FIFTY-NINTH SYMPOSIUM OF THE
SOCIETY FOR GENERAL MICROBIOLOGY
HELD AT THE UNIVERSITY OF EXETER SEPTEMBER 2000

Edited by
D. G. Allison, P. Gilbert, H. M. Lappin-Scott and M. Wilson

Community structure and co-operation in biofilms

Published for the Society for General Microbiology

CAMBRIDGE
UNIVERSITY PRESS

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

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK http://www.cup.cam.ac.uk
40 West 20th Street, New York, NY 10011–4211, USA http://www.cup.org
10 Stamford Road, Oakleigh, Melbourne 3166, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain

First published 2000

Printed in Great Britain at the University Press, Cambridge

Typeface Sabon (Adobe) 10/13.5pt *System* QuarkXPress™ [SE]

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

ISBN 0 521 79302 5 hardback

Front cover illustration: *Pseudomonas aeruginosa* PAN067 biofilm streamers growing in turbulent flow. The streamers were attached to the glass surface by the upstream 'head' while the downstream 'tails' oscillated rapidly in the flow. The scanning electron photomicrographs were provided by Paul Stoodley [Center for Biofilm Engineering (CBE), Bozeman, MT, USA, and Exeter University, UK], Frieda Jørgensen and Hilary Lappin-Scott (Exeter University, UK). Digital enhancement by Kathy Lange, CBE Graphic Design. For experimental details see Stoodley *et al.* this volume.

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An overview of biofilms as functional communities

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INTRODUCTION

A vast number of microbial aggregates fall into this 'catch-all' name – biofilm. Whether this unifying term does a great service or the opposite to a core branch of microbiology is open to some doubt – for biofilm is found in almost every environment graced with surfaces, sufficient nutrient and some water. A gentle digital examination of the waste outlet of the average kitchen sink will reveal a certain sliminess which embraces the quintessential soul of a biofilm! That 'dirt' which can block car windscreen washer jets is from the same stable. It does not seem necessary to list all possible examples of such structures. They range from growth on the leads of cardiac pacemakers, through biofilm attached to the inner surfaces of water distribution pipes, to the epilithon of rocks in streams and accumulated plaque on the surface of teeth.

There are almost as many definitions of biofilm as there are scientists working in the field or types of the structure itself. Any reasonable definition needs to incorporate the idea of a surface or interface on or at which microbes proliferate; it should also invoke the unifying effect of extracellular polymers which can envelop and probably protect the microbial colonies forming. It might also embrace a sense of community with the implication of emergent properties.

It is worth trying to classify a number of microbial systems that seem to be related to biofilm since they share many of its properties. In fact, members of the whole family of microbial aggregates have more in common than separates them (Table 1). The term microbial aggregate is chosen to mean those associations of micro-organisms that are

SGM symposium 59: Community structure and co-operation in biofilms. Editors D. Allison, P. Gilbert, H. Lappin-Scott, M. Wilson.
Cambridge University Press. ISBN 0 521 79302 5

Table 1. Some different types of microbial aggregate

Type of microbial aggregate	Description
Biofilm	Community forming at a phase boundary generally, but not always, at a liquid : solid interface. Spatially and temporally heterogeneous. May have specific mechanisms for attachment to surface. Generates EPS for adhesion, protection and to facilitate community interactions.
Bacterial colony	A group of organisms growing on a surface, often fed with nutrient from below and incorporating gas exchange from above. May be a clone formed from a single cell. Shows recognizable pattern, limited morphogenesis and spatial and temporal heterogeneity.
Effluent treatment floc	A loosely associated mixed community showing irregular radial symmetry and temporal and spatial heterogeneity
Anaerobic digester granules	A reasonably symmetrical radially organized microbial community showing spatial differentiation and metabolic co-operation, often leading to the oxidation of organic substrates, leading in the end to methane
Food-associated systems; e.g. Kefir grains, the ginger beer plant	Irregular radially organized communities often of EPS-producing lactic acid bacteria and yeast. Used in the production of low-alcohol beverages.
Marine snow	Loose associations of microbes with organic detritus
Mycelial balls	Tightly intertwined mycelia generated in fungal fermentations by carefully controlling growth conditions. Radially symmetrical, often spatially heterogeneous as conditions can become anoxic at the centre.
'Wolf-packs'	Associations, generally motile, of swarming bacteria which interact through the transmission of density-dependent signals and feed through engulfing and digesting organic detritus and other microbes
Pellicles	Predominantly two-dimensional structures forming on the surface of liquids; e.g. neuston, pellicles of bacteria including *Acetobacter* and of fungi such as *Penicillium* and *Aspergillus niger* used in the fermentation industry. Oxidant and reductants from opposite sides of the structure.
Algal mat communities	Variably dense, often layered systems whose biology is predominantly driven by sunlight

Table 2. Biofilms forming at different phase interfaces

Interface	Biofilm
Solid : liquid	Most biofilms: epilithon, medical prostheses, the inner surface of water conduits, ship hulls, marine installations, tooth and epithelial surfaces, etc., etc.
Gas : solid – though often exposed to liquids	Bacterial colonies, myxobacterial swarms, lichen, acetic acid production by the 'Quick' vinegar process, trickling filters, surface biofilms using gas phase nutrients
Gas : liquid	Neuston, vinegar production by the Orleans process, penicillin and citric acid production by traditional fungal fermentations
Liquid : liquid	Hydrocarbon oxidizing biofilm at oil : water interfaces; growth in some food emulsions
Solid : solid – though exposed to liquid phase from time to time	Endolithotrophic communities

largely microbial biomass plus varying amounts of extracellular polymeric materials produced by the microbes themselves. This definition excludes communities which are associated with significant amounts of inanimate or other materials, for example soil and many sediments.

