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Growth of Microorganisms on Surfaces

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Introduction

Traditional microbiological investigations have focused on the culture and analysis of pure cell lines of bacteria, in either batch or chemostat culture. However, it has been clearly established that in nature, disease and industry, the majority of bacteria exist attached to surfaces within biofilms (Costerton *et al.* 1978, 1987; Lappin-Scott & Costerton 1989; Characklis *et al.* 1990a). Furthermore, it has also been established that the bacteria which exist in biofilms, termed sessile bacteria, are inherently different from bacteria existing in the planktonic state. In the sessile state, bacteria may express different genes, alter their morphologies, grow at different rates, or produce extracellular polymers in large amounts (Costerton *et al.* 1978; Wright *et al.* 1988; Gilbert *et al.* 1990; Dagostino *et al.* 1991; McCarter *et al.* 1992). One significant consequence of sessile growth is that biofilm bacteria are more resistant to medical and industrial control strategies than their planktonic counterparts (Brown *et al.* 1988; Nichols 1989; Eng *et al.* 1991; Blenkinsopp *et al.* 1992).

The development of complex attached and aggregated communities is also important for the survival and reproductive success of microorganisms. These communities have been considered to act as reservoirs for diverse species, sites of specific limited niches, and protective refuges from competition, predation or harsh environmental conditions, allowing otherwise poor competitors to survive. Integration into a biofilm or bioaggregate may be regarded as a survival strategy beyond that of maximizing or increasing the

growth rate. Placed in the context of survival and integration into a community, the evolutionary and ecological rationale behind the enormous range of diversity in cell surface chemistry, cell morphology, development of life cycles, complex pathways of microcolony development, and formation of syntrophic associations or consortia can be more easily rationalized.

In part, these adaptive strategies are related to the physical nature and scale of the microbial environment. An individual adherent bacterial cell is unable to alter significantly its surrounding chemical and physical environment (Koch 1991). In addition, many of the strategies are dictated by requirements for the growth of bacteria in close associations or groupings represented by biofilms or other bioaggregations. This is meaningful because in pure cultures, the requirements for growth of some groups of bacteria, such as sulphate reducing bacteria (SRB), methanogens, and syntrophic organisms, may only be met in specialized environments. The maintenance of a stable attached bacterial population is dependent on a constant flux of nutrients and wastes to and from the cells, therefore a sessile existence may not be sufficient to ensure bacterial reproductive success if conditions become unfavourable.

Wimpenny (1992) considered that the concept of an activity domain (that is, zones under the metabolic influence of one bacterium) is fundamentally linked to biofilm growth; when substrate/product sources and sinks associated with two physiologically distinct activity domains spatially overlap, the potential for beneficial or detrimental interaction exists. Functional consortia and microcolonies within quasi-organized

biofilms provide a wide range of growth requirements, for within these systems temporal and spatial development of chemical microzones (for example, E_h and pH), positioning of syntrophic partners, and establishment of complementary metabolic pathways may all occur. Thus, while a single bacterium may live in a hostile, diffusion dominated environment, sessile bacteria overcome these limitations by modifying their surroundings through association and diversity.

While adaptations to surface colonization are widely varied, there are some traits common to all sessile bacteria. For example, all must attach to surfaces or other bacteria, utilize available resources for growth and reproduction, then redistribute over surfaces and to new locations when conditions become less favourable. Such behaviour has been reported by a number of researchers describing stages of surface colonization and biofilm formation (Lawrence *et al.* 1987, 1992; Power & Marshall 1988; Korber *et al.* 1989a). Successful growth of sessile bacteria does not necessarily mean rapid growth. Rather, sessile bacteria must be able to survive and adapt over time. They do so by remaining simple, diverse, minimizing the constraints which a large gene load would place upon them, but remaining metabolically adaptable as a group. The requirements for the formation of favourable chemical and physical microenvironments make pure culture biofilms in nature a rarity, although within a biofilm distinct microcolonies may remain identifiable.

The array of tools available for the analysis of biofilms is impressive. These include high resolution video microscopy (Kjelleberg *et al.* 1982; Power & Marshall 1988; Shapiro & Hsu 1989), environmental scanning electron microscopy (Little *et al.* 1991), microelectrode analysis (Lens *et al.* 1993; Lewandowski *et al.* 1993), microbalance applications (Nivens *et al.* 1993) and scanning confocal laser microscopy (SCLM). Of these new methods, SCLM and microelectrode analyses are widely accepted for microbiological studies (Lawrence *et al.* 1991; Caldwell *et al.* 1992a, b; Korber *et al.* 1992; Lens *et al.* 1993; Lewandowski *et al.* 1993). The data generated from these techniques have altered our understanding of biofilms. Both pure culture and mixed species biofilms are now accepted as being spatially and temporally heterogeneous systems containing microscale variations in biofilm archi-

tecture and chemistry. The extent of analyses possible using SCLM is broad, because visual information may be correlated with fluorescent signals. SCLM is amenable to *in situ* analyses on a variety of surfaces. When used in conjunction with appropriate fluorophores, SCLM has the potential to define biofilm architecture, estimate cellular viability, identify microenvironmental chemical gradients, quantify molecular transport and molecular interactions, or phylogenetically analyse population architecture (Amann *et al.* 1992).

The nature of the surface microenvironment

Organisms move from the bulk aqueous phase to microenvironments on a surface where foci of growth have established. A number of investigations have examined surface physicochemistry or the effect of adsorbed molecular films on microbial attachment success (Fletcher 1976; Fletcher & Loeb 1979). Such models of microbe-surface interactions are valuable because, in nature, newly exposed surfaces become coated with organic material present within the aqueous solution, often masking the original chemistry of the surface. These coatings may then stimulate chemotactic bacteria to colonize these surfaces, or act as a potential nutrient source.

Surface conditioning films

Surface conditioning, or molecular, films are rapidly adsorbed whenever clean surfaces are exposed to natural and *in vitro* solutions containing organic molecules. The movement of organics from the bulk phase to the surface is primarily the result of molecular diffusion, described in quiescent or laminar systems by Fick's law of diffusion (Koch 1991). Theoretical and experimental studies both indicate that molecular diffusion occurs rapidly, resulting in significant organic deposits after only 15 min (Bryers 1987; Characklis *et al.* 1990a). For example, molecular coatings have been shown to result in 0.8–15 mg organic matter per m² of exposed surface material, equating to film thicknesses ranging from 30 to 80 nm (Loeb & Neihof 1975). Experimental results obtained using Fourier-Transformed Infrared spectroscopy (FTIR), Multiple

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Attenuated Internal Reflectance Infrared spectroscopy (MAIR-IR), and Infrared spectroscopy (IR) have indicated that polymers of glycoproteins, proteins, and possibly humic acids, each of which may contain as many as 100 000 molecular units per chain, may be involved in the formation of organic films (Marshall *et al.* 1971; Baier 1980; Rittle *et al.* 1990). Thus, an abundance of reactive binding sites would be available for further reaction with either additional solute molecules or with chemical groups expressed on bacterial surfaces.

Exposure of surfaces to organic compounds has resulted in various, measurable effects on the original surface properties. For example, both negatively and positively charged surfaces acquired net negative surface charges, and zeta potentials, surface contact angles and free energy vary as a function of their original surface energy (Fletcher 1976; Fletcher & Marshall 1982; Absolom *et al.* 1983; van Loosdrecht *et al.* 1987; Sjollem *et al.* 1988; Busscher *et al.* 1990b; Vanhaecke *et al.* 1990). The manner by which molecular films influence the adsorption of bacteria to surfaces still remains unclear except for specific cases; however, the general viewpoint is that chemical groups of the organic coating interact with chemical groups on bacterial appendages, such as pili, flagella or bacterial exopolysaccharides (EPS) (Paerl 1975; Dazzo *et al.* 1984; Vesper & Bauer 1986; Sjollem *et al.* 1990b). Such appendages extend through the free energy barrier and make contact with the organic matrix where the structure may form reversible short range electrostatic, covalent, or hydrogen bonds. The presence of organic molecules on surfaces also provides an explanation for the chemotactic movement and preferential positioning of many bacterial species on solid substrata (Berg & Brown 1972; Adler 1975; Kjelleberg *et al.* 1982; Block *et al.* 1983; Berg, 1985; Hermansson & Marshall 1985).

Surface related physicochemical forces

All surfaces (coated or non-coated) possess physicochemical characteristics which may influence the adsorption of bacteria and other molecules. At relatively short distances from the surface ($\leq 1-10$ nm), attractive and repulsive forces may influence molecular sorption (Bowen & Epstein 1979), defined by the DLVO theory.

However, the critical surface tension of the surface may also dictate whether a system containing bacteria will undergo a reduction in free energy: that is, whether bacteria adsorb or stay in suspension (Absolom *et al.* 1983). These physicochemical theories have been exhaustively tested as predictive models for bacterial-surface interactions.

DLVO theory

The DLVO, or double-layer theory, was originally formulated from the works of Derjaguin & Landau (1941) and Verwey & Overbeek (1948), and equates electrostatic forces and London-van der Waals forces active at the surface with the propensity for colloidal attraction and adhesion, generally given by Equation 1:

$$V_T(l) = V_A(l) + V_R(l) \quad (1)$$

The total interaction energy (V_T) of a particle as a function of its separation distance (l) from a solid surface is the sum of the van der Waals attraction (V_A) and the electrostatic interaction (V_R) (van Loosdrecht *et al.* 1987). The theory predicts that there are two regions where particle attraction may occur, that is, regions where small distances (≤ 1 nm) separate the substratum and approaching surface, and regions where larger distances (5–10 nm) separate the two surfaces (Bowen & Epstein, 1979; Characklis *et al.* 1990b). These locations are known as the primary minimum and secondary minimum, respectively, and are quite small in scale when compared with the thickness of the hydrodynamic boundary layer (see Brading *et al.* Chapter 2). Between these two positions is located an energy level where the surfaces experience maximum repulsion (an electrostatic repulsion occurs because the cell and substratum surfaces both carry a net negative charge), the magnitude of which is dependent on the surface potential of the particle and the substratum, the separation distance, and the electrolytic strength of the aqueous medium. Furthermore, because these interactions occur within the quiescent boundary layer, convective forces and attractive events may be mathematically isolated (Adamczyk & van de Ven 1981). More detailed DLVO based mathematical expressions incorporate the radius of the approaching particle (Sjollem *et al.* 1990a). As the radius decreases, so the repulsive energy barrier decreases.

A number of problems must be addressed if the electrical double-layer theory is to be adapted for the prediction of microbial adsorption tendencies. The original theorem was developed for shear free systems (that is, within the hydrodynamic boundary layer), whereas the majority of natural systems have dynamic fluid movement applying shear force to attaching cells approaching surfaces (Christersson *et al.* 1988; Characklis *et al.* 1990a; Mittelman *et al.* 1990). Also, a significant size differential exists between colloidal particles and bacterial cells, as well as the biogenic chemical differences caused by the ion and pH gradients at the boundaries of the cell (Boone *et al.* 1989). Lastly, geometrical considerations must be taken into account (Bowen & Epstein 1979), as cellular appendages (Isaacson *et al.* 1977; Vesper & Bauer 1986) alter the cell effective diameter near the surface and hence alter the diameter dependent repulsive effects experienced within regions of maximal repulsion between the primary and secondary minimum (Sjollem *et al.* 1990a). Overall, the application of DLVO based theory remains popular during formulation of theoretical cell adhesion models, despite discrepancies between observed and predicted results.

Surface free energy/hydrophobicity theory

The initial adsorption of bacteria may also be explained in terms of system free energy. If the total free energy of a system is reduced by cell contact with a surface, then adsorption of the cell to the substratum will occur (Absolom *et al.* 1983). Thermodynamically, a free energy balance may be developed on the basis of the interfacial free energies of the surfaces involved, as shown in Equation 2:

$$\Delta F_{adh} = \gamma_{bs} - \gamma_{sl} - \gamma_{bl} \quad (2)$$

where ΔF_{adh} is the free energy of adhesion, γ_{bs} is the interfacial free energy for the bacterial-substratum interface, γ_{sl} is the interfacial free energy for the substratum-liquid interface, and γ_{bl} is the interfacial free energy for the bacterial-liquid interface. The free energy, or wetting theory, does not depend on chemical measurements, but rather relies on an appropriate method for determining critical surface tension (σ_c) of the bacteria and substratum. This is commonly performed microscopically by measuring contact angles between defined liquids and surfaces or surface

preparations of microbial cells (Dexter *et al.* 1975; Fletcher & Loeb 1979; Fletcher & Marshall 1982; Fletcher 1988; Rittle *et al.* 1990). The Young equation describes the relationship between contact angle and the interfacial energies of the involved phases and their contact angles (Equation 3):

$$\gamma_{sv} - \gamma_{sl} = \gamma_{lv} \cos \theta_c \quad (3)$$

where γ_{sv} represents the solid/vapour phase, γ_{sl} equals the solid/liquid phase, and γ_{lv} the liquid/vapour phase.

In theory, a liquid which provides a contact angle of 0° should have a surface tension equivalent to the surface free energy of the substratum, where an increase in the measured contact angle corresponds to a decrease in the surface hydrophobicity and a decrease in surface free energy. The use of dried bacterial lawns for the calculation of bacterial contact angles has been disputed, with supporters of alternative techniques maintaining that irreversible changes in the adsorbed macromolecules occur which influence the determination. Fletcher & Marshall (1982) suggested that bacterial surface free energies should be measured in a more natural state (i.e. within a liquid) using the bubble contact angle method, where the effect of adsorbed macromolecules or bacteria can be conducted in aqueous solution. Both methods use the contact angle of the liquid or bubble with the substratum to determine the critical surface tension for the test surface after conditioning. Studies utilizing these criteria to explain bacterial adhesion events have demonstrated a range of results using various substrata, bacterial species, and assay methodology (Dexter 1979; Absolom *et al.* 1983; van Pelt *et al.* 1985; Burchard *et al.* 1990; Rittle *et al.* 1990; Sorongon *et al.* 1991). This variability of experimental results is due largely to the lack of standard methods as well as the heterogeneous, physicochemical nature of the bacterial cell.

Overall, physicochemical models of surface interactions assume that the surfaces are small, smooth and energetically homogenous; however, this is rarely true for bacteria (Costerton *et al.* 1978; Busscher *et al.* 1990a). As a consequence, simple physicochemical approaches which fail to incorporate the microscopic condition of the cell outer surface or adaptive microbial behaviour will never fully explain all aspects of bacterial adhesion.

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System hydrodynamics

Bacterial surface colonization success is dependent on a flux of cells from the bulk phase to surface microenvironments where attachment may occur. Bacterial flux is largely governed by the hydrology of the fluid system (such as static, turbulent, or laminar), the concentration gradient, the nature of the organism (such as size or surface characteristics) and behaviour (Powell & Slater, 1983; Sjollem *et al.* 1988, 1989a; van Loosdrecht *et al.* 1989; Korber *et al.* 1990). Fluid hydrodynamics are important and often paramount considerations in industrial, medical and natural systems (Costerton *et al.* 1978; Lappin-Scott & Costerton 1989; Characklis *et al.* 1990a; Christensen & Characklis 1990), where cell deposition precedes metal corrosion and microbial infection, as well as a number of other deleterious and beneficial processes. In addition, the flow regime also influences the nature of the physical and chemical surface microenvironment after a biofilm has been established. It is therefore important that the dimensions of flow systems and fluid velocity be carefully controlled or defined (for example, Reynolds number) to reflect the system being modelled. Further details of these parameters, including the hydrodynamic boundary layer, are covered by Brading *et al.* (Chapter 2).

Static systems

Within quiescent environments, factors such as Brownian diffusion, gravity and motility all assist the movement of small particles, including bacteria, to surfaces (Bryers 1987). However, the relative importance of each of these mechanisms varies with specific conditions. Based on the size of most bacterial cells, Brownian motion or diffusion may contribute to the overall attachment of non-motile cells; however, the extent of these contributions is reportedly low (Characklis *et al.* 1990b). It is generally concluded that motility greatly increases the chance that a bacterium may contact a potential attachment surface (Fletcher 1977; Marmur & Ruckenstein 1986) with enough potential energy to overcome the electrical repulsive forces which exist between the bacterium and substratum. During studies where the motility of bacteria was observed to decrease with culture age, concurrent decreases in cellular

adsorption were also noted (Fletcher 1977). Similarly, cell transport resulting from gravitational cell sedimentation in static systems has also been documented as an effective surface colonization mechanism (Walt *et al.* 1985).

