediments. *Applied and Environmental Microbiology*, **55**, 1058–65.
- Balkwill, D. L. & Ghiorse, W. C. (1985). Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. *Applied and Environmental Microbiology*, **50**, 580–8.
- Beachey, E. H., Giampapa, C. S. & Abraham, S. N. (1988). Bacterial adherence. Adhesin receptor-mediated attachment of pathogenic bacteria to mucosal surfaces. *American Review of Respiratory Diseases*, **138**, S45–48.
- Bej, A. K., Mahbubani, M. H. & Atlas, R. M. (1991a). Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. *Applied and Environmental Microbiology*, **57**, 597–600.
- Bej, A. K., DiCesare, J. L., Haff, L. & Atlas, R. M. (1991b). Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. *Applied and Environmental Microbiology*, **57**, 1013–17.
- Bej, A. K., McCarty, S. C. & Atlas, R. M. (1991c). Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. *Applied and Environmental Microbiology*, **57**, 2429–32.
- Belas, M. R. & Colwell, R. R. (1982). Adsorption kinetics of laterally and polarly flagellated *Vibrio*. *Journal of Bacteriology*, **151**, 1568–80.
- Beveridge, T. J. (1981). Ultrastructure, chemistry, and function of the bacterial wall. *International Review of Cytology*, **72**, 229–317.
- Beveridge, T. J. (1989). Role of cellular design in bacterial metal accumulation and mineralization. *Annual Review of Microbiology*, **43**, 147–71.
- Beynon, L. M., Dumanski, A. J., McLean, R. J. C., MacLean, L. L., Richards, J. C. & Perry, M. B. (1992). Capsule structure of *Proteus mirabilis* (ATCC 49565). *Journal of Bacteriology*, **174**, 2172–7.
- Blenkinsopp, S. A., Khoury, A. E. & Costerton, J. W. (1992). Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, **58**, 3770–3.
- Brackenhoff, G. J., van der Voort, H. T. M., van Spronsen, E. A. & Nanninga, N. (1988). 3-Dimensional imaging of biological structures by high resolution confocal microscopy. *Scanning Microscopy*, **2**, 33.
- Bright, J. J. & Fletcher, M. (1983). Amino acid assimilation and electron transport system activity in attached and free-living marine bacteria. *Applied and Environmental Microbiology*, **45**, 818–25.
- Brock, T. D. & Madigan, M. T. (1988). *Biology of microorganisms*. Prentice Hall, Englewood Cliffs, NJ.
- Brown, M. R. W., Allison, D. G. & Gilbert, P. (1988). Resistance of bacterial biofilms to antibiotics: a growth-rate related effect. *Journal of Antimicrobial Chemotherapy*, **22**, 777–80.
- Brown, M. R. W. & Williams, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology*, **39**, 527–56.
- Bryant, R. D., Costerton, J. W. & Laishley, E. J. (1984). The role of *Thiobacillus*

- albertis* glycocalyx in the adhesion of cells to elemental sulfur. *Canadian Journal of Microbiology*, **30**, 81–90.
- Caldwell, D. E. (1977). The planktonic microflora of lakes. *Critical Reviews in Microbiology*, **5**, 306–70.
- Caldwell, D. E. (1984). Surface colonization parameters from cell density and distribution. In *Microbial adhesion and aggregation*, pp. 125–136. Ed. Marshall, K. C. Springer-Verlag Berlin.
- Caldwell, D. E. (1985). New developments in computer-enhanced microscopy (CEM). *Journal of Microbiological Methods*, **4**, 117–25.
- Caldwell, D. E. & Germida, J. J. (1985). Evaluation of difference imagery for visualizing and quantitating microbial growth. *Canadian Journal of Microbiology*, **31**, 35–44.
- Caldwell, D. E. & Lawrence, J. R. (1986). Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology*, **12**, 299–312.
- Caldwell, D. E. (1987). Microbial colonization of solid-liquid interfaces. In *Biochemical Engineering V*, pp. 274–280. New York Academy of Sciences, New York.
- Caldwell, D. E. & Lawrence, J. R. (1988). Study of attached cells in continuous-flow slide culture. In *CRC handbook of laboratory model system for microbial ecosystems, volume 1*, pp. 117–138. Ed. Wimpenny, J. W. T. CRC Press, Inc., Boca Raton, FL.
- Caldwell, D. E., Korber, D. R. & Lawrence, J. R. (1992). Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. *Journal of Microbiological Methods*, **15**, 249–61.
- Caldwell, D. E. & Tiedje, J. M. (1975a). A morphological study of bacteria in the hypolimnia of several Michigan lakes. *Canadian Journal of Microbiology*, **21**, 362–76.
- Caldwell, D. E. & Tiedje, J. M. (1975b). The structure of anaerobic bacterial communities in the hypolimnia of several lakes in Michigan. *Canadian Journal of Microbiology*, **21**, 377–85.