Aggregates all represent communities in which the microbial population is concentrated so that there is the possibility of significant interactions (exchange of substrates, products, inhibitors, deployment of signal molecules, etc.) between them. Cell density, the size and geometry of the aggregate and its metabolic activity will almost certainly lead to diffusion barriers, which may be minimal or large enough to cause significant changes in its biology. Most commonly, because of its low solubility in water and high rate of utilization by bacteria, a steep gradient in oxygen tension develops which can lead to anoxic regions and the proliferation of anaerobic species.

Biofilm itself is distinguished from other microbial aggregates since, by definition, it forms at a phase boundary (Table 2). Although most phase boundaries can be colonized, the commonest type of biofilm appears at a liquid : solid interface.

INVESTIGATIVE METHODS

As in so much scientific endeavour, research on biofilms has depended largely on the development of powerful new techniques to investigate their structure and function. These have been reviewed in the past by different authors, for example Costerton *et al.* (1994, 1995) and Caldwell *et al.* (1992, 1997). A few of these are discussed below.

Growth systems

The type of equipment needed to investigate biofilm formation is very much dependent on the type of questions asked. If *in situ* or *in vivo* investigations are required, the experimenter is obviously restricted in his choice since the biofilm itself proliferates in its natural growth system. The subject has been reviewed by Wimpenny (1996, 1999). Table 3 indicates a selection of growth systems with their main attributes.

The wide range of systems available means that there is almost certainly one that will apply to the majority of problems associated with biofilm biology. It is sensible to distinguish between experimental models and microcosms when discussing the investigation of biological systems. A model system represents the cultivation of a completely defined community (one or more species!) in whatever growth device is selected. In contrast, a microcosm is a collection of microbes from a natural community and may include some species that have not yet been isolated. More representative perhaps, but less well understood! To be quite clear, the growth system can be *either* a model or a microcosm according to the above criteria.

Microscopy

Microscopy embraces a wide range of traditional and modern techniques. The electron microscope has been a tremendous source of structural information, always remembering that on the microbial scale, preparation techniques can generate artefacts. Sutton *et al.* (1994) have compared conventional scanning electron microscopy (SEM), low temperature SEM and electroscan wet mount SEM in *Streptococcus crista* to reveal wide differences in the final image. For example, under 'environmental' conditions, one sees mostly a blanket of extracellular polymeric substances (EPS) giving no hint of the structure of the biofilm below.

The application of the confocal scanning laser optical microscope (CSLM) to biological samples (White *et al.*, 1987; Shotton, 1989) provided a powerful tool, especially in conjunction with fluorescent probe techniques. The optical geometry of the CSLM meant that coherent light beams had a very narrow depth of focus whilst all out-of-focus information was rejected. This allowed a series of narrow focal planes to be recorded at different depths throughout a sample. These images can be assembled using image-processing techniques to generate a three-dimensional digitized image. Given the latter, it becomes possible to reconstruct vertical sections through the array, generating an in-depth profile of a biofilm sample. These techniques have helped reveal the highly heterogeneous structure of microbial biofilm.

The use of fluorescent probes with the CSLM added discrimination to what was already a powerful technique. Thus it became possible to distinguish not only between classes,

Table 3. Some of the many biofilm growth systems used in the laboratory

Growth system	Attributes	Reference
Glass slide	Transparent surface allows optical microscopy. Good for attachment and early biofilm growth	Caldwell & Lawrence (1988); Bos *et al.* (1994)
Chemostat-based systems	Surfaces exposed to steady state cultures, though biofilm *not* steady state	Keevil *et al.* (1987); Keevil (1989); Marsh (1995)
Channel reactor	Models flow: channel can be open or closed as a tube. Sample ports at intervals along the channel. Example: the Robbins device.	McCoy *et al*. (1981); Ruseska *et al*. (1982)
Solid particle support	Downflow systems Trickling filter, film grows on a solid substratum with air spaces irrigated with nutrient solutions	Diz & Novak (1999)
	Upflow systems Airlift and related systems. Constant motion leads to attrition and the removal of excess biofilm from the support. System tends towards a steady state.	Gjaltema *et al*. (1994); van Loosdrecht *et al*. (1995)
Constant shear device	*The rototorque*: Two concentric cylinders, the outer stationary, the inner rotating. Removable glass slides in outer wall, constant flow of medium through the system. Growth on slides at known shear	Trulear & Characklis (1982); Bakke *et al.* (1984)
	The Gilbert rotator: Four chambers formed by four sets of intercalating cylinders, each set having fluid inputs and outputs. At a constant rotation speed, four different shear rates applied.	Allison *et al*. (1999)
	The Fowler Cell Adhesion Monitor: A stationary flat disc is aligned near a rotating disc. Cell and nutrient feed pass in near the centre. The cells attach. At a constant rotation rate a shear gradient develops across the plate and cells detach at a critical value.	Fowler & Mackay (1980)
Membrane reactors	A permeable membrane separates oxidant from reductant (e.g. air and growth medium). Biofilm grows on the membrane receiving essential nutrients from each side.	Rothemund *et al.* (1994); Wilderer (1995); Watanabe *et al*. (1997)
Rotating drums	As in the rototorque, though not designed specifically to apply reproducible shear fields. Growth on the inside of the outer cylinder and on the outside of the inner cylinder.	Arcangeli & Arvin (1995, 1997)

Table 3 (*cont.*)

