Annu. Rev. Microbiol. 1990. 44:579–602

DETERMINATION OF BACTERIAL GROWTH AND ACTIVITY AT SOLID-LIQUID INTERFACES

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KEY WORDS: microbial adhesion, boundary layer, biofilm, biofouling, sessile bacteria

CONTENTS

0066-4227/90/1001-0579$02.00

INTRODUCTION

The activities of surface-associated microorganisms have been of interest to aquatic microbiologists ever since bacteria were found to colonize microscope slides submerged in aqueous environments (12, 48, 123, 124). ZoBell (123) acutely recognized the tendency for marine bacteria to attach to the walls of sample bottles containing nutrient-poor seawater and suggested that this behavior may be a response to gain better access to nutrients concentrated at solid surfaces. Since these early observations, steady progress has been made in understanding the physiology of sessile microorganisms.

Characterization of the activities of sessile bacteria has lagged behind that of free-living bacteria, primarily because of the difficulties associated with studying the cells while they are attached to surfaces. Representative samples of surface-associated microorganisms are difficult to obtain, and differentiating between phenomena contributed by the attached cells and those contributed by the surface also presents a challenge. Nevertheless, the growing realization that immobilized microorganisms perform activities that are in some instances more diverse, efficient, and economically important than their free-living, planktonic counterparts has spawned an interest in the past decade to study cells at surfaces.

This review describes some of the approaches that have led to a better understanding of the activities of sessile microbial populations that reside in biofilms and microbial mats. We have chosen to focus our attention on techniques that have been used to characterize those activities that occur subsequent to the initial attachment step. Readers interested in additional information on the activities of biofilm populations are directed to other recent reviews on this subject (45, 46).

We hope that this review will promote a greater appreciation of the impact that surfaces have on microbial activities. We also hope that the reader will be encouraged to consider the approaches described in the following pages when studying surface-associated microbial processes. Such efforts should make possible better understanding of why a sessile existence among microorganisms is so prevalent in nature.

We dedicate this review to the late Claude E. ZoBell, who was among the first to perceive the importance of surfaces to microbial survival in natural habitats.

BEHAVIOR OF SURFACE-ASSOCIATED MICROORGANISMS

Light Microscopy

Microscopy has been and continues to be the primary method of studying attached microbial populations. Marshall (67) recently reviewed the use of

microscopic methods for the study of bacterial behavior at inert surfaces. The reader is referred to this paper for more detailed information on this subject. Transmitted light microscopy has been used extensively to study colonization of surfaces by microorganisms. The classic study of Marshall et al (68) demonstrating the reversible and irreversible stages of attachment of bacteria to transparent microscope slides submerged in a static seawater system was performed with phase-contrast photomicroscopy. Using this approach, they observed that the cells rotated around the axis of the point of cell attachment during the reversible phase. Soon thereafter, the cells were seen to either desorb or proceed into a phase of irreversible attachment to the surface.

Kjelleberg et al (56) utilized a dialysis microculture chamber described by Duxbury (20) combined with time-lapse videotape recording of bacteria observed under oil immersion phase-contrast microscopy to study bacterial colonization of a cellulose membrane in contact with an aqueous medium containing low (2–4 mg/l) concentrations of organic nutrients. Using this approach, they were able to observe small, starved vibrios attach to the membrane, increase in size, and replicate on the surface. They also observed that perpendicular attachment allowed one of the daughter cells to escape from the surface after cell division. These microscope techniques have provided a glimpse of the fascinating behavioral patterns exhibited by bacteria progressing from a planktonic to a sessile existence.

Mathematical expressions that describe colonization activities of microorganisms on glass microscope slides have also been developed using light microscope observations. Brannan & Caldwell (4) presented an equation that integrated the effects of simultaneous attachment and growth of bacteria on a surface. Using this equation, the specific growth rate of *Thermothrix thiopara* was found to be 0.38 h^{-1} during in situ colonization of glass slides in a hot spring. Later, Caldwell et al (9) and Malone & Caldwell (66) modified their mathematical approach to isolate the effects of growth and attachment on microbial surface colonization. The modified approach does not require a computer to solve for the specific growth rate and the counting procedure is simplified.