- Camper, A. K., LeChevallier, M. W., Broadaway, S. C. & McFeters, G. A. (1985). Growth and persistence of pathogens on granular activated carbon filters. *Applied and Environmental Microbiology*, **50**, 1378–82.
- Caparon, M. G., Stephens, D. S., Olsen, A. & Scott, J. R. (1991). Role of M protein in adherence of group A streptococci. *Infection and Immunity*, **59**, 1811–17.
- Carey, J. H., Fox, M. E., Brownlee, B. G., Metcalfe, J. L. & Platford, R. F. (1984). Disappearance kinetics of 2,4- and 3,4-dichlorophenol in a fluvial system. *Canadian Journal of Physiology and Pharmacology*, **62**, 971–5.
- Chan, R., Lam, J. S., Lam, K. & Costerton, J. W. (1984). Influence of culture conditions on expression of the mucoid mode of growth of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology*, **19**, 8–16.
- Chan, R. C. Y., Reid, G., Irvin, R. T., Bruce, A. W. & Costerton, J. W. (1985). Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infection and Immunity*, **47**, 84–89.
- Chang, C. C. & Merritt, K. (1991). Effect of *Staphylococcus epidermidis* on adherence of *Pseudomonas aeruginosa* and *Proteus mirabilis* to polymethyl

- methacrylate (PMMA) and gentamicin-containing PMMA. *Journal of Orthopedic Research*, **9**, 284–8.
- Cheng, K.-J. & Costerton, J. W. (1980a). Adherent rumen bacteria – their role in the digestion of plant material urea and epithelial cells. In *Digestive Physiology and Metabolism in Ruminants*, pp. 227–250. Eds Ruckebusch, Y. & Thivend, P. MTP Press, Lancaster, England.
- Cheng, K.-J. & Costerton, J. W. (1980b). The formation of microcolonies by rumen bacteria. *Canadian Journal of Microbiology*, **26**, 1104–13.
- Cheng, K.-J., McCowan, R. P. & Costerton, J. W. (1979). Adherent epithelial bacteria in ruminants and their roles in digestive tract function. *American Journal of Clinical Nutrition*, **32**, 139–48.
- Cheng, K.-J., Fay, J. P., Coleman, R. N., Milligan, L. P. & Costerton, J. W. (1981a). Formation of bacterial microcolonies of feed particles in the rumen. *Applied and Environmental Microbiology*, **41**, 298–305.
- Cheng, K.-J., Irvin, R. T. & Costerton, J. W. (1981b). Autochthonous and pathogenic colonization of animal tissues by bacteria. *Canadian Journal of Microbiology*, **27**, 461–90.
- Christersson, C. E., Dunford, R. G., Glantz, P. O. & Baier, R. E. (1989). Effect of critical surface tension on retention of oral microorganisms. *Scandinavian Journal of Dental Research*, **97**, 247–56.
- Christersson, C. E., Glantz, P. O. & Baier, R. E. (1988). Role of temperature and shear forces on microbial detachment. *Scandinavian Journal of Dental Research*, **96**, 91–98.
- Clapham, L., McLean, R. J. C., Nickel, J. C., Downey, J. & Costerton, J. W. (1990). The influence of bacteria on struvite crystal habit and its importance in urinary stone formation. *Journal of Crystal Growth*, **104**, 475–84.
- Cochrane, D. M. G., Brown, M. R. W., Anwar, H., Weller, P. H., Lam, K. & Costerton, J. W. (1988). Antibody response to *Pseudomonas aeruginosa* surface protein antigens in a rat model of chronic lung infection. *Journal of Medical Microbiology*, **27**, 255–61.
- Costerton, J. W. (1979). The role of electron microscopy in the elucidation of bacterial structure and function. *Annual Review of Microbiology*, **33**, 459–79.
- Costerton, J. W. (1990). *The removal of microbial fouling by ice nucleation*. US Patent number 4,419,248.
- Costerton, J. W., Irvin, R. T. & Cheng, K.-J. (1981a). The role of bacterial surface structures in pathogenesis. *Critical Reviews in Microbiology*, **8**, 303–38.
- Costerton, J. W., Irvin, R. T. & Cheng, K.-J. (1981b). The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology*, **35**, 299–324.
- Costerton, J. W., Lam, J., Lam, K. & Chan, R. (1983). The role of the micro-colony mode of growth in the pathogenesis of *Pseudomonas aeruginosa* infections. *Reviews of Infectious Diseases*, **5**, S867–73.
- Costerton, J. W. & Marks, I. (1977). Localization of enzymes in procaryotic cells. In *Electron Microscopy of Enzymes: Principles and Methods*, pp. 98–134. Ed. Hayat, M. A. Van Nostrand Reinhold Co., New York.