Growth system	Attributes	Reference
Steady state systems	*The Constant Depth Film Fermenter*: Generates steady state in recessed film pans by passing support ring under scraper blades. Operates aseptically under well-controlled conditions	Coombe *et al.* (1982); Peters & Wimpenny (1988)
	The Gilbert '*Baby factory*': System based on the Cooper–Helmstetter device for synchronizing cell populations by attaching them to cellulose acetate membranes, inverting and irrigating with sterile warm growth media. Mother cells attach and form a thin biofilm. Newborn cells are released as soon as the mother cell has completed a division. As in a chemostat growth rate controlled by dilution rate.	Gilbert *et al*. (1989); Gander & Gilbert (1997)

genera and species, but between the viability and even the Gram reaction of individual organisms, as well as monitoring some chemical properties within the biofilm. Probes include negative stains such as fluorescein, which provides a fluorescent background upon which the bacteria can be viewed as non-fluorescent objects. Agents such as resazurin can distinguish between 'live' and 'dead' cells. The former reduce the dye to a colourless non-fluorescent form, in contrast to non-living cells, which retain the fluorescent dye. Living and dead are contentious terms. In microbiology, a living cell can only truly be determined as one that can grow and reproduce, in the end developing a colony. The assumption here is that organisms capable of catalysing oxidation/reduction reactions are alive. Obviously this is an oversimplification. Tetrazolium and related agents operate in a similar fashion. Viability, this time based on membrane integrity, is the basis of a commercial agent, the Baclite viability probe. Here, live cells with intact membranes fluoresce green whilst cells whose membrane structure is compromised (supposed dead) fluoresce red.

Attachment of fluorophores to other agents can increase their value. For example, linked to dextrans they can be used to determine diffusion coefficients and cell distribution, and with polyanionic dextrans charge distribution. Conjugated lectins can help to reveal the distribution of oligosaccharides. Attached to polyclonal antibodies, fluorophores can be used to determine the position of species within a biofilm. A most powerful technique is to attach fluorophores to 16S rRNA probes, allowing the identification of microbes at almost any taxonomic level (see later).

Carboxyfluorescein is a probe whose fluorescence is modulated by pH and has been used to determine spatial differences in pH in biofilms of *Vibrio parahaemolyticus*

(Caldwell *et al.*, 1992). Recently, there has been a major advance in confocal microscopy with the multiphoton laser confocal microscope. The fluorescent agent is excited by two or more photons simultaneously, generally in the infrared. A powerful laser is employed in ultrashort pulses. Because of the longer wavelength used, there are fewer problems with photo-bleaching and the penetration of the laser beam is much deeper than with normal CSLM. In addition, the use of a pulsed beam means that the rate of fluorescence decay can give information which is *not* dependent on the actual concentration of the fluorophore. Using carboxyfluorescein, it is possible to determine the pH around groups of cells further into a biofilm than ever before. This has been demonstrated clearly by Vroom *et al.* (1999) using a defined 10-membered community grown in the CDFF. pH measurements were determined up to 140 μm into the biofilm. pH gradients around cell clusters revealed that values as low as 3.0 were possible.

CSLM has also been employed to determine flow rates in heterogeneous biofilm. Here, fluorescently labelled latex beads were tracked at intervals as they moved through the voids and interstices of a biofilm (Stoodley *et al.*, 1994).

Microelectrode experiments

Once it is accepted that there is heterogeneity on the microscale it is clear that the application of sensors with appropriate geometries are needed to map changes in the physico-chemical environment. Many of these now exist thanks amongst others to Bø Barker Jorgensen and Nils Peter Revsbech and more recently to Dirk deBeer and Zbigniew Lewandowski. The most commonly used are dissolved oxygen and pH electrodes, though others are available for measuring nitrogen oxides and sulphide and there are even enzyme electrodes capable of measuring glucose. Amongst the seminal work on oxygen distribution in biofilms was that of deBeer & Stoodley (1994), who mapped oxygen partial pressure in model biofilms and showed that within cell clusters pO_2 fell to zero whilst around and beneath clusters in void spaces there was always measurable oxygen. More recently, Rasmussen & Lewandowski (1998) have used oxygen probes to determine mass transfer rates in heterogeneous biofilms.

Molecular methods

Fluorescence *in situ* hybridization (FISH). The ability to identify individual bacterial cells represents an important advance in the techniques needed to understand the organization of microbial ecosystems, including biofilm. Oligonucleotide probes are made to recognize specific regions of 16S rRNA; these are then labelled with different fluorescent dyes. Whole families of probes can be generated: for example, one that recognizes prokaryotes, another for the *Archaea* and successively more specific probes for particular groups of bacteria right down to individual species. A particularly good example of the use of 16S rRNA to map the diversity of a microbial population

from earliest colonization to maturity used a river water community grown in a rotating annular reactor (Manz *et al.*, 1999).

Green fluorescent protein (GFP). The jellyfish *Aequorea victoria* synthesizes a fluorescent green pigment. The latter has proved to be a most useful probe since it can be inserted with no obvious ill effects into the genomes of many different types of organism, including animals, plants, yeasts and bacteria (Chalfie *et al.*, 1994; Anderson *et al.*, 1998). If incorporated constitutively GFP expression can be used to recognize particular species right down to the individual level. When located adjacent to specific promoters, GFP fluorescence can indicate which genes are turned on. Since its original discovery, GFP has been altered to provide a range of markers with enhanced fluorescence and/or different spectral characteristics. One or two examples of the use of GFP markers will indicate the tremendous power of the technique. Andersen *et al.* (1998) engineered the GFP protein by attaching a polypeptide sequence to its carboxy-terminus. This sequence, AANDENYALAA, is recognized in *Escherichia coli* by the tail-specific *tsp* protease, which then degrades the whole protein so that the fluorescence disappears. The authors realized that slight alterations to the AANDENYALAA tail sequence could alter the rate at which the polypeptide and hence fluorescence was degraded. They created a family of such tagged proteins which they could then use to examine time-dependent gene expression. These constructs were used to monitor growth rates in terms of the rate of synthesis of rRNA in individual cells or groups of cells in a heterogeneous community (Sternberg *et al.*, 1999).