In the absence of flow, the thickness of the hydrodynamic boundary layer (Brading *et al.* Chapter 2) can be effectively large, consequently limiting the success of organisms established in these zones by restricting nutritional transport to diffusion and convection. In nature, static aqueous systems are rare as some mechanism for mixing usually exists. However, studies applying static regimes may be useful as the conditions within biofilms are largely insulated from flow, therefore applying a static model to biofilms formed under flowing conditions (for example, removing flow after a growth period) may provide insight into nutrient depletion responses of sessile bacteria, as well as the formation or dissipation of chemical microzones.

Overall, the use of batch static systems for the study of bacterial deposition has been described by some as being poorly controlled and random (Sjollem *et al.* 1989a), dependent to a large extent on the suspension volume to total substratum area ratio, and not an appropriate model of most environments. However, the general trends observed during such studies remain valuable provided that the testable hypothesis incorporates an appropriate control where relative differences in adhesion success may be measured.

Diffusion

An understanding of diffusion is fundamental to biofilm ecology. In general terms diffusion is a process which drives motion in biology (Koch 1991). Diffusion is caused by the random, thermally energetic movement of particles from regions of high concentration to low concentration, as described by Fick's first law. This flux (dq/dt) is dependent on the solute concentration gradient (dC/dX), the area of the material through which the solute is diffusing (A), and the diffusion constant for the solute (D) (Equation 4).

$$dq/dt = -DA dC/dX \quad (4)$$

During the early stages of biofilm development in flowing microenvironments, attached organisms may be influenced by advection and flow. However, as growth proceeds, the hydrodynamic

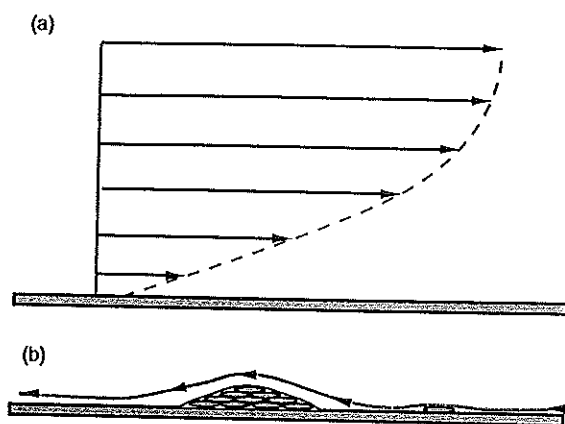


Fig. 1.1. (a) Illustration showing the distance-dependent nature of flow associated with surfaces, where bulk phase flow velocities are experienced at large distances from the surface and a no-slip condition is found at very small distances from the surface. (b) The effect of microcolony/biofilm formation on the nature of the hydrodynamic boundary layer (i.e. moves it away from the surface). Even in systems dominated by laminar flow, turbulent vortices may form in the regions of obstructions such as microcolonies or biofilm biomass.

boundary layer moves upward with the biofilm-liquid interface as the biofilm increases in depth (Fig. 1.1). Within the EPS matrix, rate limitations to growth and mass transport are now theoretically governed solely by diffusion, and as source and sink concentrations of various compounds invariably approach steady state, solute flux becomes a constant proportional to the distance of migration.

The thickness of microbial biofilms is known to influence a number of biotic and abiotic processes in flowing systems. Heat transfer efficiency, fluid frictional resistance, and chemical transformations are industrially significant examples of detrimental biofilm effects (Trulear & Characklis 1982; Characklis *et al.* 1990b). Biofilm thickness may also affect the transfer of essential nutrients such as oxygen and other metabolites into growing biofilms by increasing the diffusional length where physiological substrate consumption may limit the concentration of the metabolite before penetration to the base of the film (Sanders 1966; Howell & Atkinson 1976; Cox *et al.* 1980; Christensen & Characklis 1990). Hindered diffusion may also play a role within the biofilm cell-EPS matrix. Hindered or restricted diffusion occurs when the effective

diffusivity of a solute within a pore of comparable size is less than its value in bulk solution (Bohrer *et al.* 1984). Within aqueous solution, the diffusion of many biologically significant molecules are adequately described by Einstein's solution in Equation 5:

$$D_{\infty} = kT/f_{\infty} \quad (5)$$

where D_{∞} is the bulk solution diffusion coefficient, k is Boltzmann's constant, T is temperature, and f_{∞} is the molecular friction coefficient in bulk solution. Assuming that the diffusing molecule is spherical, f_{∞} may be determined using $f_{\infty} = 6\pi\mu r_s$, where r_s is the solute radius and μ the solvent kinematic viscosity. This conceptual model of molecular diffusion through barriers with a defined mesh size has been confirmed during biofilm and membrane diffusion studies, with reduced, or hindered diffusion being measured in the biofilm or membrane system relative to the theoretical rates of diffusion in the aqueous phase (Acker & Steere 1962; Bohrer *et al.* 1984). Hindered diffusion thus has relevance to biofilm systems with regard to molecular sieving and size dependent exclusion of molecules from the EPS matrix (Lawrence *et al.* 1994).

Fluid flow has a profound effect on diffusion even though the effective diffusion coefficient (D_e) remains constant for a given biofilm matrix (Freeze & Cherry 1979; Koch 1991). However, maintenance of a concentration gradient by flow replenishment of, for example, substrate molecules in the hydrodynamic boundary layer can lead to large increases in diffusion limited biofilm events such as growth (Characklis *et al.* 1990a). In addition to maintaining a steeper concentration gradient, high flows tend to reduce the thickness of the hydrodynamic boundary layer, decreasing the distance between molecular sources and sinks. The importance of flow to aqueous systems is much greater than within the atmosphere where much slower rates of diffusion for similar molecules in water have been reported (Vogel 1983). It thus becomes apparent that within aqueous systems of increasing viscosity, diffusion becomes a less effective mechanism of molecular transport.

Many articles examining diffusion or relevant to diffusion in biological systems have been published, the reader is directed to several of the excellent review articles on this topic (Freeze & Cherry 1979; Characklis *et al.* 1990b; Koch

1991). In addition to providing useful insight into molecular transport and nutrition, it is significant that diffusion governs the movement of bacteria to surface sites, demonstrated by predictive mathematical models of non-motile and motile bacterial transport based on Fick's law of diffusion (Jang & Yen 1985). Modifications of these formulae have also been used to predict directed chemotactic migration of bacteria (Rivero & Lauffenburger 1986; Kelly *et al.* 1988).

Quantifying behaviour and kinetics of surface colonization

The events which occur following initial attachment, including microcolony formation, aggregation, and development of syntrophic interactions or consortia, are increasingly recognized as being extremely important for understanding the adaptive significance of surface associated microbial growth. Thus, a large number of techniques can be and have been applied to these studies. The most common approach has been the use of a variety of transparent and opaque substrates for culture and subsequent observation by direct microscopic or other non-conventional techniques.

Cultural methods

Cultural methods include the use of glass slide incubation (Cholodny 1930; Henrici 1933; Bott & Brock 1970; Staley 1971), incubation of electron microscope grids (Drucker & Whittaker 1971; Hirsch & Pankratz 1971), capillary techniques (Perfil'ev & Gabe 1969), and agar block microculture (Torrella & Morita 1981). A variety of flow cells and microperfusion chambers have also been used to study attached growth by microorganisms (Duxbury 1977; Caldwell & Lawrence 1986).

Observation and analysis

Bacterial behaviour and the development of colonies and biofilms may be followed using cinemicrographic techniques (Wilkins *et al.* 1972), video, time lapse video recording, or digitization (Kjelleberg *et al.* 1982; Caldwell 1985; Power & Marshall 1988). Typically, images have been obtained using transmitted, incident or

polarizing light microscopy techniques, as outlined by Marshall (1986). These images are all amenable to digital image analysis for extraction of data on growth rates, cell size, rates of motility and a variety of other parameters (Lawrence *et al.* 1989a, b; Caldwell *et al.* 1992a, 1993). Over the past decade, computer enhanced microscopy and image analysis have been utilized to study the positioning of bacteria within surface micro-environments, permitting rapid, precise analysis and quantitative data collection. While the majority of these studies have been directed at the enumeration of cells present or being deposited on the surface of glass slides (Sieracki *et al.* 1985; Sjollem *et al.* 1988, 1989b), computer enhanced microscopy (CEM) has also been applied to the analysis of surface growth, positioning, and locomotion of cells colonizing surface environments (Gualtieri *et al.* 1985, 1988; Caldwell & Lawrence 1986; Lawrence & Caldwell 1987; Lawrence *et al.* 1987; Korber *et al.* 1989a, 1990). Other established techniques include the application of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) for observation of microcolonies on a variety of surfaces (Costerton *et al.* 1981; Handley *et al.* 1985) and studies of bioaggregates (Bochem *et al.* 1982; MacLeod *et al.* 1990).

Recent advances in techniques

More recently the application of direct microscopy utilizing scanning confocal laser microscopy (SCLM) with molecular probes and image analysis has proven an effective method for analysis of microcolonies and biofilms (Lawrence *et al.* 1991; Caldwell *et al.* 1992a, 1993). Application of SCLM technology has led to rapid developments in the study of thick biofilms without the need for disruptive or sacrificial preparation procedures (Lawrence *et al.* 1991; Caldwell *et al.* 1992a, b; Korber *et al.* 1992, 1993; Wolfaardt *et al.* 1994). Use of defined fluorescent molecular probes such as viability indicators, 16S rRNA oligonucleotides, and chemically sensitive molecules in conjunction with SCLM may help elucidate the underlying mechanisms and environmental conditions responsible for behavioural variability of sessile bacteria (Amann *et al.* 1992; Caldwell *et al.* 1992a; Rodriguez *et al.* 1992). Methods such as X-ray microanalysis, FTIR and NMR (nuclear magnetic resonance) have also

provided valuable insights into events within biofilms and microcolonies (Geesey & White 1990). Use of microelectrode techniques to study bacteria on surfaces and in aggregates also provides a wide range of data on activities within bacterial associations (Alldredge & Cohen 1987; Lewandowski *et al.* 1989, 1993; Revsbech 1989; Lens *et al.* 1993).

The techniques mentioned above have been extensively reviewed (Marshall 1986; Revsbech & Jorgenson 1986; Shotton 1989; Geesey & White 1990; Caldwell *et al.* 1992a, 1993).

Microcolony formation strategies

Bacterial behaviour and life cycles at surfaces

The behaviour of microorganisms includes interactions with their environment, such as the solutions surrounding the bacterium, the physical conditions to which the bacterium is exposed, and other bacteria inhabiting similar habitats (Lauffenburger *et al.* 1984; Lawrence & Caldwell 1987; Arnold & Shimkets 1988; Shapiro & Hsu 1989; Shimkets 1990; Lawrence *et al.* 1992). Where the conditions of study are defined and constant, any pattern evident in the growth, development and translocation of cells or colonies may be deemed as a behavioural trait characteristic of that particular bacterial strain (Lawrence & Caldwell 1987; Lawrence *et al.* 1987).

The formation of microcolonies or cellular groups is one of what may be a series of states comprising a life cycle. Life cycles of surface colonizing bacteria range from that of alternation between stalked mother cell and swarmer cell in *Caulobacter* spp. or an attached cell giving rise to free living progeny (e.g. *Rhizobium* spp.), to the complex multifaceted life cycles of myxobacteria (Arnold & Shimkets 1988; Shimkets 1990). The simplest form of the life cycle is that which separates growth and reproduction from a migratory or dispersal phase, a pattern which is repeated throughout many genera of sessile bacteria. During the attached phase of the life cycle, growth and microcolony formation proceed through a range of patterns, including the well documented behaviour of the prosthecate bacteria, as well as many not as extensively studied (Hirsch 1984; Lawrence & Caldwell 1987;

Lawrence *et al.* 1987, 1992; Power & Marshall 1988). Such behaviour represents the diversity of approaches to colonization and occupation of the substratum, redistribution on the substratum, and dispersal of daughter cells to the aqueous phase.

Motile attachment behaviour

The motile attachment hypothesis is based on behavioural and CEM analyses of cells moving along surfaces (Lawrence *et al.* 1987). During motile attachment, bacteria may travel upstream or cross stream against flow velocities exceeding the cells' maximum rate of motility or simply move along the surface while remaining in a semi-attached condition (Lawrence *et al.* 1987). Additional support for the existence of motile attachment behaviour was provided by Korber *et al.* (1989b) following observations of flow independent behaviour of *Pseudomonas fluorescens* on surfaces where the laminar flow velocity was $200 \mu\text{m s}^{-1}$, and also by Malone (1987), who reported that various *Rhizobium* spp. travelled upstream against flow rates exceeding their maximum reported rates of motility. However, the mechanisms responsible for flow independent cell movement are not known. Consequences of motile attachment include lateral colonization (also termed backgrowth) against the direction of flow. For example, motility facilitated the backgrowth of a mot⁺ *P. fluorescens* strain in continuous flow slide culture against microenvironmental flow rates greater than the maximum rate of motility (Korber *et al.* 1989b).

Gliding motility is well documented for *Myxococcus xanthus*, where locomotion is achieved by the smooth movement of cells in the direction of their long axis without the use of flagella; however, the mechanism of cell translocation is poorly understood. Overall, reports of movement of surface-associated cells are common, although the significance of such behaviour remains unclear. Explanations for motile attachment include bacterial positioning, nutrient capture strategies, and cellular migration within rapidly flowing systems. Perhaps the most obvious microbial behaviour in this context is the chemosensory and motile responses which they exhibit in natural and experimental aqueous environments (Smith & Doetsch 1969; Pilgram & Williams 1976; De Weger *et al.* 1987; Willey &

Waterbury 1989). Less apparent are the cell-cell and cell-surface interactions exhibited by motile microorganisms during colonization and growth on surfaces (Shapiro & Hsu 1989; Shimkets 1990). However, the analysis of interactive and surface associated microbial behaviour represents a relatively new area of research and further investigations are needed in this topic.

Reversible and irreversible attachment

The initial attachment of bacteria to surfaces is considered to occur in two stages: the reversible attachment phase and the irreversible attachment phase (Marshall *et al.* 1971; Lawrence *et al.* 1987; Marshall 1988). These initial attachment events frequently involve the attachment of bacteria to surfaces by a portion of the cell or flagellum while the cell continues to revolve (Meadows 1971; Lawrence *et al.* 1987; Malone 1987; Marshall 1988). Spinning behaviour may then be followed by either irreversible cell attachment or cell detachment. Powell & Slater (1983) concluded that if it was assumed that all cells which contacted the surface became irreversibly adsorbed, the resultant population of bound cells would be grossly overestimated, and that reversible attachment was predominant in nature. Marshall (1988) reported that cells became irreversibly attached to surfaces only after a period of unstable attachment, during which time cells revolved around the axis of attachment and frequently emigrated from the attachment site rather than becoming irreversibly attached. Others have made comparable observations during continuous flow slide culture of monoculture strains (Lawrence *et al.* 1987; Malone 1987) and also by surface colonizing bacteria observed in a marine microcosm (J. R. Lawrence, D. R. Korber and D. E. Caldwell, unpublished data).

Spinning behaviour by cells reversibly bound to surfaces has also been exploited to examine the mechanisms of rotation. Silverman & Simon (1974) studied rotation of motile *Escherichia coli* artificially tethered to surfaces by anti-flagellum antibodies. One explanation for the existence of spinning behaviour in nature might be the function of a chemosensory mechanism whereby the cells determine, in some manner, the suitability of potential colonization sites by binding, rotating, and sensing the ambient conditions through chemoreceptors (Lawrence *et al.* 1987).

It has been considered that the absence of a functional flagellum should limit the reversible attachment phase as well as the overall attachment, recolonization, and distribution success of non-motile organisms (Lawrence *et al.* 1987). These hypotheses have been confirmed by the studies of Korber *et al.* (1989b, 1990, 1993), during examinations of the adaptive significance of motility with respect to cell positioning, recolonization, and structural rearrangements within biofilms.

Bacterial surface colonization strategies

Strategies involving solitary cells

Some bacteria are adapted to grow at interfaces and readily attach; however, the progeny may return to the planktonic state. Lawrence & Caldwell (1987) termed this phenomenon shedding behaviour, where undifferentiated cells adhere and grow in a perpendicular manner, releasing daughter cells successively after discrete growth intervals (Lawrence & Caldwell 1987; Lawrence *et al.* 1989a). Similar behaviour has been described for attached cells by Henrici & Johnson (1935), Helmsetter (1969), Bott & Brock (1970), and Marshall & Cruickshank (1973). Bacteria utilizing this mode of attachment behaviour appear to have a some specialized polar structure, that is, the adhesins of *Caulobacter* spp. or the hydrophobic/adhesive polar regions described for *Flexibacter* sp. (Marshall & Cruickshank 1973).