Computer-Enhanced Light Microscopy

Computers have now become an essential tool in many areas of microscopic image analysis. Lawrence et al (60) used phase contrast, dark-field, and computer-enhanced microscopy to evaluate the behavior of *Pseudomonas fluorescens* that attached to the inner surface of a 1 × 3 mm glass flow-cell containing culture medium flowing at different laminar velocities. They confirmed the observations of Marshall et al (68) that the cells attached apically and rotated either clockwise or counter-clockwise about the point of

attachment for a period of time before either detaching from or becoming irreversibly attached to the surface. By continuously observing cells at an early stage of attachment, Lawrence et al (60) could demonstrate that the cells established a new orientation with the surface along their longitudinal axis before entering the irreversibly attached stage. This orientation appeared to be necessary for subsequent surface-associated growth and replication of the cell.

Computer-enhanced microscopy has also been used to evaluate bacterial colony development on surfaces. Lawrence et al (60) found that initially 100% of the cells formed two-cell colonies. Of those, only 86% formed four-cell colonies and only 77% of the four-cell colonies formed 16-cell colonies. These studies confirmed that the number of bacteria in a microcolony was 2^n and that no significant emigration or immigration occurred until the colony was past the four-cell stage of development. After four generations at one location, the microcolony became unstable. Some of the cells in the colony detached and emigrated to new locations on the surface (recolonization).

Computer-enhanced microscopy has also permitted evaluation of bacterial maneuvers on surfaces. Lawrence & Caldwell (59) showed that colonization maneuvers are required for growth of bacteria on a surface. By observing the surface continuously over time, they determined that different types of bacteria utilize different maneuvers. The packing maneuver was described for a *Pseudomonas* sp. in which the cells remain closely spaced within a cell monolayer. This maneuver was common among bacteria colonizing surfaces in aquatic habitats. Other bacteria displayed a shedding maneuver in which the cells are oriented perpendicular to the surface so that each daughter cell emigrates from the surface. Some bacteria utilize a rolling maneuver in which the replicating cell bounces across the surface during cell division and the resulting daughter cells become separated.

Lawrence & Caldwell (59) used computer-enhanced microscopy to differentiate between growth and attachment of cells to the inner surface of a glass flow cell containing flowing aqueous medium. The kinetics of attachment and replication described by cell density and distribution data obtained by microscopic evaluation applied only to those populations that utilized the packing maneuver. For *P. fluorescens*, a specific growth rate of 0.56^{-1} was determined during the period of time required to produce the first four generations.

Computer-enhanced microscopy was used to evaluate the effects of motility on the reattachment of bacteria that desorb from surfaces (57). Recolonization by motile cells of *P. fluorescens* led to the formation of a more uniform biofilm than that exhibited by nonmotile mutants. Nonmotile mutants recolonized surfaces in patterns that resembled "drifts" and "windrows". Back-

growth (colonization of surfaces against a laminar flow) occurred four times faster with motile cells than with nonmotile cells.

The growth of bacterial colonies on agar-coated microscope slides under quiescent conditions has been characterized using computer-assisted, time-lapse video microscopy (102). Intimate side-by-side associations were seen to develop between daughter cells in microcolonies that arose from isolated single bacteria and between daughter cells derived from different but nearby isolated bacteria. The results suggest that *Escherichia coli* K-12 cells respond to each other and adjust their geometric growth pattern to form multicellular groups as they proliferate on agar. These and other studies demonstrate that direct microscopic evaluation is well suited for detecting the movements and orientations of microorganisms on surfaces.

Escher & Characklis (25) used image analysis methods to obtain direct measurements of critical independent processes contributing to colonization of rectangular glass capillary tubing in a flow-through chemostat system. Sorption-related processes followed zero-order rates, while kinetic- and growth-related processes were first-order rates with respect to substrate concentration. Accumulation under constant shear stress was found to be proportional to the colony-forming-unit concentration in the bulk flow. Growth-related processes became increasingly important in accumulation of bacteria at a surface after about 100 min exposure to the surface. However, the influence of cell concentration in the bulk fluid determined the extent of accumulation with time.

Microscope techniques have been employed to study the effects of bulk fluid nutrient levels and hydrodynamics on activities of bacteria at surfaces. Using computer-enhanced microscopy, Caldwell (8) demonstrated that at low nutrient concentrations in the bulk fluid, growth of cells on a surface was flow-dependent, while at high nutrient concentrations, growth was independent of flow. Kjelleberg et al (56) utilized a dialysis microculture chamber combined with time-lapse videotape recording of bacteria observed under oil immersion, phase-contrast microscopy to study bacterial colonization of a cellulose membrane in contact with an aqueous medium containing different concentrations of organic nutrients. At low (2–4 mg/l) organic carbon concentrations, the interval between divisions of cells attached to the membrane was 57 min., whereas in nutrient rich medium (4500 mg/l), the interval was 28 min.