GFP was used to examine community interactions by the same research group (Møller *et al.*, 1998). Here, two species of bacteria were involved: *Pseudomonas putida* and an *Acinetobacter* sp. The pathway investigated was toluene and related aromatic degradation by the *pu* and the *pm* pathways. Promoters from each pathway were labelled with GFP in the pseudomonad. In pure or mixed cultures of the two species, the *pu* promoter was expressed in the presence of benzyl alcohol whilst the *pm* promoter was only expressed in the pseudomonad when both species were present. There was clearly an important interaction between the two species. The latter were individually tagged with fluorescently labelled 16S rRNA probes as well as GFP so that the identity of the bacteria as well as expression of the aromatic degradation could be monitored *independently* at the level of single cells.

Other methods

Many other investigative methods have been developed. For example, nuclear magnetic resonance imaging has been used to monitor flow regimes in biofilm communities (Lewandowski *et al.*, 1993), and Fourier transform infrared has been used to examine

attachment and growth of microbes on different surfaces. I do not plan to discuss these further.

ADHESION AND EARLY EVENTS IN BIOFILM DEVELOPMENT

It is generally accepted that a very clean surface is quickly covered with a conditioning film of organic molecules, and that this precedes attachment of bacteria to the clean surface. In a liquid : solid system, bacteria penetrate the viscous sublayer by eddy diffusion and attach to the surface through long-range, weak interactions with low specificity, namely electrostatic or van der Waals forces. Irreversible attachment follows through short-range, generally highly specific, interactions. These can be dipole, ionic, hydrogen bonding or hydrophobic interactions (see Denyer *et al.*, 1993). Some of the latter are expressed by the secretion of EPS and by the deployment of a range of fibrillar structures, including fimbriae or fibrils. Many of these are equipped with specific adhesins that can attach to elements of the conditioning film or to other bacteria, especially in complex locations such as the oral environment (Handley *et al.*, 1999).

Once the cells are attached they start to grow and to produce more EPS. At the same time, they often develop strategies for capturing space by moving from where the first few cell divisions have taken place (Caldwell *et al.*, 1992; Korber *et al.*, 1995).

The biofilm develops, generating an architecture which may be more or less porous depending on the physico-chemical characteristics of the environment in which it grows. During its formation, a succession of different species will flourish influenced by changes in the local environment. Species will be imported and exported and other organic and inorganic matter may be incorporated into the structure. At some point, due to shear forces, the development of anaerobic zones forming gas pockets, etc., pieces of the biofilm may slough off. This will be followed by recolonization and regeneration of the structure. What determines the actual three-dimensional structure of a biofilm?

BIOFILM STRUCTURE

The role of physico-chemical factors

It is simply not good enough to ignore the part that physico-chemical factors play in regulating biofilm architecture and function. Unfortunately, the biofilm world seems divided into those who posit that complete control of all aspects of biofilm development, structure, morphology and physiology is due to genetic mechanisms. On the other side are some who believe that structural determinants are completely regulated by local physico-chemical factors. Of course, the truth lies somewhere between these extremes.

A good paradigm for the role of environment in biofilm pattern formation comes from the increasingly sophisticated research into bacterial colony morphogenesis. I have described the interplay between genotype and environment regarding colonies as follows:

> 'Formation of the detailed structure of a bacterial colony is a combination of two separate factors intrinsic and extrinsic. Intrinsic factors are products of the genetics of the cell itself. They determine the morphology of the individual cell, the mode of cell reproduction, the possession of extracellular appendages (flagella, fimbriae, pili etc.) production of extracellular products (exopolysaccharides, proteins etc.) motility, energy metabolism, pigment formation and so on. Extrinsic factors include the prevailing physico-chemical environment which influences the physiology of the cell plus the transport of solutes into and out of the growing colony and the inevitable formation of solute diffusion gradients within the colony and the surrounding medium' (Wimpenny, 1992).

Whilst all of this seems still to be true, we do need to add intercellular signalling to the list of genetically controlled attributes which respond to environmental factors, including propinquity (Thomas *et al.*, 1997).

One of the earliest examples of pattern formation due to diffusion was the work of Cooper *et al.* (1968), who reported that the 'snowflake' pattern of 2-week-old colonies of *Aerobacter aerogenes* could be explained as acute substrate-limited growth. The appellation 'snowflake' indicates not only its morphological resemblance to the snow flake but a similar mechanism for its formation, since the unique and beautiful patterns of a snowflake are due to the restricted diffusion of water molecules to the developing structure. Much more recently there has been a critical examination of colonies of the Gram-positive *Bacillus subtilis* and related species and strains (Matsushita & Fujikawa, 1990; Ohgiwara *et al.*, 1992). Schindler & Rovensky (1994) created a simple computer model of bacterial colony growth. They compared different models and pointed out the resemblance of diffusion-limited aggregation (DLA) models to bacterial colony growth. Ben-Jacob *et al.* (1994) have concentrated in this and a group of related papers on the growth of *B. subtilis* as a function of substrate and agar concentration. They monitored and photographed actual bacterial colonies and compared the latter with a cellular automaton model. Through much of the range of substrate concentrations, the latter (fundamentally a DLA model) reflected the colony structure accurately. At very low substrate concentrations, the actual colony began to differ from the simulation. The difference

was interpreted as a physiological response of the bacteria, via a signalling system, to low substrate concentrations.