In some instances this surface colonization pattern has been studied in more detail. Kjelleberg *et al.* (1982) described the attachment of starved *Vibrio* DW1 adhering to a surface in a perpendicular position and following growth of the attached cells; motile daughter cells were released from the mother cells at regular intervals. In the case of *Rhizobium* spp., motile daughter cells were released which subsequently reattached at vacant surface sites and underwent growth and release of their own daughter cells. Additional variations on this behavioural theme have been reported by other authors. Power & Marshall (1988) described *Vibrio* MH3 which grows associated with the surface but completes cell division in the planktonic state.

Attached bacteria may grow and divide, with progeny gradually but continuously moving away

from other members of the spreading microcolony. Power & Marshall (1988) described this type of behaviour in *Pseudomonas* JD8. After each growth and division cycle the daughter cells of JD8 migrated away from each other while still adhering to the substratum. The mechanism proposed was related to utilization of the surface bound substrate stearic acid. When the nutrient concentration declined in the microenvironment surrounding the cell it became reversibly attached and moved until encountering more substrate. This cycle was repeated until all substrate was utilized, at which time the cells returned to the planktonic state. Organisms which exhibit such spreading surface colonization behaviour have also been observed in natural stream communities (Lawrence & Caldwell 1987). This type of behaviour can only be detected by continuous observation and thus its significance may be underestimated.

Other organisms grow and divide on surfaces while in a semi-adherent or motile state. Gliding bacteria are the most common bacteria that exhibit this type of surface association. A type of motile attachment, or rolling behaviour, has been documented by Lawrence & Caldwell (1987), where organisms were followed for extended periods with the growth, division, and release of daughter cells all conducted without the parent cell ever becoming fixed to the substratum. It is thought that the microorganisms moved in contact with the surface within the secondary minimum of the diffuse double layer, but were not immobilized.

Studies comparing the growth rates of attached bacteria indicated that solitary cells growing in the same environment exhibited higher specific growth rates than bacteria forming extensive microcolonies or groups (Lawrence & Caldwell 1987). This suggested that these pathways functioned as positioning mechanisms optimizing intercellular distances, as well as balancing substrate removal and waste accumulation (Lawrence & Caldwell 1987). However, detailed study of the growth rates of shedding cells has also indicated that the attached mother cell exhibited a declining growth rate with each subsequent generation. Observations performed on soil and marine biofilms have indicated that the presence of predation pressure virtually eliminated bacteria exhibiting these solitary behavioural patterns with the apparent exception of prosthecate bacteria (J. R. Lawrence & D. R.

Korber, unpublished data). Thus, these strategies may be those of pioneer colonizing organisms whose significance in mature biofilm communities is not known.

The budding and prosthecate bacteria

These bacteria represent a specialized group of bacteria with unique cell division and cell association strategies. However, other than the obvious division between planktonic swarmer and attached mother cell in the life cycle, and possible advantages in niche and resource exploitation, no clear understanding of the particular advantages of the strategies of these bacteria exists. Their patterns of growth have been extensively described (Hirsch 1974). For example, attached *Pedomicrobium* spp. initiated hyphal elongation with 1-4 branching hyphae arising from the central mother cell. Hyphal elongation continued with the initiation of bud formation and subsequent bud growth prior to the release of daughter cells.

Lawrence *et al.* (1989b) used computer enhanced image analysis to quantitate the surface growth and behaviour of *Pedomicrobium* spp. present within a marine microcosm and cultured using continuous flow slide culture. Behaviour similar to that reported by Hirsch (1974) was observed for *Pedomicrobium* spp., with cells attaching and developing hyphae, and later bearing daughter cells on their apical tips. The hyphae extended along the surface and also into the surrounding medium away from the attachment surface. Image analysis was used to increase the effective depth of field using images obtained from different focal planes. These allowed the determination of cell area, hyphal length, and the growth rates of mother and daughter cells. Hyphae doubled in length every 2.6 h until the onset of bud formation, during which time only slight extension of hyphae occurred. Growth rate differences between mother and daughter cells on surfaces were also documented (that is, the time to double for mother cell, 24 h; time to double for daughter cell, 1.2 h). The daughter cells were released into the bulk phase and eventually recolonized vacant sites within the flow cell system, resulting in the formation of a network of hyphae and cells. Rosette formation in these bacteria has also been extensively described and studied (Stove-Poindexter 1964; Hirsch 1984).

Colonial strategies

Hirsch (1984) documented many forms of microcolonies including coenobia, nets, mycelia, multiple parallel cell arrangements, and rosette formation. In most cases, it is not established how these various groupings of cells arise or what their adaptive significance may be. However, with the increasing interest in this aspect of microbial behaviour, some insight into pathways and even genetic and environmental control of these pathways has been obtained. Unique paths of microcolony development have been documented for a number of genera including *Pseudomonas* spp., *Rhizobium* spp., *Vibrio* spp. and *Caulobacter* spp. (Lawrence *et al.* 1989b, 1992; Caldwell *et al.* 1992a) and various unidentified bacteria in environmental samples (Torrella & Morita 1981; Lawrence & Caldwell 1987). Each of these pathways of microcolony development contains a variety of strategies for releasing daughter cells and returning them to the planktonic or migratory state.

The arrangement of cells within a microcolony is a function of the cells' shape, size, mode of cell wall growth, polymer production, and poorly understood events occurring at the moment of cell division. For example, a snapping cell division in *Corynebacterium* spp. gave rise to V-shaped daughter pairs (Hirsch 1984). Similar movements at the time of separation of daughter cells have been described for other bacteria such as *P. fluorescens* and *E. coli* (Lawrence *et al.* 1987; Shapiro & Hsu 1989). The specific alignments of cells that occur during microcolony formation have been interpreted to indicate that a variety of complex cell-cell interactions do occur within the developing microcolonies (Shapiro & Hsu, 1989). Extracellular polymers also play a critical role in microcolony development. For example, Allison & Sutherland (1987) showed that a freshwater bacterium and a non-EPS producing mutant colonized surfaces equally, but only the former could produce microcolonies.

What has been termed 'packing' colony formation behaviour is commonly observed during surface growth in natural communities by pseudomonads and vibrios (Torrella & Morita, 1981; Lawrence & Caldwell, 1987; Lawrence *et al.* 1987, 1992; Korber *et al.* 1989a). In this case, the irreversibly attached cells grow and divide while forming a tightly packed group of cells at a

discrete surface location. *P. fluorescens* strain CC840406-B followed a developmental pathway that proceeded from single cells to a monolayer microcolony containing between 8–16 cells (after 4–5 h surface growth; Lawrence *et al.* 1987). Following the 8–16 cell stage, additional growth resulted in the release of some colony members into the aqueous phase, those detached bacteria being termed 'recolonizing' or migratory cells. Cells which successfully recolonized were observed to reattach at vacant surface sites, thereby creating new focal points of growth and aiding in the formation of a confluent biofilm.

Other *Pseudomonas* spp. exhibited paths of microcolony development and timing of recolonization different to that of *P. fluorescens*. Delaquis (1990) observed that *P. fragi* microcolonies displayed a high degree of structural organization. Colonies developed via a packing manoeuvre, resulting in a monolayer of regular, circular colonies. After 7 h, detachment and recolonization of motile cells occurred, marked by an increase in the number of microcolonies per field and also by an increase in the number of motile cells present in the surface boundary layer. After approximately 8 h surface growth, it was apparent that a secondary structure of polarly attached cells had developed on microcolonies, which subsequently matured into a palisade layer. The palisade layer may remain intact and contribute to the development of a confluent biofilm structure.

Vibrio parahaemolyticus is an autochthonous estuarine organism isolated from water sediment, marine organisms and inanimate surfaces, and is also associated with gastroenteritis in humans. As such, it exhibits a broad array of adaptive flexibility. Microcolonies of *V. parahaemolyticus* formed by two pathways which were variations of the packing behaviour described above. Behavioural analysis of *V. parahaemolyticus* strains in CFSC showed that during growth on surfaces, microcolonies consisted of a variety of functional types (Lawrence *et al.* 1992). These included laterally flagellated progeny adapted for attachment in low viscosity environments or migration in high viscosity environments. In addition, polarly flagellated progeny adapted for detachment and dispersal in low viscosity environments were also produced. The mechanisms controlling the formation of these types included induction of the lateral flagella gene system (McCarter *et al.* 1992). In addition, phenotypic switching between a

translucent variant and an opaque variant occurred, the latter only having polar flagella (Lawrence *et al.* 1992). Thus, *V. parahaemolyticus* microcolonies could form opaque or translucent variants, or more commonly as mixed populations. This system provided laterally flagellated bacteria adapted for attachment to the surface in low viscosity environments and motility in high viscosity environments, or polarly flagellated progeny adapted for detachment and dispersion. The opaque variant provided a continuous source of variation, creating a population suited for planktonic growth (Lawrence *et al.* 1992). During the migration or recolonization phase which occurred after 5 h of surface growth, *V. parahaemolyticus* cells detached from microcolonies and either remained planktonic, or reattached. The sites of reattachment indicated that cell-cell interactions were also important in *V. parahaemolyticus* behaviour, because cells aggregated at the initial sites of colonization. In addition, extensive interactions were observed between laterally flagellated cells and microcolonies in high viscosity microenvironments (Lawrence *et al.* 1992).

Dispersal

In each of the cases of microcolony development described above, after a species specific period of growth on a surface, attached bacteria underwent a redistribution phase. Lawrence *et al.* (1987) termed this event 'recolonization', a phase which for *P. fluorescens* included the emigration of attached cells from the surface to the suspended phase and the subsequent reattachment of those cells to vacant surface sites. The timing of the onset of this phase varied between species as well as with the conditions under which the cells were growing. For example, attached *P. fluorescens* growing in continuous flow slide culture recolonized after approximately 5 h, whereas *V. parahaemolyticus* grown in 75% 2216 medium recolonized after approximately 4 h. A typical data set for *V. harveyi* illustrating the growth phase, increase in number of motile cells and increase in microcolony number is shown in Fig. 1.2. A series of darkfield images showing the actual growth and development of the microcolonies is shown in Fig. 1.3. In this instance, redistribution occurred following only 2 h of surface associated growth. These variations in the

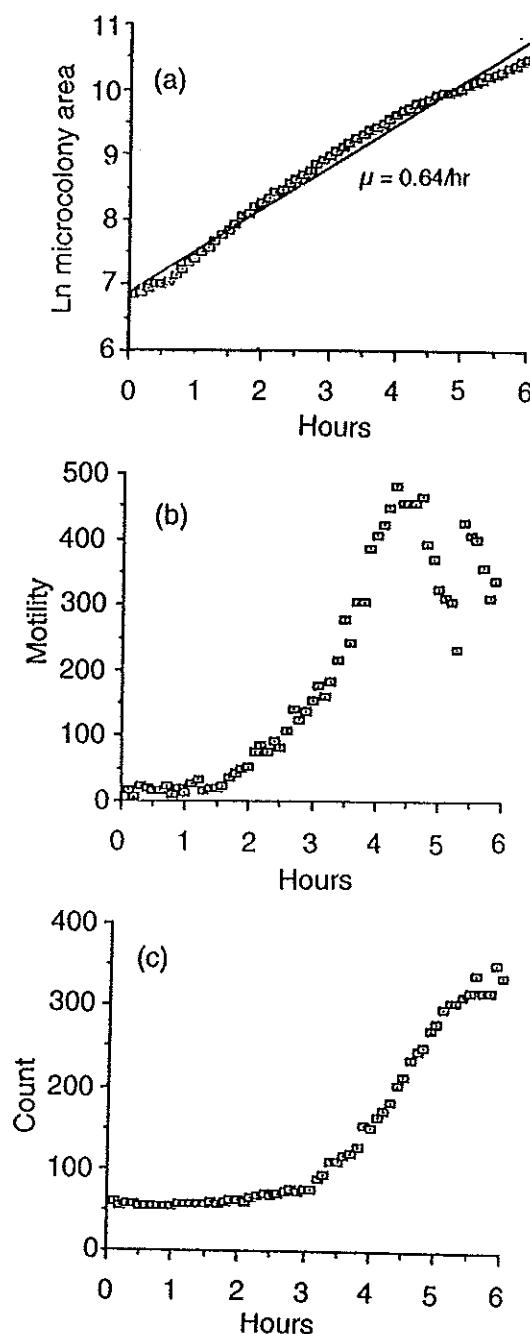


Fig. 1.2. Analysis of *Vibrio harveyi* biofilm development showing (a) biofilm development rate determined using darkfield microscopy and digital image analysis, (b) increase in the number of motile cells in the hydrodynamic boundary layer, and (c) increase in number of attached cells and microcolonies with time. Note the increase in number of motile cells at approximately 2 h and the subsequent increase in number of attached cells and microcolonies.

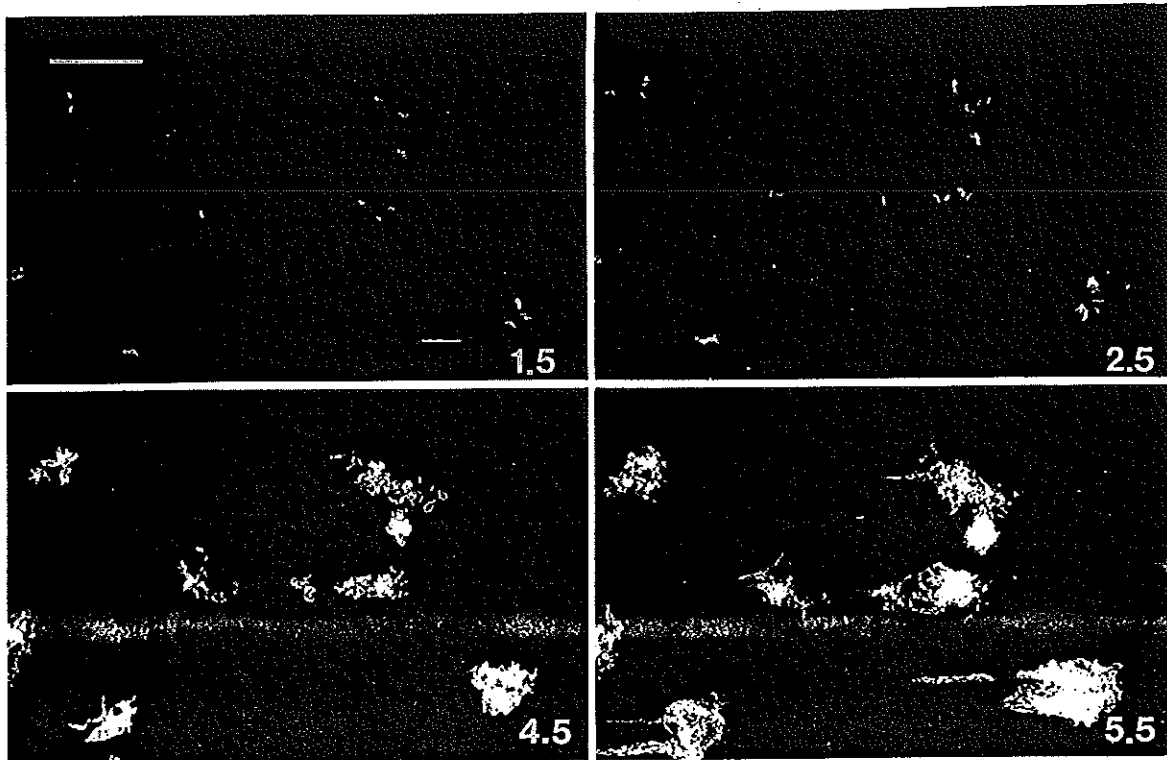


Fig. 1.3. Darkfield photomicrographs showing growth and microcolony formation during surface colonization by *V. harveyi*. Times indicated are 1.5, 2.5, 4.5 and 5.5 h after inoculation of the flow cell. The phenomena of formation of elongated cells and of aggregation around initial microcolonies are also evident in these photomicrographs. Bar = 20 μ m.

timing of cell redistribution represent important behavioural characteristics which appear to be species specific. This event provided a mechanism whereby cells emigrated from heavily colonized regions, where growth factors may be depleted, to regions more amenable to rapid growth. However, in detailed studies of individual cells within microcolonies, no evidence for a change in growth rate as a trigger has been detected (J. R. Lawrence & D. R. Korber, unpublished data). It is more likely that this phenomenon represents variable expression of traits, spontaneous variants, or other mechanisms which generate diversity allowing organisms to adapt to changing environments and providing at least two morphogenic states optimal for different circumstances.