While these bright-field and phase-contrast microscopic techniques are well-suited for the study of cells on transparent surfaces, they cannot be used to evaluate microbial behavior on opaque surfaces, which occur almost exclusively in the natural environment. Recently, Sjollema et al (103) described a method of detecting microorganisms adhering to opaque surfaces using low-angle incandescent dark-field illumination. Microscopic im-

ages were collected by a video camera, digitized, stored, and displayed on an external monitor using image analysis software run on a microcomputer. Thus, one can now study the behavior of sessile microorganisms on virtually any inert surface.

In summary, computer-enhanced, direct microscopic evaluation has proven to be a useful approach to understanding the early phases of biofilm development on submerged surfaces. For more detailed information on computer-enhanced microscopy, the readers are referred to a review by Caldwell (7).

DETERMINATION OF ATTACHED MICROBIAL BIOMASS

The biomass of microorganisms attached to surfaces has been determined using a variety of physicochemical techniques. Fletcher (28) and Pedersen (86) described simple spectroscopic methods. Microbial biomass attached to glass microscope coverslips or polystyrene dishes was determined by absorbance measurements following staining of the attached microorganisms with crystal violet. The method was valid when the bacteria were distributed evenly over the surface and control preparations contained a large number of attached cells. Absorbance values obtained at 590 nm using a spectrophotometer correlated well with protein nitrogen, dry weight, and organic carbon associated with the coverslips. The advantage of the technique was that it eliminated the tedious work involved in enumerating the bacteria under the microscope.

Microorganisms associated with biofilms can be detected by recovering specific molecules formed by bacteria on fouled surfaces. The utilization of biomarkers to define microbial consortia avoids the bias of cultural selection inherent in the classic plate count technique or the difficulties in estimating microbial biomass associated with biofilms using direct microscopic methods. When the appropriate biomarkers are evaluated, the total community may be examined without the necessity of quantitatively removing the microorganisms from the surface, thus preserving the microstructure of multi-species consortia.

Lipids

TOTAL MICROBIAL BIOMASS Polar lipids, which in bacteria are essentially phospholipids, are a particularly useful group of molecules to utilize as biomarkers (112). Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities, the bacteria contain a relatively constant proportion of their biomass as phospholipids (86). Phospholipids are not found in storage lipids and have a relatively rapid

turnover, so the assay of these lipids gives a measure of the viable cellular biomass (117). Determination of the biomass in a subsurface soil where the microbial components were confined to cocco-bacillary bacteria showed equivalence between direct counts, total ATP, muramic acid, and several measures of the membrane lipids when appropriate calibration factors were determined for bacteria at the same nutritional status (2).

BIOMASS CONTRIBUTED BY SPECIFIC PHYSIOLOGICAL GROUPS The ester-linked fatty acids in the phospholipids (PLFA) are both the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed (3, 110, 112). The specification of fatty acids that are ester-linked in the phospholipid fraction of the total lipid extract greatly increases the selectivity of this assay as most of the anthropogenic contaminants, as well as the endogenous storage lipids, are found in the neutral or glycolipid fractions of the lipids. The distribution of specific patterns of component structures in the PLFA are sufficiently limited in distribution that they may be utilized as signatures. The utilization of signatures provides an elegant and effective means of rapidly identifying isolated organisms. After growing the organisms on specified media, the amount of total acid-labile fatty acids recovered, and the patterns of the methyl esters can be determined on a standardized capillary gas chromatographic system. The bacteria can be identified by comparing the fatty acid profiles with those from thousands of known bacteria in a library (101). To fully exploit the diversity of PLFA structures that occur in microbes recovered from biofilms (as opposed to the monocultures in the Sasser method), the fatty acids can be derivatized and their specific features detected by gas chromatography/mass spectrometry (GC/MS). The determination of the configuration and position of double bonds in monoenoic fatty acids is accomplished after derivatization by dimethyldisulfide adducts (79). Derivatization for determination of the position of hydroxyl groups and cyclopropane rings also increases the specificity. The sensitivity can be greatly increased with the formation of electron-capturing derivatives that, after separation by capillary gas-liquid chromatography (GLC), can be detected using negative chemical ionization mass spectrometry at femtomolar or better sensitivities (84). A. Tunlid (unpublished data) recently conducted experiments in the detection of ethanolamine from phosphatidyl ethanolamine at the 10^{-17} molar range, which is in the range of tens to hundreds of bacteria.