The biofilm world was in some disagreement as to what constituted a 'typical' biofilm structure. Some argued that biofilms were simple stalked or irregular branching structures well separated from their neighbours; others that biofilms were mushroom- or tulip-shaped structures penetrated by large and small pores. Yet others considered biofilm to be a more or less flat, homogeneous structure. A simple cellular automaton model was used by Wimpenny & Colasanti (1997) to suggest that all three models were actually correct since the final structure was largely dependent on resource concentration. Thus the first type appeared in water distribution systems where the substrate concentration was very low (Keevil, 1989; Walker & Keevil, 1994). The second type was generated in the laboratory using media containing significant nutrient concentrations (see Costerton *et al.*, 1994, 1995). The third was dense relatively uniform biofilm (Nyvad & Fejerskov, 1989) found in habitats (for example, the human mouth) where nutrient levels are generally high, or periodically extremely high. Recent work by Wood *et al.* (2000) has indicated the presence of channels in dental plaque biofilm. Huang *et al.* (1998) describe a dense flat biofilm formed under conditions of phosphate starvation.

van Loosdrecht *et al.* (1995) proposed that resource concentration and shear rate were both determinants of biofilm morphology. Picioreanu *et al.* (1998, 1999) working in the same laboratory in Delft, Holland, extended this work with a beautiful series of hybrid computer models that agreed substantially with our own proposals. These workers made models of growth in gel beads used in the biotechnology industry and showed that they could reproduce the structures seen very accurately. They went on to investigate biofilm structure using realistic variables and parameters in the simulation. Two- and three-dimensional representations of the resulting predictions clearly showed the range from stacked tower-like configurations at low resource levels to dense confluent growth when substrate availability exceeded its utilization rate. In addition, Picioreanu included the effects of shear rate and indicated the way in which biofilm could erode and slough off as this parameter was increased. Additional support using CA models came from the USA (Hermanowicz, 1998), who presented very similar results from a simpler model.

Use of the CSLM gave interesting evidence that porous structures *were* related to substrate type as well as concentration. Wolfaardt *et al.* (1994) showed that a degradative community degrading diclofop methyl, a commercial herbicide, led to the formation of mushroom-shaped stacks of multicellular aggregates. Growth on tryptone soya broth (TSB) at low concentrations led to groups of cells separated by pores or

spaces, whilst high concentrations of TSB gave a denser confluent appearance to the biofilm.

Cellular control systems

Molecular techniques are starting to throw light on some of the processes associated with biofilm formation. Pratt & Kolter (1998) have isolated mutants of *E. coli* that can no longer attach to surfaces. They were either non-motile or could not make type 1 pili. The latter are mannose-sensitive adhesins. Both motility and the adhesin seem therefore to be associated with attachment.

Similarly, surface attachment defective (*sad*) mutants of *Pseudomonas aeruginosa* were isolated, one of which had defective flagella and hence was non-motile whilst a second could not make the type IV pilus. This last group formed a flat layer on the substratum rather than compact microcolonies (O'Toole & Kolter, 1998).

Extracellular cues seem to be involved in the expression of different regulatory systems. Davies *et al.* (1993) reported that polysaccharide production was switched on when *P. aeruginosa* contacted a surface. This organism seems to express *algC*, *algD* and *algU*::*lacZ*, all associated with alginate production (Davies & Geesey, 1995).

It has become quite clear in recent years that other cues include signalling molecules, often associated with density-dependent phenomena. Two such systems were reported in *P. aeruginosa*. LasR–LasI regulates virulence in the organisms; however, it also controls the formation of a second system, Rh1R–Rh1I, which is a regulator for a number of secondary metabolites. Both systems encode signal molecules: *rb1I*, butyryl homoserine lactone; *lasI*, 3-oxododecanoyl-homoserine lactone. Both mutant strains as well as the wild-type organism can attach to surfaces; however, LasI cannot make the step from microcolonies to a differentiated thick biofilm. Addition of the signal molecule 3-oxododecanoyl-homoserine lactone reverses the effect. Davies *et al.* (1998) conclude that signal molecules are involved in the control of biofilm formation, at least in some species.

Costerton *et al.* (1999) discuss these processes and present a scheme for possible regulation of the development of a biofilm.

Recently, Loo *et al.* (2000) isolated 18 mutants of *Streptococcus gordonii* which were unable to generate biofilm. Whilst nine of these were associated with quorum sensing, signal transduction and osmoadaptation, the remainder were of unknown function, suggesting that much remains to be learned about the processes regulating biofilm formation. Another interesting recent finding is that biofilm formation in *P. aeruginosa*

is also controlled by catabolite repression, more usually associated with regulation of carbon metabolism. O'Toole *et al*. (2000) reported that Crc mutants of this organism made only a simple monolayer film instead of the denser punctuated structure that the wild-type could generate.

COMMUNITY INTERACTIONS

Another area which has excited interest in recent years is the concept of 'community'. By community I think we mean an interacting group of living organisms in the same geographical area. The word can indicate that the sum of its activities is greater than the sum of all the activities of its constituent members. In other words, a community might have *emergent* properties.

Model communities

A productive strand in the investigation of biofilm communities has been the use of model systems. These have the advantage that the system is completely understood and interactions between species can be deduced in an unequivocal fashion. The main disadvantage is the uncomfortable feeling that one cannot properly extrapolate to the natural system since the latter may have additional unrecognized components. It is only possible to mention a few examples of model communities. Bradshaw *et al*. (1989) described a nine-member oral community which was investigated in a chemostat system containing enamel discs. This system was used later by Bradshaw *et al*. (1997) and by Kinniment *et al*. (1996), who grew the community under steady state conditions in a constant depth biofilm fermenter. Most recently, Shu *et al*. (2000) used a four-membered community of cariogenic bacteria as a model to investigate enamel and tooth root caries. Stoodley *et al*. (1999) investigated responses to shear levels on a community consisting of *Klebsiella pneumoniae*, *P. aeruginosa* and *Pseudomonas fluorescens*. In low shear fields, the community grew as roughly spherical colonies, whilst on increasing shear so that flow became turbulent, colonies elongated to generate filamentous streamers. There are many other examples of the use of model communities. It is quite clear that these have an important part to play in biofilm research; one that complements investigations into natural communities. Both approaches are necessary.