- Costerton, J. W., Rozee, K. R. & Cheng, K.-J. (1983). Colonization of particulates, mucous and intestinal tissue. *Progress in Food and Nutritional Science*, **7**, 91–105.

- Costerton, J. W. (1984a). The formation of biocide-resistant biofilms in industrial, natural, and medical systems. *Developments in Industrial Microbiology*, **25**, 363–72.
- Costerton, J. W. (1984b). The etiology and persistence of cryptic bacterial infections: a hypothesis. *Reviews of Infectious Diseases*, **6**, S608–16.
- Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annual Review of Microbiology*, **41**, 435–64.
- Cowan, M. M., Taylor, K. G. & Doyle, R. J. (1987). Energetics of the initial phase of adhesion of *Streptococcus sanguis* to hydroxylapatite. *Journal of Bacteriology*, **169**, 2995–3000.
- Cusack, F., Brown, D. R., Costerton, J. W. & Clementz, D. M. (1987). Field and laboratory studies of microbial/fines plugging of water injection wells: mechanism, diagnosis and removal. *Journal of Petroleum Science and Engineering*, **1**, 39–50.
- Dart, J. K. & Seal, D. V. (1988). Pathogenesis and therapy of *Pseudomonas aeruginosa* keratitis. *Eye*, **2**, S46–55.
- Dazzo, F. B. & Truchet, G. L. (1983). Interactions of lectins and their saccharide receptors in the *Rhizobium*–legume symbiosis. *Journal of Membrane Biology*, **73**, 1–16.
- Dean-Ross, D. & Mills, A. L. (1989). Bacterial community structure and function along a heavy metal gradient. *Applied and Environmental Microbiology*, **55**, 2002–9.
- Doig, P., Smith, N. R., Todd, T. & Irvin, R. T. (1987). Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. *Infection and Immunity*, **55**, 1517–22.
- Doig, P., Todd, T., Sastry, P. A., Lee, K. K., Hodges, R. S., Paranchych, W. & Irvin, R. T. (1988). Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. *Infection and Immunity*, **56**, 1641–6.
- Driessens, F. C. M., Borggreven, J. M. P. M., Verbeeck, R. M. H., van Dijk, J. W. E. & Feagin, F. F. (1985). On the physicochemistry of plaque calcification and the phase composition of dental calculus. *Journal of Periodontal Research*, **20**, 329–36.
- Duguid, J. P., Smith, I. W., Demster, G. & Edwards, P. N. (1955). Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli*. *Journal of Pathology and Bacteriology*, **70**, 335–48.
- Eighmy, T. T., Maratea, D. & Bishop, P. L. (1983). Electron microscopic examination of wastewater biofilm formation and structural components. *Applied and Environmental Microbiology*, **45**, 1921–31.
- Eighmy, T. T. & Bishop, P. L. (1984). Multiplicity of aspartate transport in thin wastewater biofilms. *Applied and Environmental Microbiology*, **48**, 1151–8.
- Eisenstein, B. I. (1987). Fimbriae. In *Escherichia coli* and *Salmonella typhimurium Cellular and Molecular Biology*, volume 1, pp. 84–90. Eds Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umberger, H. E. American Society for Microbiology, Washington DC.
- Ellen, R. P., Schwarz-Faulkner, S. & Grove, D. A. (1988). Coaggregation among periodontal pathogens, emphasizing *Bacteroides gingivalis*–*Actinomyces*

- viscosus* cohesion on a saliva-coated mineral surface. *Canadian Journal of Microbiology*, **34**, 299–306.
- Elliot, E. T., Coleman, D. C., Ingham, R. E. & Trofymow, J. A. (1984). Carbon and energy flow through microflora and microfauna in the soil subsystem of terrestrial ecosystems. In *Current Perspectives in Microbial Ecology*, pp. 424–33. Eds. Klug, M. J. & Reddy, C. A. American Society for Microbiology, Washington.
- Fader, R. C. & Davis, C. P. (1980). Effect of piliation on *Klebsiella pneumoniae* infection in rat bladders. *Infection and Immunity*, **30**, 554–61.
- Ferris, F. G., Beveridge, T. J. & Fyfe, W. S. (1986). Iron-silica crystallite nucleation by bacteria in a geothermal sediment. *Nature*, **320**, 609–11.
- Ferris, F. G., Schultze, S., Witten, T. C., Fyfe, W. S. & Beveridge, T. J. (1989a). Metal interactions with microbial biofilms in acidic and neutral pH environments. *Applied and Environmental Microbiology*, **55**, 1249–57.
- Ferris, F. G., Fyfe, W. S., Witten, T., Schultze, S. & Beveridge, T. J. (1989b). Effect of mineral substrate hardness on the population density of epilithic microorganisms in two Ontario rivers. *Canadian Journal of Microbiology*, **35**, 744–7.
- Finlay, B. B. & Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiological Reviews*, **53**, 210–30.