Aggregation and coaggregation

Behaviour such as aggregation appears to play an important role in the development of micro-

colonies, and subsequently, in biofilm architecture. Kolenbrander (1989) noted that nearly all human oral bacteria participate in intergeneric coaggregation. Sjollem *et al.* (1990a) used the term cooperative effects among oral bacteria to describe a microbial process by which adhering cells can modify their surrounding environment into a more favourable one for further attachment. In addition, a unanimous conclusion from surveys on more than 700 strains of oral bacteria was that partner recognition during bacterial coaggregation is very specific (Kolenbrander 1989). Coaggregation may occur intergenerically, intragenerically and multigenerically, and these interactions are extremely important in the colonization and occupation of substrata. These phenomena have been reviewed in detail by Kolenbrander (1989) and Kolenbrander & London (1992).

The existence of extensive aggregation during the development of *V. parahaemolyticus* micro-

colonies has been described (Lawrence *et al.* 1992). In addition, cell aggregation was still evident in the form of distinct microcolonies attached to the surface at the base of the biofilms after 12 and 24 h growth. However, the architecture of the biofilms was also influenced by migration within the biofilm, a phenomenon apparent in the development of a diffuse basal layer with numerous channels in *V. parahaemolyticus* biofilms after 24 h growth (Lawrence *et al.* 1991). Similarly, observations of the growth and development of *P. fragi* microcolonies indicated that after onset of migration an extensive layer of vertically attached cells developed on the surface of the initial microcolonies. This layer has also been observed in biofilms of *P. fluorescens*, suggesting that some cells may aggregate to form specialized structural elements during biofilm development (Korber *et al.* 1993; Lawrence & Korber 1993). The nature of the cellular interactions in biofilm formation may be further investigated through the use of non-motile mutants.

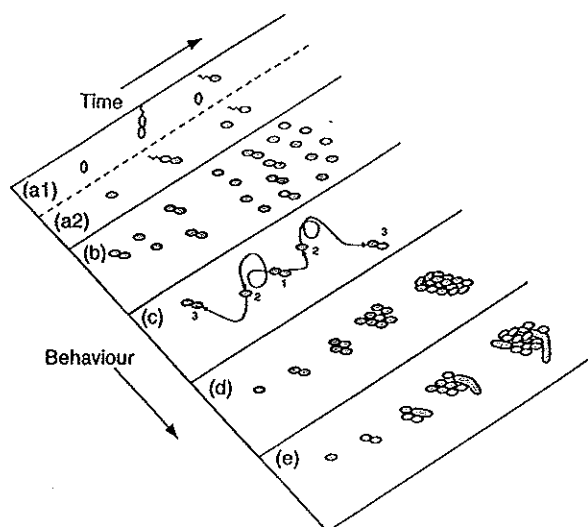


Fig. 1.4. Illustration showing the strategies exhibited by various surface-colonizing bacteria. (a1) and (a2), the attachment of a mother cell, in polar and horizontal orientations, with growth and subsequent release of the daughter cell to the planktonic state. (b) the attachment, growth, and slow migration of daughter cells (spreading). (c), growth and division during motile attachment. (d), typical microcolony development (packing) exhibited by *Pseudomonas* and other species. (e), microcolony development with variant production and aggregation exhibited by *Vibrio* spp.

Studies of this nature have been carried out using wild type and *mot⁻* mutants of *P. fluorescens* (Korber *et al.* 1989b, 1992, 1993). These latter studies have concluded that many of the structures which develop in microcolonies and within biofilms require active, motility dependent, cell-cell interactions. The range of surface colonization behaviour exhibited by various bacteria is summarized in Fig. 1.4.

Control of microcolony formation

Surfaces may stimulate, inhibit, or have no effect on the activity of microorganisms at interfaces (van Loosdrecht *et al.* 1990). However, microscopy has shown that in many cases surface associated growth results in the formation of microcolonies, following developmental pathways that are species specific and modulated by a number of factors such as flow rates, viscosity, nutrient status, starvation and light intensity (Hirsch 1968, 1974; Kjelleberg *et al.* 1982; Marshall 1988; Power & Marshall 1988; Lawrence *et al.* 1989a, 1992). In contrast, *P. fluorescens* strain CC840406-E, when grown in either minimal or complex medium, followed the same developmental pathway. This pattern of surface growth by *P. fluorescens* has also been shown to be unaffected by factors such as initial cell density, nutrition, and laminar flow velocity (Korber *et al.* 1989b).

However, behavioural sequences have been shown to vary with respect to environmental conditions or induction of specific genes. Nutritional status affects the sequence of events which occur during surface associated growth by *P. syringae*, where the formation of microcolonies in CFSC was inhibited by growth in defined, glucose amended minimal medium. Under these conditions, *P. syringae* initially adhered to the surface and then detached without a growth phase. In contrast, *P. syringae* cells cultured using complex media attached, grew to form microcolonies, and exhibited a recolonization phase after four generations. With *Vibrio* DW1, overcrowding on the surface resulted in nutrient depletion, cessation of growth, and induction of a starvation survival phase (Kjelleberg *et al.* 1982; Marshall 1989). In the case of a natural stream community (Lawrence & Caldwell 1987), one morphological type responded to the cessation of flow by immediately emigrating from the surface. The micro-

colony in question had developed over a period of 10–12 h without any previous dispersal of daughter cells, indicating a possible response to changes in the microenvironment as a consequence of loss of flow.

Studies of *V. parahaemolyticus* represent the most comprehensive investigations of surface induced gene expression (McCarter *et al.* 1992). The genetic control of *V. parahaemolyticus* lateral flagellum production has been shown to be physical, that is, increasing the viscosity of the medium was sufficient to induce the laterally flagellated phenotype. In this instance, the polar flagellum has been shown to act as a tactile sensory device informing the bacterium about its ability to move. Additional studies have shown that iron deficiency is also required to initiate production of swarmer cells (McCarter *et al.* 1988; McCarter & Silverman 1989). Dagostino *et al.* (1991) reported that genes related to other specific functions may only be switched on at surfaces and not while the bacteria are growing planktonically. All of the above observations suggest that some surface colonizing bacteria respond to a broad set of environmental factors, including concentrations of nutrients, micronutrients, O₂, CO₂, etc. It is also possible that substances such as pheromones or signal proteins similar to those active in myxobacteria (Nealson 1977; Kim & Kaiser 1990) may be involved in controlling these surface growth related phenomena.

Thus, bacteria have a variety of strategies for adapting to life in a surface habitat and also for releasing progeny from the attached to the planktonic state. Behavioural adaptations are an integral part of surface colonization and represent: (i) adaptations for colonization and occupation of the substratum, (ii) optimization of substrate availability, (iii) strategies for reproductive success, (iv) balancing of intra- and interspecific competition, and (v) responses to environmental stress. However, more information is required regarding the quantitative relationships between growth rate, reproductive success, resistance to stress, and the relative merits of specific behavioural patterns. In addition, it is clear that cell-cell interactions play a role in the establishment of the structural matrix of microcolonies and biofilms.

Advantages of sessile growth

It is considered that surface colonization provides an advantage for bacteria that exist in oligotrophic environments (Kjelleberg *et al.* 1982; Kjelleberg & Hermansson 1984). The open ocean is representative of such an environment as the concentration of dissolved organic matter in these habitats is commonly less than 10 mg L⁻¹ (ZoBell 1943; Wahl 1989). Surface conditioning films adsorb to surfaces and tend to concentrate scarce nutrients, making attached growth by bacteria nutritionally advantageous.

However, eutrophic conditions do not necessarily result in the exclusive existence of planktonic bacteria, suggesting that the role of bacterial attachment cannot be solely based on nutrient capture (Paerl 1975; Robinson *et al.* 1984; MacLeod *et al.* 1990). Alternative explanations for bacterial surface colonization include resistance from washout from the surface, predator avoidance behaviour, heterotrophic relationships, co-metabolism of recalcitrant compounds, positioning behaviour, syntrophic dependencies and optimization of solution chemistry (Caldwell & Caldwell 1978; Boone & Bryant 1980; McInerney *et al.* 1981; Brannan & Caldwell 1983; Lawrence *et al.* 1987; Thiele *et al.* 1988; Korber *et al.* 1989a; Decho 1990; Wolfaardt *et al.* 1994). In many cases, attachment behaviour may not be easily explained in terms of a single advantage, but rather by the combined effect of a number of factors.

Nutrient scavenging

One explanation for the attachment of bacteria existing in nutrient limiting environments is to gain access to the film of organic molecules adsorbed to the substratum (ZoBell 1943; Kjelleberg 1980; Kefford *et al.* 1982; Kjelleberg *et al.* 1982; Hermansson & Marshall 1985). Heukelekian & Heller (1940) observed that *E. coli* failed to grow in suspension where the concentration of organic nutrients was less than 0.5–2.5 mg L⁻¹ except in the proximity of solid surfaces. At higher nutrient concentrations (>25 mg L⁻¹), no surface dependent growth effects were observed. Fletcher (1986) measured the uptake of radiolabelled glucose by attached and free living cells, determining that the attached populations assimilated 2–5 times more

glucose and also that the respiration of surface associated cells occurred at greater rates than for suspended cells. This confirmed earlier observations made by Bright & Fletcher (1983), and led to explanations for increased activity of cells on surfaces which included altered cell physiological responses triggered by: (i) induction of surface induced pathways (e.g. increased substrate transport), or (ii) increased flux of nutrients at the surface through formation of organic films or improved nutrient movement at the surface as a result of fluid hydrodynamics.

Results have been more conclusive within flowing environments. Caldwell & Lawrence (1986) demonstrated flow dependent growth kinetics of a *P. fluorescens* strain in continuous flow slide culture. At high nutrient concentrations (1 g L^{-1} glucose), it was determined that laminar flow could be halted without affecting the rate of microcolony development. At lower nutrient concentrations (100 mg L^{-1} glucose), microcolony growth ceased when flow was halted, suggesting that glucose depletion within the hydrodynamic boundary layer rather than oxygen depletion was responsible for growth limitation. Similarly, Confer & Logan (1991) provided evidence that shear strength resulted in significant increases in the utilization of radiolabelled macromolecules, suggesting that diffusion limitation through the zone surrounding the cell (termed the dead zone), in combination with enzymatic hydrolysis, decreased the rate of uptake by up to 12.5 times. In contrast, low molecular weight compounds were demonstrated to show little shear dependency on uptake. Thus, constant replenishment of the surface microenvironment surrounding attached bacteria may provide a mechanism for maintaining a steep concentration gradient (such as of nutrients and wastes) required for rapid diffusion and growth.

An alternative mechanism for nutrient scavenging on surfaces is based on the non-adhesive association of bacteria with the surface. Hermansson & Marshall (1985) reported the surface associated behaviour of marine isolate *Vibrio* MH3 which reversibly adhered to surfaces coated with stearic acid without becoming permanently attached. It was determined that *Vibrio* MH3 had to be in close proximity with the surface to take up the fatty acid, even though only 6% of the measured cell respiration was due to irreversibly

bound MH3 cells. This provided evidence that irreversible binding was not a prerequisite for the uptake of surface bound nutrients.

Microbially produced EPS is considered to play a significant role in the nutrition of sessile bacteria. The sorptive capacity of EPS for dissolved organic matter and metals has been widely demonstrated for sewage and marine systems (Brown & Lester 1982; Lion *et al.* 1988; Ferris *et al.* 1989). Thus, organisms can concentrate scarce nutrients extracellularly and utilize these compounds via the activity of exoenzymes. As a consequence, the uptake of dissolved organic matter from the suspending solution has been shown to be up to 60% greater for sessile biofilms than for planktonic bacteria. The storage of nutrients within EPS has also been suggested; however, there is little direct evidence that bacteria are capable of utilizing either their EPS or sorbed nutrients on their EPS when conditions for growth become inadequate. In addition to protecting cells from surface grazing predators, EPS, which are rich in sugars, may also constitute an important energy source for protozoa (Sibbald & Albright 1988). Examination of the amount of cellular biomass present within multiple trophic level systems has demonstrated that there is not enough bacterial biomass to support the measured biomass of grazing protozoans (for example, only <10–25%: Juniper 1987), and since there always remains a fairly large microbial population associated with surfaces even in the presence of extensive grazing, another mechanism of carbon transfer to higher trophic levels must exist. This supports epifluorescent microscopy observations made by J. R. Lawrence, D. R. Korber & D. E. Caldwell (unpublished data), where flagellate and ciliates actively feeding in a marine microcosm containing attached bacteria stained with fucose specific, FITC (fluorescein isothiocyanate) conjugated lectin were observed to have large amounts of fluorescent material in their food vacuoles. It was considered that the predators in this system were removing the abundant bacterial EPS but not the bacteria, thereby transferring nutrients up the food chain without detrimentally affecting populations of primary producers. The reactivity of microbial exopolymeric substances in a broad range of microbially associated events has been described by Decho (1990).

Positioning and resistance to washout

Bacteria persist in locations conducive to their growth and development. Motile responses to variations in light and solution chemistry, such as photo- and chemotaxis (Willey & Waterbury 1989) provide well documented examples of position maintenance by bacteria, as does the formation of intracellular gas vacuoles (Caldwell & Tiedje 1975). A wide range of indigenous bacteria also rely on adherent behaviour to position themselves where the supply of organic nutrients is optimal, the pH allows utilization of specific growth factors, oxygen requirements are met, or where temperature ranges are favourable (Caldwell & Caldwell 1978; Caldwell *et al.* 1983). The colonization of sulphur springs by *Thermothrix thiopara* provides an example of bacterial positioning as a result of cellular attachment (Brannan & Caldwell 1983; Caldwell *et al.* 1983). *T. thiopara* is a sulphur oxidizing bacterium which lives and grows in sulphur springs at temperatures of 74 °C. The maintenance of *T. thiopara* filaments at sulphide-oxygen interfaces permits the use of reduced sulphur as a source of energy. Cells growing beyond the interface into oxidizing conditions are continuously shed, reattaching where favourable conditions are encountered.

Adherent positioning mechanisms utilized by bacteria may involve either attachment to substrata or coaggregation, adaptations responsible for the formation of marine snow and sewage sludge granules (Conrad *et al.* 1985; LeChevallier *et al.* 1987; Alldredge & Silver 1988; Merker & Smit 1988; Thiele *et al.* 1988; MacLeod *et al.* 1990). In high rate, anaerobic waste treatment reactors, sludge must be immobilized or retained by some mechanism in order to decrease the dependence of sludge retention on the rate of flow within the system. It has been shown that upflow reactors may bear several times the loading factors utilized in systems lacking the upflow design, and that the pivotal factor responsible for this increased loading is the formation and persistence of large (diameter *c.* 3 mm) bioactive granules. The factors contributing to the formation of such granules have been a topic for conjecture: however, flow resistance, microcolony positioning, and syntrophic cross-feeding are all potential advantages for degradative bacteria which exist in a granular consortium. Adherent behaviour is

also observed within the bovine rumen (Russel *et al.* 1981; Rasmussen *et al.* 1989), where bacteria with cellulolytic capabilities adhere to cellulose fibres, causing the release of glucose monomers for growth by the attached organism and preventing the rapid loss of bacterial cells to bovine digestive processes. Similarly, the adherence of heterotrophic bacteria to cyanobacterial filaments may optimize the uptake of O₂ by the bacteria while simultaneously producing CO₂ for cyanobacterial photosynthesis (Caldwell & Caldwell 1978). Similar observations have been made by Lupton & Marshall (1981), where EPS produced by cyanobacterial heterocysts were observed to be populated by bacteria. Positioning behaviour is also common in streams, at aerobic-anaerobic interfaces in iron rich waters, on the surfaces of pipes and water supply equipment, in biological reactors, and in model flow systems (Kinner *et al.* 1983; Robinson *et al.* 1984; Lawrence & Caldwell 1987; Lawrence *et al.* 1987; Characklis *et al.* 1990a). Thus, adherent colonization strategies are indirectly responsible for the formation of diverse and unique microhabitats.

Resistance to predation

Bacterial attachment also improves the survival of microorganisms which might otherwise be preyed upon by protozoa feeding on plankton or bacteria on the surface of the biofilm (Porter 1976; Decho & Castenholtz 1986; Decho 1990). Cell aggregation constitutes another form of cellular adherence which may similarly improve species survival. Caron (1987) observed that attached or aggregated bacteria were grazed to a lesser extent by microflagellates than planktonic cells. Heijnen *et al.* (1991) investigated the feeding success of a flagellate, *Bodo saltans*, on *Rhizobium leguminosarum* in liquid suspension amended with bentonite clay. The authors found that addition of the clay strongly reduced the predation success of *B. saltans*, and ascribed these effects to coating of either the rhizobia or flagellates with clay particles. Thus, by association with surfaces or other cells, bacteria may become less susceptible to the action of predators. However, there have been reports of protozoan feeding behaviour adapted to surfaces exhibiting selective grazing on aggregated bacteria.