Community structure has been the subject of considerable discussion. Caldwell and colleagues (e.g. Caldwell *et al*., 1997) have developed a most interesting *proliferation* theory. Caldwell points out that success at any level amongst living things comes as a result of the ability to grow and reproduce. Success results not from Darwinian concepts of competition, survival of the fittest and so on, but rather just by the ability to grow and survive with inevitably those that grow fastest in a particular set of physico-chemical conditions surviving best. Proliferation is an iterative process: it can apply at

the molecular level, at the level of subcellular structures, at the cellular, organ and species level. What is more, proliferation is not, as Darwin would have it, restricted to 'species'. A group of species can form a community, and the latter may have emergent properties which allow it greater success by increasing its genetic potential, allowing it to use a wider range of resources, modify its own environment possibly in a favourable direction and in the end to proliferate faster. The Caldwell reference is an interesting, detailed, thought-provoking article, which discusses microbial communities in all their disparate forms, including homogeneous chemostat and gradostat cultures as well as gel-stabilized gradient systems. In addition, the physiology of anaerobic digester granules as communities is discussed. The concept of 'proliferation' seems perfectly acceptable and raises interesting suggestions that a community can become, under steady state conditions, an entity with some kind of deterministic stoichiometry. It might be true under rigidly controlled conditions though even here, as Caldwell points out, the genotypic flexibility of constituent species will ensure that continual changes occur. For example, the Dalapon community grown in a chemostat over prolonged periods consisted of primary Dalapon degraders and secondary organisms. In the end, a secondary organism mutated to be able to use the herbicide (Senior *et al.*, 1976).

There are a number of other interesting observations, both structural and functional, concerning biofilm communities. Oral bacteria were first shown to coaggregate by Gibbons & Nygaard (1970). Kolenbrander (1988) and most recently Kolenbrander *et al*. (1999) have carried the analysis of coaggregation to a highly sophisticated level, demonstrating complex patterns of possible structures that have been deduced first from simple test-tube coaggregation studies, and secondly by a careful analysis of specific receptor sites and adhesins on oral species. Here we should recognize the idea of a *structural* community where the members are arranged in a specific spatial order. Events in oral communities have progressed further, perhaps in an understanding of these complex interactions, than for most other biofilm systems. In the same environment, there is good evidence for a *functional* community. Bradshaw *et al*. (1994) demonstrated metabolic co-operation between a selected model community of oral bacteria, which together could degrade hog mucin better than the sum of activities of all the individual species. This was due to the collaborative efforts of community members, each of which was able to degrade one step in the breakdown of the substrate. This work has most recently been reviewed by Marsh & Bradshaw (1999), who discuss both the sequential cleavage of sugars of the oligosaccharide side chain and the concerted attack on host proteins and glycoproteins using sulphatases, glycosidases and proteinases.

Community structure has been elegantly investigated by Molin and his group using GFP plus fluorescent rRNA probes to visualize the manner in which species in

microbial aggregates in a biofilm are organized. This group has recently shown that two bacterial species, a *Pseudomonas* and a *Burkholderia*, growing on citrate would develop as separate individual microcolonies. However, only the *Burkholderia* was capable of growing on the aromatic compound chlorobiphenyl, which was oxidized to chlorobenzoate, which the pseudomonad could use. Under these conditions, the pseudomonad moved, presumably chemotactically, towards the *Burkholderia*, leading to a mixed colony functioning as a 'working' community at the microcolony level (Nielsen *et al.*, 2000). A beautiful example of ecology in action!

Stochastic or deterministic factors?

Molin (1999) has pointed out that there are two quite different approaches to biofilm community structure. The first view is that such communities are merely random accretions of bacteria which can, if spatial constraints permit, associate and interact in a manner which benefits the community. A second point of view is that all communities are evolving structures associated in a more or less deterministic fashion as a specific answer to environmental problems.

Perhaps the truth is somewhere between these two extremes. Stochastic processes precede deterministic ones. Thus the colonization of clean surfaces is essentially random, consisting of a subset of all possible microbes that have the ability first to attach loosely and then firmly to a surface. These cells may start to grow or may be washed off the surface due to shear forces. Organisms capable of interaction *may* be located close enough to each other that interactions are possible. This is the *propinquity* factor (Thomas *et al.*, 1997). Now deterministic factors come into play. Interactions may lead to a hybrid association forming, where the two organisms co-operate. Chemoattractants, including pheromone molecules, may lead to the movement or even the growth of microbes towards one another. The emerging community might recruit additional contributing members by a combination of chance and necessity. So under reasonably stable environmental conditions, a community is formed whose properties *may* represent the best solution to immediate environmental problems/opportunities. One should not really be under the illusion that such a community could be compared to a *tissue* in the sense that a multicellular organism has a precise structure with only relevant cell types present. No, our microbial community will consist of a melange of types. These will include primary resource converters; secondary and subsequent species relying on products of a food chain; scavengers that do not contribute to the efficiency of the community or may even detract from it; parasites, predators and competitors, none of which represent added value for the association. What is more, as time goes by, other species will be imported or exported so that the community will change in ways that may or may not be energetically favourable. Some of these processes are indicated in Fig. 1.