- Fletcher, M. (1988). Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium–substratum separation distance. *Journal of Bacteriology*, **170**, 2027–30.
- Geesey, G. G., Mutch, R., Costerton, J. W. & Green, R. B. (1978). Sessile bacteria: an important component of the microbial population in small mountain streams. *Limnology and Oceanography*, **23**, 1214–23.
- Geesey, G. G. & White, D. C. (1990). Determination of bacterial growth and activity at solid–liquid interfaces. *Annual Review of Microbiology*, **44**, 579–602.
- Ghiorse, W. C. & Wilson, J. T. (1988). Microbial ecology of the terrestrial subsurface. *Advances in Applied Microbiology*, **33**, 107–72.
- Gibbons, R. J. (1989). Bacterial adhesion to oral tissues: a model for infectious diseases. *Journal of Dental Research*, **68**, 750–60.
- Gibbons, R. J. & Nygaard, M. (1970). Interbacterial aggregation of plaque bacteria. *Archives of Oral Biology*, **15**, 1397–400.
- Gilbert, P., Collier, P. J. & Brown, M. R. W. (1990). Influence of growth rate on susceptibility to antimicrobial agents: Biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial Agents and Chemotherapy*, **34**, 1865–8.
- Godwin, S. L., Fletcher, M. & Burchard, R. P. (1989). Interference reflection microscopic study of sites of association between gliding bacteria and glass substrata. *Journal of Bacteriology*, **171**, 4589–94.
- Gristina, A. G. & Costerton, J. W. (1985). Bacterial adherence to biomaterials and tissue. The significance of its role in clinical sepsis. *American Journal of Bone and Joint Surgery*, **67**, 264–73.
- Hagberg, L., Lam, J., Svanborg Edén, C. & Costerton, J. W. (1986). Interaction of a pyelonephritogenic *Escherichia coli* strain with the tissue components of the mouse urinary tract. *Journal of Urology*, **136**, 165–72.
- Hamilton, W. A. (1985). Sulphate-reducing bacteria and anaerobic corrosion. *Annual Review of Microbiology*, **39**, 195–217.

- Hamilton, W. A. (1987). Biofilms: microbial interactions and metabolic activities. In *Ecology of microbial communities*, pp. 361–385. Ed. Hamilton, W. A. University Press, Cambridge, UK.
- Haugland, R. P. (1992). *Handbook of Fluorescent Probes and Research Chemicals*. Molecular Probes, Inc., Eugene, OR.
- Holt, S. C. & Beveridge, T. J. (1982). Electron microscopy: its development and application to microbiology. *Canadian Journal of Microbiology*, **28**, 1–53.
- Humphries, M., Nemcek, J., Cantwell, J. B. & Gerrard, J. J. (1987). The use of graft copolymers to inhibit the adhesion of bacteria to solid surfaces. *FEMS Microbial Ecology*, **45**, 297–304.
- Hungate, R. E. (1966). *The Rumen and its Microbes*. Academic Press, New York.
- Iriberry, J., Unanue, M., Ayo, B., Barcina, I. & Egea, L. (1990). Bacterial production and growth rate estimation from [³H]thymidine incorporation for attached and free-living bacteria in aquatic systems. *Applied and Environmental Microbiology*, **56**, 483–7.
- Jacques, M., Marrie, T. J. & Costerton, J. W. (1987). Review: Microbial colonization of prosthetic devices. *Microbial Ecology*, **13**, 173–91.
- Jacques, M., Olson, M. E., Crichlow, A. M., Osborne, A. D. & Costerton, J. W. (1986). The normal microflora of the female rabbit's genital tract. *Canadian Journal of Comparative Medicine*, **50**, 272–4.
- Jacques, M., Roy, G. & Mittal, K. R. (1988). Hemagglutinating properties of *Actinobacillus pleuropneumoniae*. *Canadian Journal of Microbiology*, **34**, 1046–9.
- Kenne, L. & Lindberg, B. (1983). Bacterial polysaccharides. In *The polysaccharides*, volume 2, pp. 287–363. Ed. Aspinall, G. O. Academic Press, New York.
- Kieft, T. L. & Caldwell, D. E. (1984). Chemostat and in-situ colonization kinetics of *Thermothrix thiopara* on calcite and pyrite surfaces. *Geomicrobiology Journal*, **3**, 217–29.
- Kjelleberg, S., Hermansson, M., Marden, P. & Jones, G. W. (1987). The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annual Review of Microbiology*, **41**, 25–49.
- Koga, T., Toda, Y., Moro, I. & Hamada, S. (1988). Electron-microscopic observation of adherence of serotype c *Streptococcus mutans* to the enamel surface due to glucan synthesis. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene, Series A*, **269**, 492–500.