The formation of a complex EPS coating by

the bacterium provides strong adhesive forces which physically inhibit removal of the cell from the attachment surface (Porter 1976; Costerton *et al.* 1985, 1987; Decho & Castenholz 1986), and may also slow the penetration of digestive enzymes secreted by surface predators.

Development of heterogenous biofilm communities

Concept of the microenvironment

The ambient conditions associated with clean surfaces are temporally and spatially modified by the colonization, growth and activity of bacteria. Microbial consumption of nutrients, production of wastes, and synthesis of cellular and extracellular materials all act in concert to define physicochemically what is known as the microbial microenvironment (Hamilton 1987). These microenvironments persist over time due to diffusion limitations in combination with sustained metabolic activity. The presence of concentration gradients can influence bacterial population diversity and spatial distribution as well as microbial metabolic activity within biofilms, allowing the survival of other organisms with specific, and often stringent, growth requirements. The microenvironment is therefore believed to be the foundation

for much of the observed microbial diversity in nature. Although this is a fundamental concept, its presence has proven difficult to directly verify. However, application of pH sensitive probes has provided some information on microenvironments, indicating 1–3 μm zones of reduced fluorescence surrounding cells and

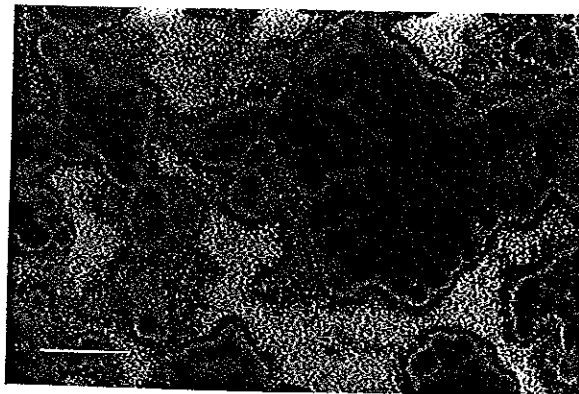


Fig. 1.5. Scanning confocal laser image of fluorescence intensity of the pH sensitive probe 5 and 6 carboxyfluorescein in the voids and spaces surrounding cells and microcolonies of *V. parahaemolyticus* in a 48 h biofilm. The image shows a dark boundary layer approximately 1–3 μm thick surrounding individual cells and microcolonies. This zone represents the approximate dead volume surrounding bacterial cells and may represent the scale of microzonal influence for these cells. Bar = 5 μm .

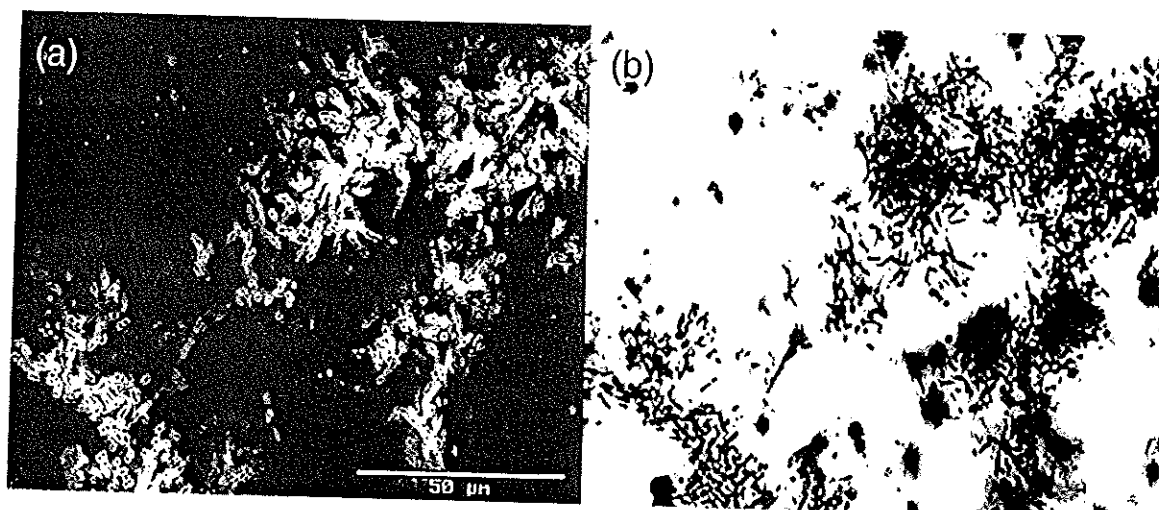


Fig. 1.6. (a), SCLM horizontal optical thin section showing the position of exopolysaccharide material produced by a microcolony within a sludge degrading biofilm. A polyanionically charged fluorescently labelled dextran (70 kD.) was found to bind this colony type preferentially when compared with other consortium members, indicating spatial variability in the polymer chemistry of the biofilm. (b) is a negatively stained image of the same region, showing the position of the microcolony bacteria which produced the EPS. Bar = 50 μm .

microcolonies within biofilms as shown in the SCLM micrograph (Fig. 1.5). Distribution of unique extracellular polymers surrounding different members of biofilm consortia may also contribute to the development of chemical microzones (Fig. 1.6a, b).

One of the best examples of temporal/chemical modification of biofilms results from the activity of aerobic heterotrophic bacteria. Within a newly formed biofilm, rapid utilization of available nutrients lead to an increase in microbial biomass with a concurrent increase in the demand for oxygen. Thus, anaerobic zones form due to metabolic oxygen depletion and diffusion limitation, potentially allowing proliferation of anaerobic microorganisms while limiting the success of aerobes. In pure laboratory cultures, the cultivation of strict anaerobes involves reducing the growth medium and eliminating any exposure to oxidized equivalents during transfer, growth or subculture. Within the biofilm microenvironment, these same organisms flourish because the conditions are met within microniches created chemically or biologically.

Development of biofilm architecture - spatial heterogeneity

Microbial biofilms consist of a spatially heterogeneous arrangement of bacteria, biogenic extracellular material, and void space (McFeters *et al.* 1984; Gujer 1987; Lawrence *et al.* 1991; Keevil & Walker 1992; Stewart *et al.* 1993). Repeating patterns of cells and EPS in biofilms formed by pure bacterial cultures indicate that this spatially defined architecture is often species specific, and that distinct microcolony morphology evident at early stages of development is sometimes detectable following prolonged biofilm growth (Lawrence *et al.* 1991). Biofilm biomass distribution can exhibit species specific trends (Lawrence *et al.* 1991; Korber *et al.* 1993). For example, significant differences in the distribution of biomass within biofilms of *P. fluorescens*, *P. aeruginosa* and *V. parahaemolyticus* have been determined using SCLM and image analysis (Lawrence *et al.* 1991). These trends in biomass distribution were likely to be a reflection of the different surface colonization strategies used by these different bacteria (Lawrence & Caldwell 1987; Korber *et al.* 1989a; Lawrence *et al.* 1992).

Motility also affects the distribution and

arrangement of biomass within biofilms. For example, the amount of cell biomass present at different depths in motile and non-motile strains of 24 h *P. fluorescens* biofilms differed (Korber *et al.* 1993). At the base of the mot⁻ biofilm (depth 0 μm) the measured mean cell area value was $4.2 \pm 1.5 \mu\text{m}^2$ per $50 \mu\text{m}^2$, at the 8 μm depth, $2.6 \pm 2.0 \mu\text{m}^2$ per $50 \mu\text{m}^2$, and at the 16 μm depth, $1.8 \pm 2.1 \mu\text{m}^2$ per $50 \mu\text{m}^2$. This arrangement of non-motile biofilm cells culminated in formation of typical pyramidal biofilm colonies, with the highest measured cell areas present at the base. In contrast, the mot⁺ strain formed thicker biofilms (depth = $19 \pm 10 \mu\text{m}$ vs $42 \pm 19 \mu\text{m}$ for the mot⁻ and mot⁺ strains, respectively), they were relatively less variable, and possessed greater amounts of cellular biomass at the attachment surface and the biofilm-liquid interface. Interestingly, the 8 μm section depth of the mot⁺ *P. fluorescens* biofilms was diffuse, relative to over- and underlying regions, and may have played a role in facilitating diffusion of nutrients to the base of the biofilm.

Analyses have also shown mixed species biofilms to be quite variable (Robinson *et al.* 1984; Caldwell *et al.* 1992a; Wolfaardt *et al.* 1994). For example, Keevil & Walker (1992) reported that natural biofilms, observed with DIC and epifluorescence microscopy, consisted of a basal cell layer approximately 5–10 μm thick, with stacks of polymer associated cells extending from the surface into the bulk aqueous phase at regular intervals. These structures were presumed to have facilitated the flux of nutrients to the basal biofilm layers, thereby supporting the growth of aerobic heterotrophs, while creating chemical microenvironments which would support the growth of anaerobic organisms as well.

This general theme of cell aggregates interspersed by channels has been confirmed during other pure culture and mixed species investigations, with biofilm structure varying for different hydrodynamic regimes, incubation times, species composition, or nutrient status (Santos *et al.* 1991; Korber *et al.* 1994b; Stewart *et al.* 1993). For example, Bakke & Olsson (1986) detected channels within *P. aeruginosa* biofilms using low magnification microscopy. Depth measurements indicated that variability increased with age, although the average depth of the biofilm did not. The channels were considered by the authors to have increased surface roughness and

decreased diffusional length, thus facilitating mixing and nutrient exchange. An SCLM study of flow velocity effects (flow range, 0.01–0.21 cm s⁻¹) on the architecture of *P. fluorescens* biofilms determined that the extent of channel formation was flow velocity dependent, with increased flow velocities causing an increase in the average depth of the biofilms and a concurrent decrease in the variability of depth measurements (Korber *et al.* 1994b). This study, performed over large biofilm regions (>1000000 µm²) and at low magnification, demonstrated that at low flow rates biofilms were less deep and contained relatively more channels (0.01 cm s⁻¹; mean depth, 12.3 ± 6.8 µm) than at high flow rates (0.21 cm s⁻¹; mean depth, 83.7 ± 10.2 µm). The increase in depth and decrease in the total area of channels suggested that limitations to growth present at low flow may be offset by increased exchange of nutrients and/or oxygen across the hydrodynamic boundary layer at high flow rates. Figure 1.7 shows the presence of channels and cell aggregates within a typical *P. fluorescens* biofilm using SCLM at low magnification in the xy plane (showing the pattern of cell aggregates and channels), as well as at high magnification (Fig. 1.8) in the xz plane (showing in sagittal profile, individual channels and cell aggregates). In addition to pores and channels, other unique architectural features have also been observed. For example, bridging structures, formed as part of biofilms developed under flowing conditions, have been observed (Korber *et al.* 1993b). The basal layer of a biofilm (Fig. 1.9a) and the bridge structure extending across the channel can also be observed (Fig. 1.9b). Pore development also constitutes a situation where the biofilm has overgrown a small channel, resulting in a void space not connected to the bulk aqueous phase. Similar structures have been described for many biofilms including those on rotating biological contactors and other systems (Kinner *et al.* 1983). The significance of such structures is as yet not clear.

The presence of channels within biofilms (Figs. 1.7, 1.8) may be analogous to the growth habit of barnacle colonies, which form relatively high hummocks about 5 cm across and are also interspersed by water channels at regular intervals (Barnes & Powell 1950; Knight-Jones & Moyse 1961). The largest barnacle individuals are found at the peak of the hummocks, where more nutrients are available for growth as a consequence of

more rapidly flowing water. Hummocks would also cause turbulent mixing thereby preventing buildup of a nutrient depleted boundary layer, with associated benefits for other members of the barnacle community. For biofilm bacteria, channels and cell aggregates may function similarly, with flow and advection replenishing nutrients and facilitating diffusion in regions not directly exposed to the bulk phase. Depth dependent variability in growth rates for biofilm bacteria has previously been inferred by Korber *et al.* (1994a), where treatment with feroxacin, a DNA gyrase inhibiting fluoroquinolone, caused greater cell elongation at the bulk-liquid interface than at the base of the biofilm, even though visual determinations of feroxacin penetration indicated that migration of this compound was not significantly impeded by the bacterial-EPS matrix. Similar observations have been made for penetration of antimicrobial agents (Nichols 1989). Consequently, the increase in cell length following application of feroxacin should be proportional to the amount of cell growth. These observations agree with the premise that biofilms grow mainly at the biofilm-liquid interface and not from the biofilm base, and have implications not only for understanding the ecology of biofilm systems, but also for interpreting the effects of antimicrobial agents on biofilms (Gilbert *et al.* 1990; Eng *et al.* 1991).

Population architecture of biofilms

While studies of the physical architecture of microbial biofilms are now routinely conducted, population architecture may also be directly studied using fluorescent conjugates of oligonucleotide probes, using phylogenetic probes and PCR (polymerase chain reaction) methods (Stahl *et al.* 1988; Amann *et al.* 1992). If microchemical gradients are also examined, valuable information describing chemical and spatial requirements of highly structured biofilm communities may be obtained. Recently, Amann *et al.* (1992) conducted studies using natural populations of SRB within multispecies biofilms, demonstrating that two types of cell distributions were observed when biofilms were stained with SRB specific oligonucleotides. One probe distribution pattern indicated a microcolony growth habit for *D. vulgaris*-like sequences, whereas *D. desulfuromonas* was more randomly distributed. Time course

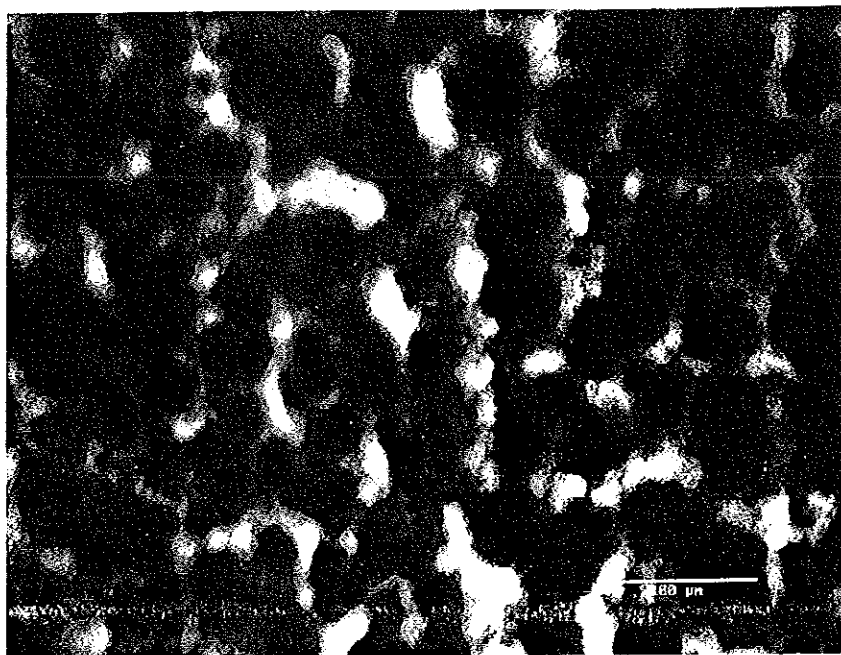


Fig. 1.7. Low magnification SCLM optical thin section (c. 80 000 μm^2) clearly showing a spatial pattern of channels and cell aggregates. Bar = 100 μm .

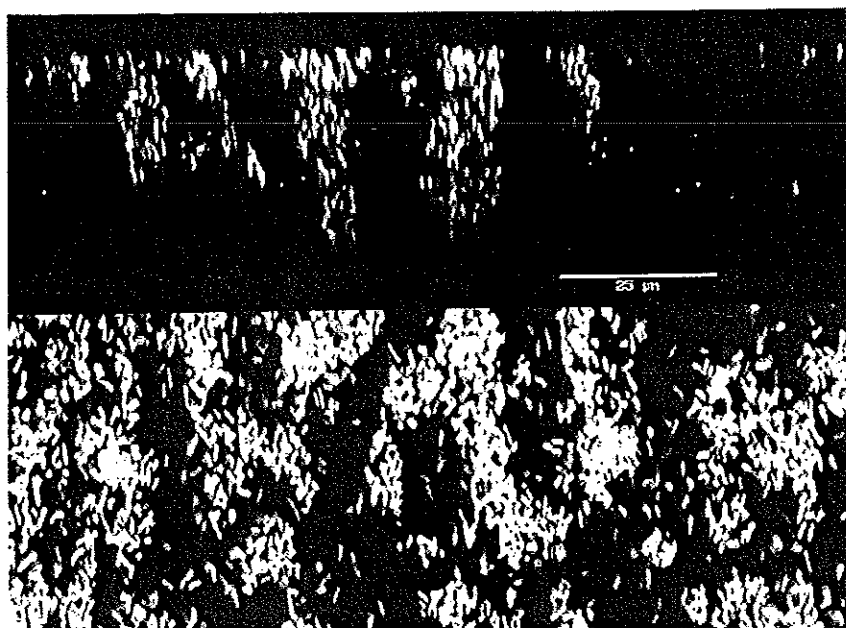


Fig. 1.8. SCLM optical thin section showing in sagittal profile (xz) channels and cell aggregates present within a positively-stained 24 h *P. fluorescens* biofilm (top). The bottom portion of the micrograph is a horizontal optical thin section, the top portion of which corresponds with the position of xz sectioning. Bar = 25 μm .