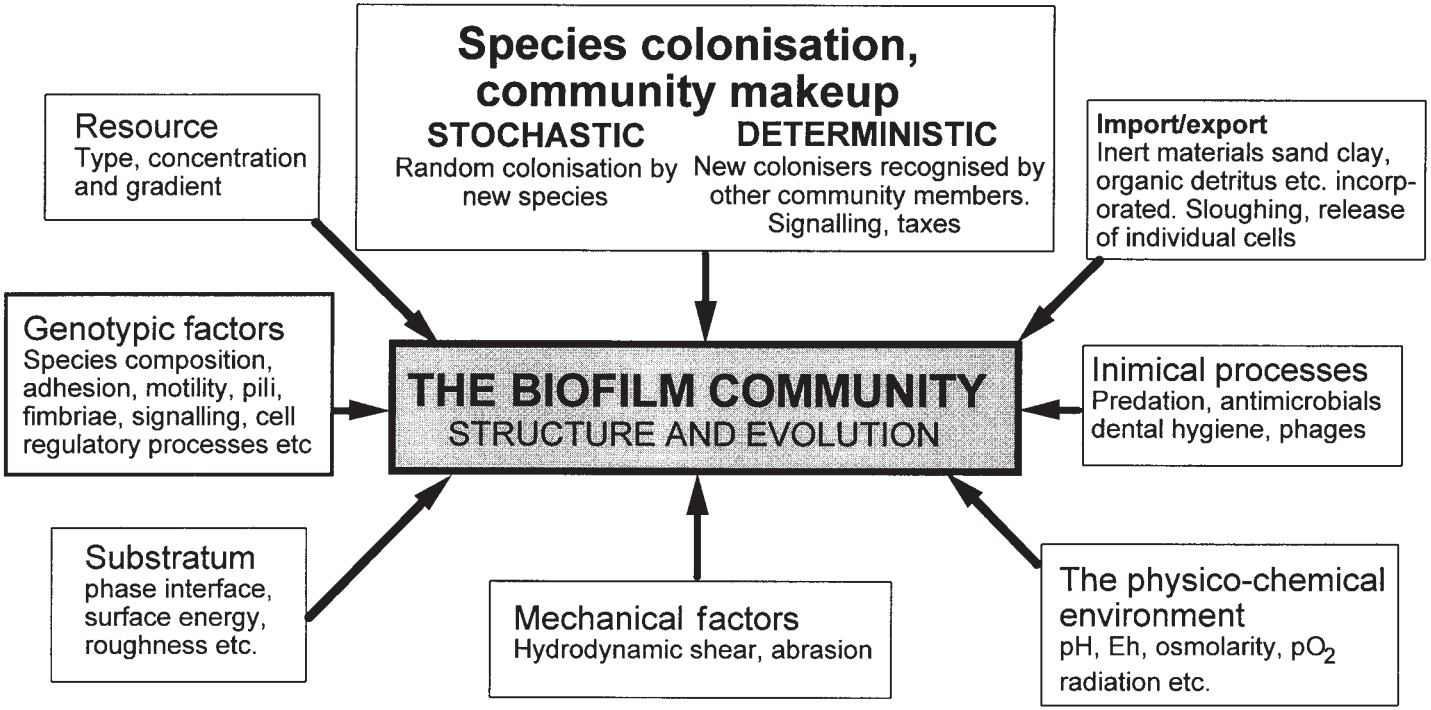

Fig. 1. Some of the many factors that influence the development and biology of a biofilm.

Factors affecting possible levels of community interaction

Even a cursory examination of microbial systems reveals that there are many different levels of association between microbial communities. At one end of the scale are obligate endosymbiotic associations like mitochondria or chloroplasts; at the other, very loose temporary associations in dynamically changing temporally and spatially heterogeneous systems. There seem to be two dominant themes that govern the process of organization into a community. The first of these is the benefit of the association; the second is stability in terms of space and time. The energetic value of possessing prokaryote-derived organelles transcends most other considerations as far as eukaryotic microbes and metazoans are concerned. From this extreme form of co-operation one can discern a spectrum of levels of association. This is illustrated in Fig. 2. Below organelle development come endosymbionts, some of which can exist apart from their hosts. These might be green algae associated with *Hydra*, or possibly some sulphur-oxidizing species allied to marine animals found around deep submarine black-smokers. Tight associations between cellular types epitomize lichens as well as the two bacteria that make up '*Methanobacillus omelianski*'. Other syntrophic associations can be found in anaerobic communities, where one organism donates hydrogen and a second scavenges it to generate methane. Some of these lead to discrete organized structures like anaerobic digester granules. At the other end of the scale are very loose associations, such as those found in many biofilm communities, though even here there is a wide range of levels of interdependence. For instance, high in the interdependency stakes are dental plaque communities.

There may be some lessons for us in this spectrum of dependency amongst microbial communities. For many microbial habitats there is not much evidence that recognizable tightly organized consortia exist, suggesting that we might look for other explanations for the loose associations which seem to be the *sine qua non* of microbial ecology. One explanation could be based on two main factors: micro-scale heterogeneity on the one hand and response time on the other.

At the micro-scale, the physico-chemical universe is very different from the way we perceive it at the macro-scale. Diffusion is the main transport process and is extraordinarily fast when cell–cell distances are short. At the one or a few microbes level, diffusion gradients around the cell are meaningless, since mass transfer rates exceed the cell's ability to use a substrate or generate a product. However, larger multicellular associations *can* generate meaningful solute gradients, leading to structural and metabolic differentiation or the accumulation of molecules (for example, digestive enzymes or quorum-sensing products). Again it follows that the development of a structure means that processes of chemotaxis and of intercellular signalling can operate successfully.