Criticism has been widespread of the use of PLFA patterns to identify groups of bacteria in view of evidence demonstrating changes in PLFA patterns in monocultures exposed to varying nutrient levels or temperature. Where actual natural biofilms have been utilized, the detection of specific groups of bacteria such as the sulfate-reducing bacteria, methane-forming or

methane-oxidizing bacteria, sulfur-oxidizing bacteria, specific pathogens in wounds or soils, and others have been reliably determined. The validation of the PLFA technique in biofilms has been reviewed (116). Manipulations of biofilms utilizing antibiotics and cultural conditions produced the expected morphological and biochemical changes (119). Other validations such as the isolation and analysis of specific organisms and their detection in appropriate environmental consortia and changes in the local environment such as light intensity or predation have been summarized (81, 82, 97, 114, 118).

EVALUATION OF NUTRITIONAL STATUS The lipid analysis also provides specific information about the nutritional status of the biofilm populations. Some bacteria that are starved for periods of time decrease in size and exhibit an absolute increase in the ratio of *trans* to *cis* monoenoic PLFA (42). Certain bacteria form the endogenous lipid poly beta-hydroxyalkanoate (PHA) under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (78).

Cell Wall Components

The peptidoglycan of the bacterial cell wall contains several components not found in other organisms that can be utilized as indicators of bacterial biomass on surfaces. The glycan chains of N-acetyl glucosamine and N-acetyl muramic acid (MA) are interconnected with short chains of specific amino acids with unique structures such as D-alanine and diaminopimelic acid (DAP). MA and D-alanine are essentially universal cell wall components and DAP is found in gram-negative and some gram-positive bacteria. The wall components require strong acid hydrolysis and purification by chromatography. Detection of MA is based on the detection of lactate following its release from the MA molecule (72). Lactate can be detected with high sensitivity using GC or high-pressure liquid chromatography (10, 26, 34, 50, 74, 107). DAP analysis has been simplified from more cumbersome procedures (19). D-alanine detection requires either separation on a chiral chromatography column or derivatization with an optically active agent (107, 108). The composition of the wall components does not vary much with growth cycle or nutrient input (24). Wall components persist in soil and aquatic environments much longer than cytoplasmic components by complexing with humic acid polymers (19, 76, 111).

The contribution of gram-positive and gram-negative bacteria to surface microbial populations can be determined on the basis of unique cell wall components. Gram-positive organisms in biofilms can be detected by analysis of teichoic acids based on selective hydrolysis of phosphate esters by concentrated hydrofluoric acid (40). Gram-negative bacteria can be detected by the lipopolysaccharide (LPS) components. The LPS contains several unique

components for analysis, but the extraction of LPS from the biofilm requires harsh reagents such as hot phenol/water or trichloroacetic acid (99, 113).

LPS in biofilm extracts has been determined using the *Limulus* amebocyte lysate (LAL) test (23, 31, 71, 83, 105) or by GC analysis of ketodeoxyoctonate (KDO) (6, 99). The LAL test is subject to interference. GC determination of the hydroxy fatty acids that are covalently bound to the Lipid A portion of the LPS is more sensitive than other methods. In addition, the hydroxy fatty acids are easier to recover from biofilms than other LPS components. Typically, the lipids are extracted, acid hydrolyzed, the hydroxy fatty acids are re-extracted and analyzed after derivatization by GC/MS (85). The LPS-fatty acids are detected with high sensitivity using chemical ionization mass spectroscopy with negative ions (65, 104). Many different groups of gram-negative bacteria can be identified from the patterns of the hydroxy fatty acids (121).

DETERMINATION OF METABOLIC ACTIVITIES OF SURFACE-ASSOCIATED MICROBIAL POPULATIONS

Microcalorimetry

The expenditure of energy is a prerequisite of all microbial activities. A nonspecific measure of energy expenditure is heat output. Microcalorimetry has been used in several studies to measure heat output by surface-associated microorganisms.

Lock & Ford (62) described a relatively inexpensive flow microcalorimeter that could be used to measure heat output from any attached or sedimentary microbial community over or through which a small volume of water is passed. They found that the measurement of heat output of an attached community was accurate as long as the flow of water through the instrument was precisely controlled. The instrument could detect heat outputs as low as 3 μW.