- Kolenbrander, P. E. (1988). Intergeneric coaggregation among human oral bacteria and ecology of dental plaque. *Annual Review of Microbiology*, **42**, 627–56.
- Korber, D. R., Lawrence, J. R., Sutton, B. & Caldwell, D. E. (1989). Effect of laminar flow velocity on the kinetics of surface recolonization by Mot⁺ and Mot⁻ *Pseudomonas fluorescens*. *Microbial Ecology*, **18**, 1–19.
- Kudo, H., Cheng, K.-J. & Costerton, J. W. (1987). Electron microscopic study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. *Canadian Journal of Microbiology*, **33**, 267–72.
- Ladd, T. I., Schmiel, D., Nickel, J. C. & Costerton, J. W. (1985). Rapid method for detection of adherent bacteria on Foley urinary catheters. *Journal of Clinical Microbiology*, **21**, 1004–6.

- Ladd, T. I., Schmiel, D., Nickel, J. C. & Costerton, J. W. (1987). The use of a radiorespirometric assay for testing the antibiotic sensitivity of catheter-associated bacteria. *Journal of Urology*, **138**, 1451–6.
- Lappin-Scott, H. M., Cusack, F., MacLeod, A. & Costerton, J. W. (1988a). Starvation and nutrient resuscitation of *Klebsiella pneumoniae* isolated from oil well waters. *Journal of Applied Bacteriology*, **64**, 541–9.
- Lappin-Scott, H. M., Cusack, F. & Costerton, J. W. (1988b). Nutrient resuscitation and growth of starved cells in sandstone cores: a novel approach to enhanced oil recovery. *Applied and Environmental Microbiology*, **54**, 1373–82.
- Lappin-Scott, H. M. & Costerton, J. W. (1989). Bacterial biofilms and surface fouling. *Biofouling*, **1**, 323–42.
- Larsen, S. H., Reader, R. W., Kort, E. N., Tso, W.-W. & Adler, J. (1974). Change in direction of flagellar rotation is the basis of the chemotactic response in *Escherichia coli*. *Nature*, **249**, 74–7.
- Lawrence, J. R. & Caldwell, D. E. (1987). Behavior of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology*, **14**, 15–27.
- Lawrence, J. R., Delaquis, P. J., Korber, D. & Caldwell, D. E. (1987). Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology*, **14**, 1–14.
- Lawrence, J. R., Korber, D. R., Hoyle, B. D., Costerton, J. W. & Caldwell, D. E. (1991). Optical sectioning of microbial biofilms. *Journal of Bacteriology*, **173**, 6558–67.
- LeChevallier, M. W., Babcock, T. M. & Lee, R. G. (1987). Examination and characterization of distribution system biofilms. *Applied and Environmental Microbiology*, **53**, 2714–24.
- LeChevallier, M. W., Cawthon, C. D. & Lee, R. G. (1988a). Factors promoting survival of bacteria in chlorinated water supplies. *Applied and Environmental Microbiology*, **54**, 649–54.
- LeChevallier, M. W., Cawthon, C. D. & Lee, R. G. (1988b). Inactivation of biofilm bacteria. *Applied and Environmental Microbiology*, **54**, 2492–9.
- Leung, J. W. C., Sung, J. Y. & Costerton, J. W. (1989). Bacteriological and electron microscopy examination of brown pigment stones. *Journal of Clinical Microbiology*, **27**, 915–21.
- Liljemark, W. F., Bloomquist, C. G., Coulter, M. C., Fenner, L. J., Skopek, R. J. & Schachtele, C. F. (1988). Utilization of a continuous streptococcal surface to measure interbacterial adherence *in vitro* and *in vivo*. *Journal of Dental Research*, **67**, 1455–60.
- Lytle, M. S., Adams, J. C., Dickman, D. G. & Bressler, W. R. (1989). Use of nutrient response techniques to assess the effectiveness of chlorination of rapid sand filter gravel. *Applied and Environmental Microbiology*, **55**, 29–32.
- MacLeod, F. A., Lappin-Scott, H. M. & Costerton, J. W. (1988). Plugging of a model rock system by using starved bacteria. *Applied and Environmental Microbiology*, **54**, 1365–72.
- MacLeod, F. A., Guiot, S. R. & Costerton, J. W. (1990). Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Applied and Environmental Biology*, **56**, 1598–1607.

- MacNab, R. M. (1987). Flagella. In *Escherichia coli* and *Salmonella typhimurium* cellular and molecular Biology, volume 1, pp. 70–83. Eds Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umberger, H. E. American Society for Microbiology, Washington DC.
- Mann, S. (1988). Molecular recognition in biomineralization. *Nature*, **332**, 119–24.
- Marshall, K. C., Stout, R. & Mitchell, R. (1971). Mechanisms of the initial events in the sorption of marine bacteria to solid surfaces. *Journal of General Microbiology*, **68**, 337–48.