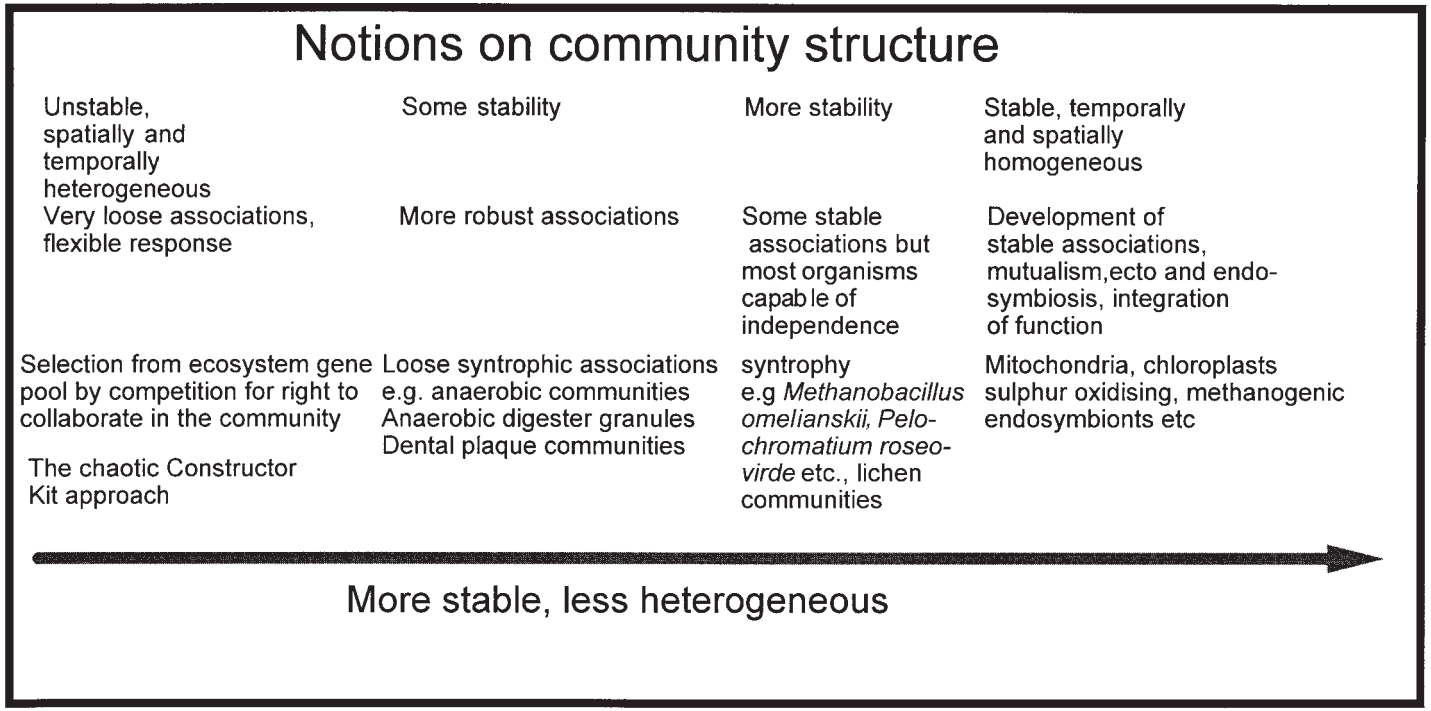

Fig. 2. One interpretation of the range of microbial communities found in nature.

In most microbial habitats, spatial heterogeneity on the micro-scale can lead to a multiplicity of microscopic niches, allowing the proliferation of different species. In a soil system, there are steep pH gradients around colloidal clay and humic materials. Organic debris means that localized 'hot spots' containing good substrate resources or conversely inhibitors may be present. Water potential, temperature, light, substrate and product gradients can alter quickly, sometimes in an irregular manner with very short time constants, sometimes as part of daily or yearly cycles.

For all these reasons, living cells at this scale must be capable of a rapid response. There is therefore a premium on very small units of metabolism (cells) which have high surface area to volume ratios, a simple structure and the minimum of diffusion restriction in their internal transport structures.

Biological constructor kits. The strategic consideration at this level is that of the Meccano (constructor) set! In the latter, it is possible to generate a crane from amongst the store of basic components (nuts, bolts, wheels, axles, frames, etc., etc.), or if later a truck is needed, the crane can be taken to pieces and the new vehicle generated from the same building blocks. Similarly, local physico-chemical and biotic factors determine the kind of biological 'machine' that is needed and the pieces are selected to put it together. As conditions change, so different 'machines' are needed and they duly form. It might be interesting to collect together some of the parts of the constructor kit. Naturally, it is tempting to regard each species in the microbial world as one such building block and in a way this is almost certainly true. The divisions of labour seen in microbiology are endlessly intriguing. Examples include the separation of ammonia oxidation from nitrite oxidation amongst nitrifying species. The dental plaque community seems to be quite a good example of a construction kit having emergent properties, as does the community found in anaerobic digester granules.

Of course the concept of a construction kit is too simplistic, since, as discussed earlier, our machines are much more chaotic that the mechanical variety just mentioned. Imagine the crane or the truck being *approximately* right for the job, however the mad creativity of Nature has led to the bolting on of all sorts of different bits, some with function, others rather hindering its ability to do its professed job, yet others hell bent on destroying it! Now *that's* a microbial community!

Biofilms are just one, albeit the most important, of a group of microbial aggregates that can operate as integrated communities. Community is a relatively novel idea in microbial ecology, and now we have the tools to investigate this properly, the future looks tremendously exciting!