Using microcalorimetry, Lock & Ford (63, 64) grew epilithic microorganisms on glass beads inside plastic tubes submerged in streams. After sufficient time for establishment of a stable community, the beads were transferred to two cells in the microcalorimeter. The difference in heat output between the cell containing beads of acid-killed microorganisms and the cell containing an equal number of beads colonized with live microorganisms provided an estimate of total metabolic activity. Using this approach, they found that heterotrophic activity was 51% of the total combined autotrophic and heterotrophic metabolic activity.

Microcalorimetry was also used to determine which size fractions of the naturally occuring dissolved organic carbon (DOC) pool supported lotic epilithon activity. Lock & Ford (63, 64) found that the fraction with a weight

of less than 1 kd contributed up to 15% of the metabolic activity of the epilithon. However, when the > 1-kd size fraction of DOC was removed from the stream water, the attached community exhibited the same heat output as those exposed to the complete range of size fractions contributing the dissolved organic carbon in the stream. The lack of response of the epilithon community to radical changes in the exogenous organic energy supply was attributed to utilization of endogenous reserve material, exogenous energy stored within the polysaccharide matrix of biofilm, or cryptic growth. When similar experiments were conducted over longer periods of time, removal of the > 1000-kd size fraction from the water resulted in a stimulation of epilithon activity over that observed in the presence of the complete DOC pool (32). These results indicated that the higher molecular weight fractions contained compounds that were inhibitory to heat output–related epilithon activities.

Humphrey & Marshall (51) used a microcalorimetry reaction vessel to study dissolved nutrient uptake by attached bacteria. The presence of strips of cellulose dialysis tubing stimulated heat output from starving, surface-associated bacteria during glutamic acid uptake. Combining microcalorimetry with microscopic examination of the starved bacteria on the cellulose surface revealed that the cells exhibited a decrease in volume at the time of increased heat output. Although calorimetric determinations provide little information on specific biofilm processes, they do afford a measure of overall surface-associated metabolic activity.

Microelectrodes

One of the most promising approaches for the study of surface-associated microbial activities involves microelectrodes. Developed primarily for determining variations in pH and oxygen concentration in structured microbial mats, microelectrodes have proven to be useful probes for dissecting the different physiological activities carried out by microbial populations that develop on surfaces.

Microelectrodes capable of oxygen, hydrogen sulfide, and pH determinations have been positioned with a micromanipulator in thick microbial mats in a hypersaline pond to obtain a respiratory budget for the mat community (93). The amount of sulfide produced was found to correspond to the mineralization of an amount of organic carbon equal to the total amount produced by primary production, as determined by oxygen measurements. Because the electrodes are very thin (5–200 μm) at the tip, minimal disturbance occurred in the area of the mat where readings were obtained. Measurements taken at 100-μm depth intervals produced smooth changes in oxygen, sulfide, and pH, indicating that diffusion occurred rapidly within the mat (53).

Oxygen microelectrodes have been useful in defining the chemical environment of a surface covered by a biofilm. The oxygen concentration at the surface of the brown algae *Fucus serratus* was found to decrease to zero when a thin layer of fine detritus covered the algae (54). Lewandowski et al (61) determined the oxygen profile in an artificial biofilm of bacterial cells in agar that coated the surface of stainless steel coupons. They too found that the respiratory activities of the biofilm bacteria promoted a gradual decline in dissolved oxygen concentration from 8 ppm at the agar surface to 0 ppm at a depth of 3.5 mm. They proposed that oxygen removal at the metal surface by the microorganisms promotes corrosion of the metal by inhibiting the deposition of a protective calcareous layer.

The oxygen concentration was mapped in a 0.2- to 2.0-mm-thick epiphyte layer on different marine and freshwater macrophytes with a microelectrode during light and dark cycles (100). Large fluctuations in oxygen concentration were recorded at the macrophyte surface under the epiphyte layer. During the photosynthetically active period, the oxygen concentration at the macrophyte surface was three times the saturation level, then dropped to zero during periods of darkness. The investigators concluded that the epiphytic population attenuated light and increased resistance to the transfer of dissolved substances between the bulk phase and the plant surface.

High-resolution profiles of photosynthesis and oxygen concentration in a microbial mat were achieved using an oxygen microelectrode (91). Photosynthetic rate was calculated from the decrease in oxygen concentration that occurred during the first second of the dark period. The maximum concentration of oxygen occurred at a depth in the mat just below that which exhibited maximum photosynthetic activity. Because the size of the electrode tip (10 μm) was significantly smaller than the sensing distance (100 μm), the researchers concluded that the electrode had little effect on the spatial resolution of the method. However, the significant temperature coefficient of the oxygen electrode required that the temperature of the mat be closely monitored.