- Marshall, K. C. (1985). Bacterial adhesion in oligotrophic habitats. *Microbiological Science*, **2**, 321–6.
- Marshall, K. C. (1988). Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian Journal of Microbiology*, **34**, 503–6.
- Marshall, P. A., Loeb, G. L., Cowan, M. M. & Fletcher, M. (1989). Response of microbial adhesives and biofilm matrix polymers to chemical treatments as determined by interference reflection microscopy and light section microscopy. *Applied and Environmental Microbiology*, **55**, 2827–31.
- Matin, A., Auger, E. A., Blum, P. H. & Schultz, J. E. (1989). Genetic basis of starvation survival in nondifferentiating bacteria. *Annual Review of Microbiology*, **43**, 293–316.
- McEldowney, S. & Fletcher, M. (1986). Variability of the influence of physico-chemical factors affecting bacterial adhesion to polystyrene substrata. *Applied and Environmental Microbiology*, **52**, 460–5.
- McEldowney, S. & Fletcher, M. (1987). Adhesion of bacteria from mixed cell suspension to solid surfaces. *Archives of Microbiology*, **148**, 57–62.
- McKinley, V. L., Costerton, J. W. & White, D. C. (1988). Microbial biomass, activity, and community structure of water and particulates retrieved by backflow from a waterflood injection well. *Applied and Environmental Microbiology*, **54**, 1383–93.
- McLean, R. J. C., Beauchemin, D. & Beveridge, T. J. (1992). Influence of oxidation state on iron binding by *Bacillus licheniformis* capsule. *Applied and Environmental Microbiology*, **58**, 405–8.
- McLean, R. J. C., Beauchemin, D., Clapham, L. & Beveridge, T. J. (1990). Metal binding characteristics of the gamma-glutamyl capsule polymer of *Bacillus licheniformis* ATCC 9945. *Applied and Environmental Microbiology*, **56**, 3671–7.
- McLean, R. J. C. & Beveridge, T. J. (1990). Metal binding capacity of bacterial surfaces and their ability to form mineralized aggregates. In *Microbial Mineral Recovery*, pp. 185–222. Eds Ehrlich, H. L. & Brierley, C. L. McGraw-Hill, New York.
- McLean, R. J. C., Cheng, K.-J., Gould, W. D., Nickel, J. C. & Costerton, J. W. (1986). Histochemical and biochemical urease localization in the periplasm and outer membrane of two *Proteus mirabilis* strains. *Canadian Journal of Microbiology*, **32**, 772–8.
- McLean, R. J. C., Lawrence, J. R., Korber, D. R. & Caldwell, D. E. (1991). *Proteus mirabilis* biofilm protection against struvite crystal dissolution and its implications in struvite urolithiasis. *Journal of Urology*, **146**, 1138–42.
- McLean, R. J. C. & Nickel, J. C. (1991). Bacterial colonization behaviour: a new virulence strategy in urinary infections? *Medical Hypotheses*, **36**, 269–72.

- McLean, R. J. C., Nickel, J. C., Cheng, K.-J. & Costerton, J. W. (1988). The ecology and pathogenicity of urease-producing bacteria in the urinary tract. *Critical Reviews in Microbiology*, **16**, 37–79.
- Mergenhagen, S. E., Sandberg, A. L., Chassy, B. M., Brennan, M. J., Yeung, M. K., Donkersloot, J. A. & Cisar, J. O. (1987). Molecular basis of bacterial adhesion in the oral cavity. *Reviews of Infectious Diseases*, **9**, S467–74.
- Mills, A. L. & Maubery, R. (1981). Effect of mineral composition on bacterial attachment to submerged rock surfaces. *Microbial Ecology*, **7**, 315–22.
- Millward, T. A. & Wilson, M. (1989). The effect of chlorhexidine on *Streptococcus sanguis* biofilms. *Microbios*, **58**, 155–64.
- Morita, R. Y. (1982). Starvation-survival of heterotrophs in the marine environment. *Advances in Microbial Ecology*, **6**, 171–98.
- Morita, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Canadian Journal of Microbiology*, **34**, 436–41.
- Moyer, C. L. & Morita, R. Y. (1989). Effect of growth rate and starvation-survival on the viability and stability of a psychophilic marine bacterium. *Applied and Environmental Microbiology*, **55**, 1122–7.
- Murray, R. E., Cooksey, K. E. & Priscu, J. C. (1987). Influence of physical disruption on growth of attached bacteria. *Applied and Environmental Microbiology*, **53**, 2997–9.
- Nichols, P. D., Henson, J. M., Guckert, J. B., Nivens, D. E. & White, D. C. (1985). Fourier transform-IR spectroscopic methods for microbial ecology analysis of bacteria, bacteria-polymer mixtures and biofilms. *Journal of Microbiology Methods*, **4**, 79–94.