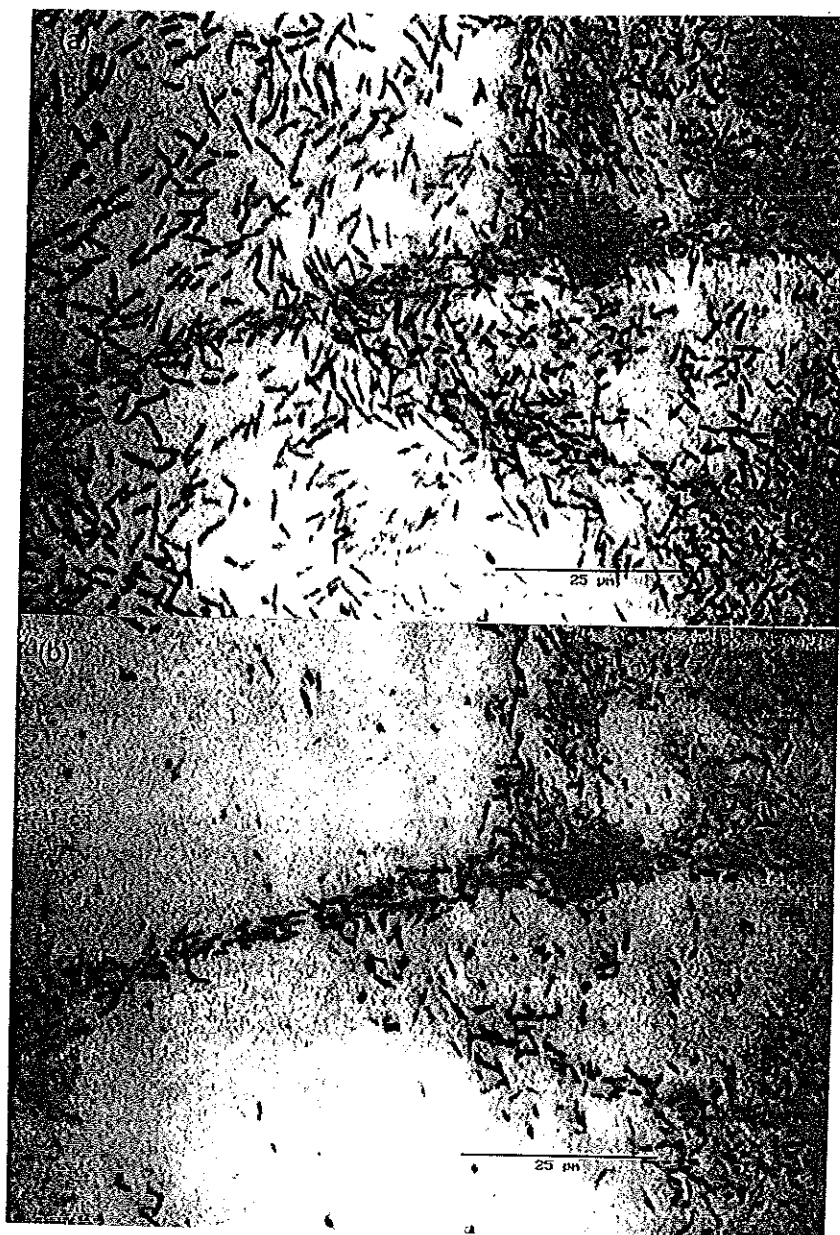


Fig. 1.9. A projection of four SCLM optical thin sections obtained from the basal region of a negatively stained *P. fluorescens* biofilm (a), showing the distribution of cells near the attachment surface, as well as a bridge structure evident near the 6 μm sectioning depth. (b) is a single optical thin section from the 6 μm section depth, showing the bridge structure alone. Bar = 25 μm .

investigations using these techniques may provide important information regarding the temporal success of organisms which have specific chemical requirements. For example, development of anaerobic zones may be found to corre-

late with the occurrence of SRB microcolonies.

Investigations using 16S, 5S and 23S rRNA probes are now being used to study a wide range of natural systems, allowing investigations into community structure without the need for culti-

vation (Stahl *et al.* 1988; Amann *et al.* 1990, 1992). This is important because members of a biofilm community isolated from the environment may not be easily culturable. While still undergoing rapid development, these methods, used in conjunction with either SCLM or epifluorescence microscopy, will provide valuable insight into the spatial contiguity of obligate or syntrophic cell associations.

Competition and succession in biofilms

In natural systems, there exists a high degree of cellular interaction and competitive behaviour (Connell & Slatyer 1977; Fredrickson 1977). Much of this interactive behaviour results from the competition of cells for available resources, whether those resources be attachment sites or growth substrate. Higher organisms may also influence the outcome of microbial surface colonization, and events such as grazing by predators have the potential to limit the success of one strain while having relatively little effect on other consortium members. Few studies have focused on the effect of competitive pressure during the development of microbial biofilms. Cowan *et al.* (1991) utilized image analysis to measure the extent of surface coverage during colonization of pure cultures and mixed species over a 27 day period; in the presence of *Xanthomonas maltophilia*, greater surface colonization was observed during coculture experiments than where *X. maltophilia* was absent. The researchers also noted limitations of their system in that three dimensional cell aggregates could not effectively be discriminated from two dimensional growth and development using phase contrast image analysis. Also noted was the inability to discriminate between bacterial species in order to determine dominance of any particular colonizing species within the system.

Radiochemical labelling of bacteria has also been used to evaluate competitive behaviour in biofilm cocultures (Banks & Bryers 1991). Discrimination between the two strains used during this study was possible because a *Pseudomonas putida* strain metabolized glucose exclusively whereas *Hyphomicrobium* sp. metabolized only methanol. Replicate culture systems were used to cultivate either mixed species biofilms grown in the presence of ^{14}C -glucose and unlabelled methanol, or ^{14}C -methanol and unlabelled glucose. Success of coculture members was deter-

mined by comparison of radioactivity accumulated over time, sacrificing flow cells for analytical purposes. When both strains were simultaneously added to flow cells, the *P. putida* isolate outnumbered the *Hyphomicrobium* sp. after 24 and 48 h. Similarly, when *P. putida* was added to an established *Hyphomicrobium* sp. biofilm, the *P. putida* cells became the dominant biofilm species after only 48 h. In both cases, the *P. putida* strain outnumbered the *Hyphomicrobium* sp. by approximately 5 to 1 after 50 h of biofilm growth. The authors attributed these results to the differences in growth rates of the two species, whereas the effect of microbial colonization behaviour was not addressed. Current limitations to the study of complex microbial communities lie in the inability to discriminate visually between consortium members that have similar shapes. Use of SCLM in conjunction with fluorescently labelled poly/monoclonal antibodies has potential in this area of study, as laser optical thin sectioning may then be used to help elucidate the position of fluorescent antibody stained bacteria present within the biofilm matrix (James *et al.* 1993).

Observations of adherent populations in natural habitats provides evidence that these systems are in constant flux (Connell & Slatyer 1977; Baier 1984; Wahl 1989). A sequence of events occurs during surface fouling, resulting in a succession of surface colonizing species which dominate at various times following immersion of clean surfaces in natural waters. In a process termed epibiosis (Wahl 1989), the initial colonizing species are subsequently colonized by organisms before others, with defined requirements, become involved with the surface consortium. The succession is based on a sequence of physical and biological events, initiated by adsorption of organic films and closely followed by surface colonization by bacterial species (Fletcher & Loeb 1979; Characklis 1981; Baier 1984). The time frames for these two initial phases of surface colonization are relatively short, with the molecular film forming over a period of minutes, and with significant bacterial colonization occurring within 24 h. J. R. Lawrence, D. R. Korber & D. E. Caldwell (unpublished data) noted that only brief periods were required for the onset of bacterial deposition from a simulated marine microcosm, and that common marine species (*Hyphomicrobium*, *Pedomicrobium*, *Pseudomonas*, *Caulobacter* spp.) were primarily involved.

While bacteria continue to adsorb to exposed surfaces, larger fouling organisms (including amoeba, flagellates, ciliates, diatoms and larvae) subsequently colonize the surface over a period of days for predatory eukaryotes and diatoms, or weeks for larval and spore deposition (Wahl 1989; Decho 1990). This successional pattern of surface colonization has been frequently observed during long term observation of immersed surfaces in flowing or static systems. Researchers have reported that rod-shaped bacteria are frequently the primary colonizers following exposure of clean surfaces to natural aqueous systems (Marshall *et al.* 1971; Marszalek *et al.* 1979), followed by stalked bacteria such as *Caulobacter* spp. (Dempsey 1981). Subsequent colonization by filamentous algae, diatoms, larvae, etc. has reportedly been observed in stream, river and oceanic environments, with eventual attraction of predators which feed on the biofilm (Marszalek *et al.* 1979; Baier 1985; Wahl 1989; Rittle *et al.* 1990).

The recent developments in studying biofilms described in this chapter have allowed us to describe them as both variable and heterogeneous (Keevil & Walker 1992; Stewart *et al.* 1993; Korber *et al.* 1993). In addition, biofilms form through a variety of strategies based on cell-cell interactions that function as multicellular behaviour of microorganisms. The techniques are now available to study the microenvironment in real time using non-destructive methods. They also allow analysis of the spatial arrangements of cells within the context of their natural surroundings. SCLM permits optical sectioning of thick biological specimens without interference from light scattering (Heertje *et al.* 1987; White *et al.* 1987) and has recently been utilized to examine the internal structure and architecture of mature biofilms without diffraction based limitations in microscopy resolution (Lawrence *et al.* 1991; Caldwell *et al.* 1992a, b; Korber *et al.* 1992, 1993; Wolfaardt *et al.* 1994). This, combined with the wide range of environmentally sensitive reporter molecules, means that the microenvironment may be defined and quantitatively analysed. The application of molecular techniques provides the ability to analyse natural communities (Amann *et al.* 1992). These techniques allow the study of biofilms and help to relate the occurrence of bacterial populations to the measurement of specific processes.

Application of these tools should provide new understanding of the functioning of biofilms in the diverse industrial, agricultural, medical and natural environments where they are of major importance as discussed in later chapters.

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References

- Absolom, D. R., Lamberti, F. V., Policova, Z., Zingg, W., van Oss, C. J. & Neumann, A. W. (1983). Surface thermodynamics of bacterial adhesion. *Applied and Environmental Microbiology*, 46, 90-7.
- Acker, G. K. & Steere, R. L. (1962). Restricted diffusion of macromolecules through agar-gel membranes. *Biochimica et Biophysica Acta*, 59, 137-49.
- Adamczyk, Z. & van de Ven, T. G. M. (1981). Deposition of particles under external forces in laminar flow through parallel-plate and cylindrical channels. *Journal of Colloid and Interface Science*, 80, 340-57.
- Adler, J. (1975). Chemotaxis in bacteria. *Annual Reviews of Biochemistry*, 44, 341-55.
- Allredge, A. L. & Cohen, Y. (1987). Can microscale chemical patches persist at sea? Microelectrode study of marine snow, fecal pellets. *Science*, 235, 689-91.
- Allredge, A. L. & Silver, M. W. (1988). Characteristics, dynamics, and significance of marine snow. *Progress in Oceanography*, 20, 41-82.
- Allison, D. G. & Sutherland, I. W. (1987). The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology*, 133, 1319-27.
- Amann, R. I., Krumholz, L. & Stahl, D. A. (1990). Fluorescent oligonucleotide probing of whole cells for determinative phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, 172, 762-70.
- Amann, R. I., Stromley, J., Devereux, R., Key, R. & Stahl, D. A. (1992). Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Applied and Environmental Microbiology*, 48, 614-23.
- Arnold, J. W. & Shimkets, L. J. (1988). Inhibition of cell-cell interactions in *Myxococcus xanthus*. *Journal of Bacteriology*, 170, 5765-70.

- Baier, R. E. (1980). Substrate influence on adhesion of microorganisms and their resultant new surface properties. In *Adsorption of microorganisms to surfaces*, ed. G. Bitton & K. C. Marshall, pp. 59–104. New York: Wiley.
- Baier, R. E. (1984). Initial events in microbial film formation. In *Marine biodeterioration: an interdisciplinary approach*, ed. J. D. Costlow & R. C. Tipper, pp. 57–62. London: E. & F. N. Spon Ltd.
- Baier, R. E. (1985). Adhesion in the biologic environment. *Biomaterials Medical Devices and Artificial Organs*, 12, 133–59.
- Bakke, R. & Olsson, P. Q. (1986). Biofilm thickness measurements by light microscopy. *Journal of Microbiological Methods*, 5, 93–8.
- Banks, M. K. & Bryers, J. D. (1991). Bacterial species dominance within a binary culture biofilm. *Applied and Environmental Microbiology*, 57, 1874–9.
- Barnes, H. & Powell, H. T. (1950). The development, general morphology, and subsequent elimination of barnacle populations, *Balanus crenatus* and *B. balanoides* after a heavy initial settlement. *Journal of Animal Ecology*, 19, 175–9.
- Berg, H. C. (1985). Physics of bacterial chemotaxis. In *Sensory perception and transduction in asexual organisms*, ed. G. Colombetti, F. Linci & P.-S. Song, pp. 19–30. London: Plenum Press.
- Berg, H. C. & Brown, D. A. (1972). Chemotaxis in *Escherichia coli* analyzed by three-dimensional tracking. *Nature*, 239, 500–4.
- 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.
- Block, S. M., Segall, J. E. & Berg, H. C. (1983). Adaptation kinetics in bacterial chemotaxis. *Journal of Bacteriology*, 154, 312–24.
- Bochem, H. P., Schoberth, S. M., Sprey, B. & Wengler, P. (1982). Thermophilic biomethanation of acetic acid: morphology and ultrastructure of a granular consortium. *Canadian Journal of Microbiology*, 28, 500–10.
- Bohrer, M. P., Patterson, G. D. & Carroll, P. J. (1984). Hindered diffusion of dextran and ficoll in microporous membranes. *Macromolecules*, 17, 1170–3.
- Boone, D. R. & Bryant, M. P. (1980). Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. *Applied and Environmental Microbiology*, 40, 626–32.
- Boone, D. R., Johnson, R. L. & Liu, Y. (1989). Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. *Applied and Environmental Microbiology*, 55, 1735–41.
- Bott, T. L. & Brock, T. D. (1970). Growth and metabolism of periphytic bacteria: methodology. *Limnology and Oceanography*, 20, 191–7.
- Bowen, B. D. & Epstein, N. (1979). Fine particle deposition in smooth parallel-plate channels. *Journal of Colloid and Interface Science*, 72, 81–97.
- Brannan, D. K. & Caldwell, D. E. (1983). Growth kinetics and yield coefficients of the extreme thermophile *Thermothrix thiopara* in continuous culture. *Applied and Environmental Microbiology*, 45, 169–73.
- Bright, J. J. & Fletcher, M. (1983). Amino acid assimilation and respiration by attached and free-living populations of a marine *Pseudomonas* sp. *Microbial Ecology*, 9, 215–26.
- Brown, M. R. W., Allison, D. G. & Gilbert, G. (1988). Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *Journal of Antimicrobial Chemotherapy*, 22, 777–80.
- Brown, M. J. & Lester, J. N. (1982). Role of bacterial extracellular polymers in metal uptake in pure bacterial culture and activated sludge-II: Effects of mean cell retention time. *Water Research*, 16, 1549–60.
- Bryers, J. D. (1987). Biologically active surfaces: processes governing the formation and persistence of biofilms. *Biotechnology Progress*, 3, 57–68.
- Burchard, R. P., Rietschhof, D. & Bonaventura, J. (1990). Adhesion and motility of gliding bacteria on substrata with different surface free energies. *Applied and Environmental Microbiology*, 56, 2529–34.
- Busscher, H. J., Bellon-Fontaine, M.-N., Mozes, N. et al. (1990a). Deposition of *Leuconostoc mesenteroides* and *Streptococcus thermophilus* to solid substrata in a parallel plate flow cell. *Biofouling*, 2, 55–63.
- Busscher, H. J., Bellon-Fontaine, M.-N., Sjollem, J. & van Der Mei, H. C. (1990b). Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solid surfaces. In *Microbial cell surface hydrophobicity*, ed. R. J. Doyle & M. Rosenberg, pp. 335–59. Washington, DC: American Society for Microbiology.
- Caldwell, D. E. (1985). New developments in computer-enhanced microscopy. *Journal of Microbiological Methods*, 4, 117–25.
- Caldwell, D. E., Brannan, D. K. & Kieft, T. L. (1983). *Thermothrix thiopara*: Selection and adaptation of a filamentous sulfur-oxidizing bacterium colonizing hot spring tufa at pH 7.0 and 74 °C. *Environmental Biogeochemical Ecology Bulletin*, 35, 129–34.
- Caldwell, D. E. & Caldwell, S. J. (1978). A *Zoogloea* sp. associated with blooms of *Anabaena flos-aquae*. *Canadian Journal of Microbiology*, 24, 922–31.
- Caldwell, D. E., Korber, D. R. & Lawrence, J. R. (1992a). Confocal laser microscopy and digital image analysis in microbial ecology. *Advances in Microbial Ecology*, 12, 1–67.