- Nichols, W. W., Dorrington, S. M., Slack, M. P. & Walmsley, H. L. (1988). Inhibition of tobramycin diffusion by binding to alginate. *Antimicrobial Agents and Chemotherapy*, **32**, 518–23.
- Nickel, J. C., Wright, J. B., Ruseska, I., Marrie, T. J., Whitfield, C. & Costerton, J. W. (1985a). Antibiotic resistance of *Pseudomonas aeruginosa* colonizing a urinary catheter *in vitro*. *European Journal of Clinical Microbiology*, **4**, 213–18.
- Nickel, J. C., Ruseska, I., Wright, J. B. & Costerton, J. W. (1985b). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy*, **27**, 619–24.
- Nielsen, P. H. (1987). Biofilm dynamics and kinetics during high-rate sulfate reduction under anaerobic conditions. *Applied and Environmental Microbiology*, **53**, 27–32.
- Noorda, W. D., Purdell-Lewis, D. J., van Montfort, A. M. & Weerkamp, A. H. (1988). Monobacterial and mixed bacterial plaques of *Streptococcus mutans* and *Veillonella alcalescens* in an artificial mouth: development, metabolism, and effect on human dental enamel. *Caries Research*, **22**, 342–7.
- Nyström, T., Olsson, R. M. & Kjelleberg, S. (1992). Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. *Applied and Environmental Microbiology*, **58**, 55–65.
- Ofek, I. & Perry, A. (1985). Molecular basis of bacterial adherence to tissues. In *Molecular Basis Oral Microbial adhesion*, pp. 7–13. Eds. Mergenhagen, S. E. & Rosan, B. American Society for Microbiology, Washington, DC.

- Olson, G. J. (1986). Microbial intervention in trace element-containing industrial process streams and waste products. In *The Importance of Chemical 'speciation' in Environmental Processes*, pp. 493–512. Eds. Bernhard, M., Brinckman, F. E. & Sadler, P. J. Springer-Verlag, Berlin.
- Op den Camp, H. J. M., Oosterhof, A. & Veerkamp, J. H. (1985). Interaction of Bifidobacterial lipoteichoic acid with human intestinal epithelial cells. *Infection and Immunity*, **47**, 332–4.
- Payment, P. (1989). Bacterial colonization of domestic reverse-osmosis water filtration units. *Canadian Journal of Microbiology*, **35**, 1065–7.
- Pedersen, K., Holmstrom, C., Olsson, A.-K. & Pedersen, A. (1986). Statistical evaluation of the influence of species variation, culture conditions, surface wettability, and fluid shear on attachment and biofilm development of marine bacteria. *Archives of Microbiology*, **145**, 1–8.
- Poirier, T. P., Kehoe, M. A., Whitnack, E., Dockter, M. E. & Beachey, E. H. (1989). Fibrinogen binding and resistance to phagocytosis of *Streptococcus sanguis* expressing cloned M protein of *Streptococcus pyogenes*. *Infection and Immunity*, **57**, 29–35.
- Pratt-Tepstra, I. H., Weerkamp, A. H. & Busscher, H. J. (1989). The effects of pellicle formation on streptococcal adhesion to human enamel and artificial substrata with various surface free-energies. *Journal of Dental Research*, **68**, 463–7.
- Pringle, J. H. & Fletcher, M. (1986). Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces. *Applied and Environmental Microbiology*, **51**, 1321–5.
- Reid, G., Jewett, M. A. S., Nickel, J. C., McLean, R. J. C. & Bruce, A. W. (1990). Effect of extra corporeal shock wave lithotripsy on bacterial viability: relationship to the treatment of struvite stones. *Urological Research*, **18**, 425–7.
- Reid, G., McGroarty, J. A., Angotti, R. & Cook, R. L. (1988). *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Canadian Journal of Microbiology*, **34**, 344–51.
- Ridgway, H. F., Kelly, A., Justice, C. & Olson, B. H. (1983). Microbial fouling of reverse-osmosis membranes used in advanced wastewater treatment technology: chemical, bacteriological, and ultrastructural analyses. *Applied and Environmental Microbiology*, **45**, 1066–84.
- Rittmann, B. E., Crawford, L., Tuck, C. K. & Namkung, E. (1986). In-situ determination of kinetic parameters for biofilms isolation and characterization of oligotrophic biofilms. *Biotechnology and Bioengineering*, **28**, 1753–60.
- Rosan, B., Eifert, R. & Golub, E. (1985). Bacterial surfaces, salivary pellicles, and plaque formation. In *Molecular Basis of Oral Microbial Adhesion*, pp. 69–76. Eds Mergenhausen, S. E. & Rosan, B. American Society for Microbiology, Washington, DC.
- Roszak, D. B. & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews*, **51**, 365–79.
- Ruseska, I., Robbins, J., Lashen, E. S. & Costerton, J. W. (1982). Biocide testing against corrosion-causing oilfield bacteria helps control plugging. *Oil and Gas Journal*, **3**, 253–64.
- Shaw, J. C., Bramhill, B., Wardlaw, N. C. & Costerton, J. W. (1985). Bacterial fouling in a model core system. *Applied and Environmental Microbiology*, **49**, 693–701.

- Siegele, D. A. & Kolter, R. (1992). Life after log. *Journal of Bacteriology*, **174**, 345–8.
- Stahl, D. A., Flesher, B., Mansfield, H. R. & Montgomery, L. (1988). Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Applied and Environmental Microbiology*, **54**, 1079–84.
- Staley, J. T. & Fuerst, J. A. (1989). Budding and/or appendaged bacteria. In *Bergey's Manual of Systematic Bacteriology*, volume 3, pp. 1890–1993. Eds Staley, J. T., Bryant, M. P., Pfennig, N. & Holt, J. G. Williams and Wilkins, Baltimore.
- Steffan, R. J., Breen, A., Atlas, R. M. & Sayler, G. S. (1989). Application of gene probe methods for monitoring specific microbial populations in freshwater ecosystems. *Canadian Journal of Microbiology*, **35**, 681–5.
- Steffan, R. J., Goksoyr, J., Bej, A. K. & Atlas, R. M. (1988). Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology*, **54**, 2908–15.
- Stenström, T. A. (1989). Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. *Applied and Environmental Microbiology*, **55**, 142–7.
- Stickler, D. J., Clayton, C. L. & Chawla, J. C. (1987). Assessment of antiseptic bladder washout procedures using a physical model of the catheterised bladder. *British Journal of Urology*, **60**, 413–8.
- Sutherland, I. W. (1977). Bacterial exopolysaccharides – their nature and production. In *Surface Carbohydrates of the Prokaryotic Cell*, pp. 27–96. Ed. Sutherland, I. W. Academic Press, London.
- Sutherland, I. W. (1985). Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. *Annual Review of Microbiology*, **39**, 243–70.
- Sutherland, I. W. (1988). Bacterial surface polysaccharides: structure and function. *International Review of Cytology*, **113**, 187–231.
- Svanborg Edén, C., Andersson, B., LeMer, H., Magnusson, G. & Noori, G. (1985). Specificity for glycolipid receptors of adhering bacteria: Experimental differences between uropathogenic *Escherichia coli* and *Streptococcus pneumoniae*. In *Molecular Basis of Oral Microbial Adhesion*, pp. 14–17. Eds Mergenhagen, S. E. & Rosan B. American Society for Microbiology, Washington, DC.
- Tormo, A., AlmirQorn, M. & Kolter, R. (1990). *sur A*, an *Escherichia coli* gene essential for survival in stationary phase. *Journal of Bacteriology*, **172**, 4339–47.
- Troy, F. A. (1973). Chemistry and biosynthesis of the poly (γ -D-glutamyl) capsule in *Bacillus licheniformis*. *Journal of Biological Chemistry*, **248**, 305–15.
- Tsai, Y.-L. & Olson, B. H. (1992). Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology*, **58**, 754–7.
- van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G. & Zehnder, A. J. (1987). Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology*, **53**, 1898–1901.
- van Loosdrecht, M. C., Lyklema, J., Norde, W. & Zehnder, A. J. (1990). Influence of interfaces on microbial activity. *Microbiological Reviews*, **54**, 75–87.

- Voordouw, G., Voordouw, J. K., Karkhoff-Schweizer, R. R., Fedorak, P. M. & Westlake, D. W. S. (1991). Reverse sample genome probing, a new technique for identification of bacteria in environmental samples by DNA hybridization, and its application to the identification of sulfate-reducing bacteria in oil field samples. *Applied and Environmental Microbiology*, **57**, 3070–8.
- White, D. C. & Findlay, R. H. (1988). Biochemical markers for measurement of predation effects on the biomass, community structure, nutritional status, and metabolic activity of microbial biofilms. *Hydrobiologia*, **159**, 119–32.
- Whitfield, C. (1988). Bacterial extracellular polysaccharides. *Canadian Journal of Microbiology*, **34**, 415–20.
- Wolin, M. J. & Miller, T. L. (1983). Carbohydrate fermentation. In *Human Intestinal Microflora in Health and Disease*, pp. 147–165. Ed. Hentges, D. J. Academic Press, New York.
- Wrangstadh, M., Conway, P. L. & Kjelleberg, S. (1986). The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. *Archives of Microbiology*, **145**, 220–7.
- Wrangstadh, M., Conway, P. L. & Kjelleberg, S. (1989). The role of an extracellular polysaccharide produced by the marine *Pseudomonas* sp. S9 in cellular detachment during starvation. *Canadian Journal of Microbiology*, **35**, 309–12.