- Caldwell, D. E., Korber, D. R. & Lawrence, J. R. (1992b). Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. *Journal of Microbiological Methods*, 15, 249-61.
- Caldwell, D. E., Korber, D. R. & Lawrence, J. R. (1993). Analysis of biofilm formation using 2-D versus 3-D digital imaging. *Journal of Applied Bacteriology* (Symposium Supplement), 74, 52S-66S.
- 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. & Tiedje, J. M. (1975). The structure of anaerobic bacterial communities in the hypolimnia of several lakes in Michigan. *Canadian Journal of Microbiology*, 21, 377-85.
- Caron, D. A. (1987). Grazing of attached bacteria by heterotrophic microflagellates. *Microbial Ecology*, 13, 203-18.
- Characklis, W. G. (1981). Bioengineering report. Fouling biofilm development: a process analysis. *Biotechnology and Bioengineering*, 23, 1923-60.
- Characklis, W. G., McFeters, G. A. & Marshall, K. C. (1990a). Physiological ecology in biofilm systems. In *Biofilms*, ed. W. G. Characklis & K. C. Marshall, pp. 341-93. New York: Wiley.
- Characklis, W. G., Turakhia, M. H. & Zilver, N. (1990b). Transfer and interfacial transport phenomena. In *Biofilms*, ed. W. G. Characklis & K. C. Marshall, pp. 265-40. New York: Wiley.
- Cholodny, N. (1930). Über eine neue Method zur Untersuchung der Bodenflora. *Archiv für Mikrobiologie*, 1, 620-52.
- Christensen, B. E. & Characklis, W. G. (1990). Physical and chemical properties of biofilms. In *Biofilms*, ed. W. G. Characklis & K. C. Marshall, pp. 93-130. New York: Wiley.
- Christersson, C. E., Glantz, P.-O. J. & Baier, R. E. (1988). Role of temperature and shear forces on microbial detachment. *Scandinavian Journal of Dental Research*, 96, 91-8.
- Confer, D. R. & Logan, B. E. (1991). Increased bacterial uptake of macromolecular substrates with fluid shear. *Applied and Environmental Microbiology*, 57, 3093-100.
- Connell, J. H. & Slatyer, R. O. (1977). Mechanisms of succession in natural communities and their role in community stability and organization. *American Naturalist*, 111, 1119-44.
- Conrad, R., Phelps, T. J. & Zeikus, J. G. (1985). Gas metabolism evidence in support of the juxtaposition of hydrogen-producing and methanogenic bacteria in sewage sludge and lake sediments. *Applied and Environmental Microbiology*, 50, 595-601.
- Costerton, J. W., Cheng, K.-J., Geesey, G. G. et al. (1987). Bacterial biofilms in nature and disease. *Annual Reviews of Microbiology*, 41, 435-64.
- Costerton, J. W., Geesey, G. G. & Cheng, K.-J. (1978). How bacteria stick. *Scientific American*, 238, 86-95.
- Costerton, J. W., Irvin, R. T. & Cheng, K.-J. (1981). The bacterial glycocalyx in nature and disease. *Annual Reviews of Microbiology*, 35, 299-324.
- Costerton, J. W., Marrie, T. J. & Cheng, K.-J. (1985). Phenomena of bacterial adhesion. In *Bacterial adhesion: mechanisms and physiological significance*, ed. D. C. Savage & M. Fletcher, pp. 3-43. New York: Plenum Press.
- Cowan, M. M., Warren, T. M. & Fletcher, M. (1991). Mixed-species colonization of solid surfaces in laboratory biofilms. *Biofouling*, 3, 23-34.
- Cox, D. J., Bazin, M. J. & Gull, K. (1980). Distribution of bacteria in a continuous-flow nitrification column. *Soil Biology and Biochemistry*, 12, 241-6.
- Dagostino, L., Goodman, A. E. & Marshall, K. C. (1991). Physiological responses induced in bacteria adhering to surfaces. *Biofouling*, 4, 113-19.
- Dazzo, F. B., Truchet, G. L., Sherwood, F. E., Hrabak, E. M., Abe, M. & Pankratz, S. H. (1984). Specific phases of root hair attachment in the *Rhizobium trifolii*-clover symbiosis. *Applied and Environmental Microbiology*, 48, 1140-50.
- Decho, A. W. (1990). Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. *Oceanography and Marine Biology Annual Review*, 28, 73-153.
- Decho, A. W. & Castenholtz, R. W. (1986). Spatial patterns & feeding of meiobenthic harpacticoid copepods in relation to resident microbial flora. *Hydrobiologica*, 131, 87-96.
- Delaquis, P. J. (1990). Colonization of model and meat surfaces by *Pseudomonas fragi* and *Pseudomonas fluorescens*. Ph.D. thesis, University of Saskatchewan, Saskatoon, Canada.
- Dempsey, M. J. (1981). Marine bacterial fouling: A scanning electron microscope study. *Marine Biology*, 61, 305-15.
- Derjaguin, B. V. & Landau, L. (1941). Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physicochimica URSS*, 14, 633-62.
- Dexter, S. C. (1979). Influence of substratum critical surface tension on bacterial adhesion - *in situ* studies. *Journal of Colloid and Interface Science*, 70, 346-53.
- Dexter, S. C., Sullivan, J. D. Jr, Williams, J. & Watson, S. W. (1975). Influence of substrate wettability on the attachment of marine bacteria to various surfaces. *Applied Microbiology*, 30, 298-308.
- De Weger, L. D., van Der Vlugt, C. I. M., Wijffes, A. H. M., Bakker, P. A. H. M., Schippers, B. &

- Lugtenberg, B. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *Journal of Bacteriology*, 169, 2769-73.
- Drucker, D. B. & Whittaker, D. K. (1971). Microstructure of colonies of rod-shaped bacteria. *Journal of Bacteriology*, 108, 515-25.
- Duxbury, T. (1977). A microperfusion chamber for studying growth of bacterial cells. *Journal of Applied Bacteriology*, 42, 247-51.
- Eng, R. H. K., Padberg, F. T., Smith, S. M., Tan, E. N. & Cherubin, C. E. (1991). Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrobial Agents and Chemotherapy*, 35, 1824-28.
- Ferris, F. G., Schultze, S., Witten, T. C., Fyfe, W. S. & Beveridge, T. J. (1989). Metal interactions with microbial biofilms in acidic and neutral pH environments. *Applied and Environmental Microbiology*, 55, 1249-57.
- Fletcher, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology*, 94, 400-4.
- Fletcher, M. (1977). The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. *Canadian Journal of Microbiology*, 23, 1-6.
- Fletcher, M. (1986). Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces. *Applied and Environmental Microbiology*, 52, 672-76.
- Fletcher, M. (1988). Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium-substratum separation distance. *Abstracts of the 88th Annual Meeting of the American Society for Microbiology*, Washington DC, USA.
- Fletcher, M. & Loeb, G. I. (1979). Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Applied and Environmental Microbiology*, 37, 67-72.
- Fletcher, M. & Marshall, K. C. (1982). Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Applied and Environmental Microbiology*, 44, 184-192.
- Fredrickson, A. G. (1977). Behaviour of mixed cultures of microorganisms. *Annual Reviews of Microbiology*, 33, 63-87.
- Freeze, R. A. & Cherry, J. A. (1979). Groundwater. Englewood Cliffs, NJ: Prentice Hall.
- Geesey, G. G. & White, D. C. (1990). Determination of bacterial growth and activity at solid-liquid interfaces. *Annual Review of Microbiology*, 44, 579-602.
- 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, 1856-68.
- Gualtieri, P., Colombetti, G. & Lenci, F. (1985). Automatic analysis of the motion of microorganisms. *Journal of Microscopy*, 139, 57-62.
- Gualtieri, P., Francesco, G., Passarelli, V. & Barsanti, L. (1988). Microorganism track reconstruction: an image processing approach. *Computers in Biology and Medicine*, 18, 57-63.
- Gujer, W. (1987). The significance of segregation of biomass in biofilms. *Water Science Technology*, 19, 495-503.
- Hamilton, W. A. (1987). Biofilms: microbial interactions and metabolic activities. In *Ecology of microbial communities*, ed. M. Fletcher, T. R. G. Gray & J. G. Jones, Symposium of the Society for General Microbiology 41. Cambridge: Cambridge University Press.
- Handley, P. S., Carter, P. L., Wyatt, J. E. & Hesketh, L. M. (1985). Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. *Infections and Immunity*, 47, 217-27.
- Heertje, I., van der Vlist, P., Blonk, J. C. G., Hendrickx, H. A. C. M. & Brakenhoff, G. J. (1987). Confocal scanning laser microscopy in food research: some observations. *Food Microstructure*, 6, 115-20.
- Heijnen, C. E., Hok-A-Hin, C. H. & van Veen, J. A. (1991). Protection of rhizobium by bentonite clay against predations by flagellates in liquid culture. *FEMS Microbial Ecology*, 85, 65-72.
- Helmsetter, C. E. (1969). Sequence of bacterial reproduction. *Annual Reviews of Microbiology*, 23, 223-38.
- Henrici, A. T. (1933). Studies of freshwater bacteria. I. A direct microscopic technique. *Journal of Bacteriology*, 25, 277-86.
- Henrici, A. T. & Johnson, D. E. (1935). Studies of freshwater bacteria. II. Stalked bacteria, a new order of Schizomycetes. *Journal of Bacteriology*, 30, 61-93.
- Hermansson, M. & Marshall, K. C. (1985). Utilization of surface localized substrate by non-adhesive marine bacteria. *Microbial Ecology*, 11, 91-105.
- Heukelekian, H. & Heller, A. (1940). Relationship between food concentration and surface for bacterial growth. *Journal of Bacteriology*, 40, 547-58.
- Hirsch, P. (1968). Biology of budding bacteria. *Archiv für Mikrobiologie*, 60, 201-16.
- Hirsch, P. (1974). Budding bacteria. *Annual Reviews of Microbiology*, 28, 391-433.
- Hirsch, P. (1984). Microcolony formation and consortia. In *Microbial adhesion and aggregation*, ed. K. C. Marshall, pp. 373-93. New York: Springer-Verlag.
- Hirsch, P. & Pankratz, S. H. (1971). Studies on

- bacteria populations in natural environments by use of submerged electron microscope grids. *Zeitschrift für Allgemeine Mikrobiologie*, 10, 589-605.
- Howell, J. A. & Atkinson, B. (1976). Sloughing of microbial film in trickling filters. *Water Research*, 10, 307-15.
- Isaacson, R. E., Nagy, B. & Moon, H. W. (1977). Colonization of porcine small intestine by *Escherichia coli*: colonization and adhesion factors of pig enteropathogens that lack K88. *Journal of Infectious Diseases*, 135, 531-38.
- James, G. A., Caldwell, D. E. & Costerton, J. W. (1993). Spatial relationships between bacterial species within biofilms. Abstract, Canadian Society of Microbiologists/Society for Industrial Microbiology annual meeting, Toronto, Canada.
- Jang, L. K. & Yen, T. F. (1985). A theoretical model of diffusion of motile and nonmotile bacteria toward solid surfaces. In *Microbes and oil recovery*, 1, ed. J. E. Zajt & E. C. Donaldson, pp. 226-46. International Bioresources Journal.
- Juniper, S. K. (1987). Deposit-feeding ecology of *Amphibola crenata*. I. Long-term effects of deposit feeding on sediment microorganisms. *New Zealand Journal of Marine and Freshwater Research*, 21, 235-46.
- Keevil, C. W. & Walker, J. T. (1992). Nomarski DIC microscopy and image analysis of biofilms. *Binary: Computing in Microbiology*, 4, 93-5.
- Kefford, B., Kjelleberg, S. & Marshall, K. C. (1982). Bacterial scavenging: utilization of fatty acids localized at a solid-liquid interface. *Archives of Microbiology*, 133, 257-60.
- Kelly, F. X., Dapsis, K. J. & Lauffenburger, D. A. (1988). Effect of bacterial chemotaxis on dynamics of microbial competition. *Microbial Ecology*, 16, 115-31.
- Kim, S. K. & Kaiser, D. (1990). C-factor; a cell-cell signalling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell*, 61, 19-26.
- Kinner, N. E., Balkwill, D. L. & Bishop, P. L. (1983). Light and electron microscopic studies of microorganisms growing in rotating biological contactor biofilms. *Applied and Environmental Microbiology*, 45, 1659-69.
- Kjelleberg, S. (1980). Effects of interfaces on survival mechanisms of copiotrophic bacteria in low-nutrient environments. In *Microbial adhesion to surfaces*, ed. R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter & B. Vincent, pp. 151-9. Chichester: Ellis Horwood.
- Kjelleberg, S. & Hermansson, N. (1984). The effect of interfaces on small starved marine bacteria. *Applied and Environmental Microbiology*, 48, 497-503.
- Kjelleberg, S., Humphrey, B. A. & Marshall, K. C. (1982). The effect of interfaces on small, starved marine bacteria. *Applied and Environmental Microbiology*, 43, 1166-72.
- Knight-Jones, E. W. & Moyse, E. (1961). Intraspecific competition in sedentary marine animals. *Symposium of the Society for Experimental Biology*, 15, 72-95.
- Koch, A. L. (1991). Diffusion: the crucial process in many aspects of the biology of bacteria. In *Advances in microbial ecology*, ed. K. C. Marshall, pp. 37-70. New York: Plenum Press.
- Kolenbrander, P. E. (1989). Surface recognition among oral bacteria: multigeneric coaggregations and their mediators. *Critical Reviews in Microbiology*, 17, 137-59.
- Kolenbrander, P. E. & London, J. (1992). Ecological significance of coaggregation among oral bacteria. In *Advances in Microbial Ecology*, 12, 183-217.
- Korber, D. R., James, G. A., Costerton, J. W. (1994a). Evaluation of flevoxacillin efficacy against established *Pseudomonas fluorescens* biofilms. *Applied and Environmental Microbiology*, 60, 1663-9.
- Korber, D. R., Lawrence, J. R., Cooksey, K. E., Cooksey, B. & Caldwell, D. E. (1989a). Computer image analysis of diatom chemotaxis. *Binary*, 1, 155-68.
- Korber, D. R., Lawrence, J. R., Hanson, K. G., Caldwell, D. E. & Costerton, J. W. (1994b). The effect of environmental laminar flow velocities on the architecture of *Pseudomonas fluorescens* biofilms. Abstract, American Society of Microbiology 94th annual meeting, Las Vegas.
- Korber, D. R., Lawrence, J. R., Hendry, M. J. & Caldwell, D. E. (1992). Programs for determining representative areas of microbial biofilms. *Binary*, 4, 204-10.
- Korber, D. R., Lawrence, J. R., Hendry, M. J. & Caldwell, D. E. (1993). Analysis of spatial variability within mot^+ and mot^- *Pseudomonas fluorescens* biofilms using representative elements. *Biofouling*, 7, 339-58.
- Korber, D. R., Lawrence, J. R., Sutton, B. & Caldwell, D. E. (1989b). The effect of laminar flow on the kinetics of surface recolonization by mot^+ and mot^- *Pseudomonas fluorescens*. *Microbial Ecology*, 18, 1-19.
- Korber, D. R., Lawrence, J. R., Zhang, L. & Caldwell, D. E. (1990). Effect of gravity on bacterial deposition and orientation in laminar flow environments. *Biofouling*, 2, 335-50.
- Lappin-Scott, H. M. & Costerton, J. W. (1989). Bacterial biofilms and surface fouling. *Biofouling*, 1, 323-42.
- Lauffenburger, D., Grady, M. & Keller, K. H. (1984). An hypothesis for approaching swarms of myxobacteria. *Journal of Theoretical Biology*, 110, 257-74.
- Lawrence, J. R. & Caldwell, D. E. (1987). Behaviour 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. R. & Caldwell, D. E. (1987). Behaviour of *Pseudomonas*

- fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology*, 14, 1-14.
- Lawrence, J. R., Korber, D. R. & Caldwell, D. E. (1992). Behavioural analysis of *Vibrio parahaemolyticus* variants in high- and low-viscosity microenvironments by use of digital image processing. *Journal of Bacteriology*, 174, 5732-9.
- Lawrence, J. R., Korber, D. R. & Caldwell, D. E. (1989a). Computer-enhanced darkfield microscopy for the quantitative analysis of bacterial growth and behavior on surfaces. *Journal of Microbiological Methods*, 10, 123-38.
- 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.
- Lawrence, J. R., Malone, J. A., Korber, D. R. & Caldwell, D. E. (1989b). Computer image enhancement to increase depth of field in phase contrast microscopy. *Binary*, 1, 181-85.
- Lawrence, J. R., Wolfaardt, G. M. & Korber, D. R. (1994). Monitoring diffusion in biofilm matrices using confocal laser microscopy. *Applied and Environmental Microbiology*, 60, 1166-73.
- LeChevallier, M. W., Babcock, T. M. & Lee, R. G. (1987). Examination and characterization of distribution system biofilms. *Applied and Environmental Microbiology*, 53, 2714-24.
- Lens, P. N. L., De Beer, D., Cronenberg, C. C. H., Houwen, F. P., Ottengraf, S. P. P. & Verstraete, W. H. (1993). Heterogeneous distribution of microbial activity in methanogenic aggregates: pH and glucose microprofiles. *Applied and Environmental Microbiology*, 59, 3803-15.
- Lewandowski, Z., Altobelli, S. A. & Fukushima, E. (1993). NMR and microelectrode studies of hydrodynamics and kinetics in biofilms. *Biotechnology Progress*, 9, 40-5.
- Lewandowski, Z., Lee, W. C., Characklis, W. G., & Little, B. (1989). Dissolved oxygen and pH microelectrode measurements at water immersed metal surfaces. *Corrosion*, 45, 92-8.
- Lion, L. W., Shuler, M. L., Hsieh, K. M. & Costerton, W. C. (1988). Trace metal interactions with microbial biofilms in natural and engineered systems. *CRC Critical Reviews in Environmental Control*, 17, 273-305.
- Little, B., Wagner, P., Ray, R., Pope, R. & Scheetz, R. (1991). Biofilms: an ESEM evaluation of artifacts introduced during SEM preparation. *Journal of Industrial Microbiology*, 8, 213-22.
- Loeb, G. I. & Neihof, R. A. (1975). Marine conditioning films. *Advances in Chemistry Series*, 145, 319-35.
- Lupton, F. S. & Marshall, K. C. (1981). Specific adhesion of bacteria to heterocysts of *Anabaena* spp. and its ecological significance. *Applied and Environmental Microbiology*, 42, 1085-92.
- MacLeod, F. A., Guiot, S. R. & Costerton, J. W. (1990). Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed reactor. *Applied and Environmental Microbiology*, 56, 1598-607.
- Malone, J. A. (1987). Colonization of surface microenvironments by *Rhizobium* spp. M.Sc. thesis, University of Saskatchewan, Saskatoon, Canada.
- Marmur, A. & Ruckenstein, E. (1986). Gravity and cell adhesion. *Journal of Colloid and Interface Science*, 114, 261-66.
- Marshall, K. C. (1986). Microscopic methods for the study of bacterial behaviour at inert surfaces. *Journal of Microbiological Methods*, 4, 217-27.
- Marshall, K. C. (1988). Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian Journal of Microbiology*, 34, 503-6.
- Marshall, K. C. (1989). Growth of bacteria on surface-bound substrates: significance in biofilm development. In *Recent advances in microbial ecology*, ed. T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita & A. Uchida, pp. 146-50. Tokyo: Japan Scientific Societies Press.
- Marshall, K. C. & Cruickshank, R. H. (1973). Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. *Archiv für Mikrobiologie*, 91, 29-40.
- 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.
- Marszalek, D. S., Gerchakov, S. M. & Udey, L. R. (1979). Influence of substrate composition on marine microfouling. *Applied and Environmental Microbiology*, 38, 987-95.
- McCarter, L., Hilmen, M. & Silverman, M. (1988). Flagellar dynamometer controls swarmer cell differentiation of *Vibrio parahaemolyticus*. *Cell*, 54, 345-51.
- McCarter, L. L., Showalter, R. E. & Silverman, M. R. (1992). Genetic analysis of surface sensing in *Vibrio parahaemolyticus*. *Biofouling*, 5, 163-75.
- McCarter, L. L. & Silverman, M. (1989). Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus*. *Journal of Bacteriology*, 171, 731-6.
- McPeters, G. A., Bazin, M. J., Bryers, J. D. *et al.* (1984). Biofilm development and its consequences: group report. In *Microbial adhesion and aggregation*, ed. K. C. Marshall, pp. 109-24. Berlin: Springer-Verlag.
- McInerney, M. J., Bryant, M. P., Hespell, R. B. & Costerton, J. W. (1981). *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. *Applied and Environmental Microbiology*, 41, 1029-39.

- Meadows, P. S. (1971). The attachment of bacteria to solid surfaces. *Archiv für Mikrobiologie*, 75, 374-81.
- Merker, R. I. & Smit, J. (1988). Characterization of the adhesive holdfast of marine and freshwater Caulobacters. *Applied and Environmental Microbiology*, 54, 2078-85.
- Mittelman, M. W., Nivens, D. E., Low, C. & White, D. C. (1990). Differential adhesion, activity, and carbohydrate:protein ratios of *Pseudomonas atlantica* monocultures attaching to stainless steel in a linear shear gradient. *Microbial Ecology*, 19, 269-78.
- Nealson, K. H. (1977). Autoinduction of bacterial luciferase: occurrence, mechanism and significance. *Archives of Microbiology*, 112, 73-79.
- Nichols, W. W. (1989). Susceptibility of biofilms to toxic compounds. In *Structure and function of biofilms*, ed. W. G. Characklis & P. A. Wilderer, pp. 321-31. New York: Wiley.
- Nivens, D. E., Chambers, J. Q., Anderson, T. R. & White, D. C. (1993). Long-term, on-line monitoring of microbial biofilms using a quartz crystal microbalance. *Analytical Chemistry*, 65, 65-9.
- Paerl, H. W. (1975). Microbial attachment to particles in marine and freshwater ecosystems. *Microbial Ecology*, 2, 73-83.
- Perfil'ev, B. V. & Gabe, D. R. (1969). *Capillary methods of investigating micro-organisms* (translated by J. M. Shewan). Toronto: University of Toronto Press.
- Pilgram, W. K. & Williams, F. D. (1976). Survival value of chemotaxis in mixed cultures. *Canadian Journal of Microbiology*, 22, 1771-3.
- Porter, K. G. (1976). Enhancement of algal growth and productivity by grazing zooplankton. *Science*, 192, 1332-4.
- Powell, M. S. & Slater, N. K. H. (1983). The deposition of bacterial cells from laminar flows onto solid surfaces. *Biotechnology and Bioengineering*, 25, 891-900.
- Power, K. & Marshall, K. C. (1988). Cellular growth and reproduction of marine bacteria on surface-bound substrates. *Biofouling*, 1, 163-74.
- Rasmussen, M. A., White, B. A. & Hespell, R. B. (1989). Improved assay for quantitating adherence of ruminal bacteria to cellulose. *Applied and Environmental Microbiology*, 55, 2089-91.
- Revsbech, N. P. (1989). Diffusion characteristics of microbial communities determined by use of oxygen microsensors. *Journal of Microbiological Methods*, 49, 111-22.
- Revsbech, N. P. & Jørgensen, B. B. (1986). Microelectrodes: their use in microbial ecology. *Advances in Microbial Ecology*, 9, 252-93.
- Rittle, K. H., Helmstetter, C. E., Meyer, A. E. & Baier, R. E. (1990). *Escherichia coli* retention on solid surfaces as functions of substratum surface energy and cell growth phase. *Biofouling*, 2, 121-30.
- Rivero, M. & Lauffenburger, D. A. (1986). Analysis of the capillary assay for determination of bacteria motility and chemotaxis parameters. *Biotechnology and Bioengineering*, 28, 1178-83.
- Robinson, R. W., Akin, D. E., Nordstedt, R. A., Thomas, M. V. & Aldrich, H. C. (1984). Light and electron microscopic examinations of methane-producing biofilms from anaerobic fixed-bed reactors. *Applied and Environmental Microbiology*, 48, 127-36.
- Rodriguez, G. G., Phipps, D., Ishiguro, K. & Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied and Environmental Microbiology*, 58, 1801-8.
- Russel, J. B., Cotta, M. A. & Dombrowski, D. B. (1981). Rumen bacterial competition in continuous culture: *Streptococcus bovis* versus *Megasphaera elsdenii*. *Applied and Environmental Microbiology*, 41, 1394-9.
- Sanders, W. M. (1966). Oxygen utilization by slime organisms in continuous-culture. *Journal of Air and Water Pollution*, 10, 253-76.
- Santos, R., Callow, M. E. & Bott, T. R. (1991). The structure of *Pseudomonas fluorescens* biofilms in contact with flowing systems. *Biofouling*, 4, 319-36.
- Shapiro, J. A. & Hsu, C. (1989). *Escherichia coli* K-12 cell-cell interactions seen by time-lapse video. *Journal of Bacteriology*, 171, 5963-74.
- Shimkets, L. J. (1990). Social and developmental biology of the myxobacteria. *Microbiological Reviews*, 54, 473-501.
- Shotton, D. M. (1989). Confocal scanning optical microscopy and its applications for biological specimens. *Journal of Cell Science*, 94, 175-206.
- Sibbald, M. J. & Albright, L. J. (1988). Aggregated and free bacteria as food sources for heterotrophic microflagellates. *Applied and Environmental Microbiology*, 54, 613-16.
- Sieracki, M. E., Johnson, P. W. & Sieburth, J. M. (1985). Detection, enumeration, and sizing of planktonic bacteria by image-analyzed epifluorescence microscopy. *Applied and Environmental Microbiology*, 49, 799-810.
- Silverman, M. & Simon, M. (1974). Flagellar rotation and the mechanism of bacterial motility. *Nature*, 249, 73-4.
- Sjollema, J., Busscher, H. J. & Weerkamp, A. H. (1988). Deposition of oral streptococci and polystyrene latices onto glass in a parallel plate flow cell. *Biofouling*, 1, 101-12.
- Sjollema, J., Busscher, H. J. & Weerkamp, A. H. (1989a). Experimental approaches for studying adhesion of microorganisms to solid substrata: applications and mass transport. *Journal of Microbiological Methods*, 9, 79-90.
- Sjollema, J., Busscher, H. J. & Weerkamp, A. H. (1989b). Real-time enumeration of adhering

- microorganisms in a parallel plate flow cell using automated image analysis. *Journal of Microbiological Methods*, 9, 73-8.
- Sjollema, J., van der Mei, H. C., Uyen, H. M. & Busscher, H. J. (1990a). Direct observations of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. *FEMS Microbiology Letters*, 69, 263-70.
- Sjollema, J., van der Mei, H. C., Uyen, H. M. W. & Busscher, H. J. (1990b). The influence of collector and bacterial cell surface properties on the deposition of oral streptococci in a parallel plate flow cell. *Journal of Adhesion Science and Technology*, 4, 765-77.
- Smith, J. L. & Doetsch, R. N. (1969). Studies on negative chemotaxis and the survival value of motility in *Pseudomonas fluorescens*. *Journal of General Microbiology*, 55, 379-91.
- Sorongon, M. L., Bloodgood, R. A. & Burchard, R. P. (1991). Hydrophobicity, adhesion, and surface-exposed proteins of gliding bacteria. *Applied and Environmental Microbiology*, 57, 3193-9.
- 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. (1971). Growth rates of algae determined *in situ* using an immersed microscope. *Journal of Phycology*, 13-17.
- Stewart, P. S., Peyton, B. M., Drury, W. J. & Murga, R. (1993). Quantitative observations of heterogeneities in *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, 59, 327-9.
- Stove-POindexter, J. (1964). Biological properties and classification of the caulobacter group. *Bacteriological Reviews*, 28, 231-95.
- Thiele, J. H., Chartrain, M. & Zeikus, J. G. (1988). Control of interspecies electron flow during anaerobic digestion: role of floc formation in syntrophic methanogenesis. *Applied and Environmental Microbiology*, 54, 10-19.
- Torrella, F. & Morita, R. Y. (1981). Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. *Applied and Environmental Microbiology*, 41, 518-27.
- Trulear, M. G. & Characklis, W. G. (1982). Dynamics of biofilm processes. *Journal of the Water Pollution Control Federation*, 54, 1288-301.
- Vanhaecke, E., Remon, J.-P., Moors, M., Raes, F., de Rudder, D. & van Peteghem, A. (1990). Kinetics of *Pseudomonas aeruginosa* adhesion to 304 and 316-L stainless steel, role of cell surface hydrophobicity. *Applied and Environmental Microbiology*, 56, 788-95.
- van Loosdrecht, M. C. M., Lyklema, J., Norde, W. & Zehnder, A. J. B. (1989). Bacterial adhesion: a physicochemical approach. *Microbial Ecology*, 17, 1-15.
- van Loosdrecht, M. C. W., Lyklema, J., Norde, W. & Zehnder, A. J. B. (1990). Influence of interfaces on microbial activity. *Microbiological Reviews*, 54, 75-87.
- van Loosdrecht, M. C. M., Norde, W. & Zehnder, A. J. B. (1987). Influence of cell surface characteristics on bacterial adhesion to solid surfaces. *Proceedings of the 4th European Congress on Biotechnology*, pp. 575-80.
- van Pelt, A. W. J., Weerkamp, A. H., Uyen, M. H. W. J. C., Busscher, H. J., de Jong, H. P. & Arends, J. (1985). Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. *Applied and Environmental Microbiology*, 49, 1270-5.
- Verwey, E. J. W. & Overbeek, J. T. G. (1948). *Theory of the stability of lyophobic colloids*. Amsterdam: Elsevier.
- Vesper, S. J. & Bauer, W. D. (1986). Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* to soybean roots. *Applied and Environmental Microbiology*, 52, 134-41.
- Vogel, S. (1983). *Life in moving fluids: the physical biology of flow*. Princeton, NJ: Princeton University Press.
- Wahl, M. (1989). Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Marine Ecology Progress Series*, 58, 175-89.
- Walt, D. R., Smulow, J. B., Turesky, S. S. & Hill, R. G. (1985). The effect of gravity on initial microbial adhesion. *Journal of Colloid and Interface Science*, 107, 334-6.
- White, J. G., Amos, W. B. & Fordham, M. (1987). An evaluation of confocal versus conventional imaging of biological structure by fluorescence light microscopy. *Journal of Cell Biology*, 105, 41-8.
- Wilkins, J. R., Darnell, W. L. & Boykin, E. H. (1972). Cinemicrographic study of the development of subsurface colonies of *Staphylococcus aureus* in soft agar. *Applied Microbiology*, 24, 786-97.
- Willey, J. M. & Waterbury, J. B. (1989). Chemotaxis toward nitrogenous compounds by swimming strains of marine *Synechococcus* spp. *Applied and Environmental Microbiology*, 55, 1888-94.
- Wimpenny, J. W. T. (1992). Microbial systems: patterns in time and space. In *Advances in microbial ecology*, ed. K. C. Marshall, pp. 469-522. New York: Plenum Press.
- Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D., Caldwell, S. J. & Caldwell, D. E. (1994). Multicellular organization in a degradative biofilm community. *Applied and Environmental Microbiology*, 60, 434-46.
- Wright, J. B., Costerton, J. W. & McCoy, W. F. (1988). Filamentous growth of *Pseudomonas aeruginosa*. *Journal of Industrial Microbiology*, 3, 139-46.
- ZoBell, C. E. (1943). The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology*, 46, 39-56.