Microelectrodes were also employed to study photosynthetic activities that occurred in a phototrophic mat in an alkaline hot spring (96). Using a micromanipulator mounted on a heavy rack to introduce pH and oxygen microelectrodes into the mat, a spatial resolution of 100–150 μm was obtained for photosynthetic activity within regions of the mat containing the phototrophic microorganisms. The highest pH and oxygen concentrations occurred at a depth in the mat that corresponded to the depth that yielded the highest photosynthetic activity. Oxygen concentration and pH within the mat were different from those measured in the overlying water. Large fluctuations in pH and oxygen concentration within the mat suggested that the chemical environment within the mat is strongly affected by the activities of the mi-

croorganisms present. Computer models have been used to simulate the combined effects of oxygen consumption, photosynthesis, and diffusion of oxygen in microbial mats based on microelectrode measurements (94).

Hydrogen sulfide and oxygen microelectrodes were used to demonstrate transitions from anoxygenic to oxygenic photosynthesis in a *Microcoleus chthonoplastes* cyanobacterial mat (52). A shift from anoxygenic photosynthesis with H_2S as the electron donor occurred when H_2S concentration dropped to levels below 100–300 μmol/l. Oxygen build-up occurred in the cyanobacterial mat, even in the presence of 500 μmol/l H_2S in the overlying water. In situ experiments with a sulfide microelectrode demonstrated the uptake of sulfide by a mat of *Chloroflexus aurantiacus* in a hot spring (41). Net sulfide uptake in the light and net sulfide production in the dark suggested the presence of a functional sulfur cycle.

The rates of production and consumption of dissolved chemical species within a biofilm, as well as their diffusion rates in and out of the biofilm, can be calculated from concentration profiles measured with microelectrodes (89). However, the diffusion characteristics of the solute in the biofilm must be known. Revsbech (89) described a method using an oxygen microelectrode to determine the diffusion coefficient in agar and in a glass-bead sediment. The product of the diffusion coefficient and the porosity were also determined in a glass bead sediment, a riverine sediment, and a diatom biofilm. It was concluded that it is necessary to know the porosity and diffusion coefficient at different depths in the biofilm in order to obtain accurate flux information.

Recent advances in microelectrode design have enhanced their usefulness in evaluating biofilm activities. A new oxygen microelectrode has been described that is more rugged than previous sensors and does not require stirring (90). This should prove to be useful in evaluating changes across the biofilm–bulk water interface. One microsensor containing two gold cathodes and one silver cathode measures oxygen and N_2O simultaneously (95). Depth profiles of oxygen and N_2O have been obtained in a one- to two-mm thick biofilm on a metal surface submerged in river water using the electrode. This microsensor should be useful in evaluating denitrification activities of biofilm microbial populations. Additional information on the types of microelectrodes available and their applications in evaluating activities of sessile microorganisms may be found in a review by Revsbech & Jorgensen (92).

DETERMINATION OF SPECIFIC SURFACE-ASSOCIATED MICROBIAL ACTIVITIES

Metabolic activity of biofilm organisms can be determined by exposing the microbes to labeled precursors and then determining the formation of labeled lipids or cell walls. Incorporation of substrates labeled with ^{32}P phosphate,

^{14}C or ^{13}C acetate, or ^{15}N ammonia into lipids from nonlipid precursors has proved to be a very sensitive measure of activity (27, 75, 87, 106, 109).

Fourier transforming infrared (FT/IR) spectroscopy provides a nondestructive means to detect biofilm formation and gain insight into the chemical nature of the constituents (80). FT/IR examination by diffuse reflectance (DRIFT) of freeze-dried biofilms shows differences in ratios of bacterial proteins to exopolymer polysaccharides (73), providing a rapid analysis with a DRIFT microscope of biofilm heterogeneity to localize microbial biomass and composition. Living biofilms can be monitored using attenuated total reflectance apparatus (ATR-FT/IR) to observe the formation and succession of living biofilms (80, 115). Biofilm formation on a 10-cm diameter coupon in a laminar flow gradient can be observed in a sterilizable continuous flow system (33). In this system, a monoculture of *Pseudomonas atlantica* showed a marked decrease in per-cell metabolic activity (lipid synthesis) and an increase in the carbohydrate/protein ratio (DRIFT) with an increasing shear gradient (73). This system provides a means to examine biofilm formation and stability under defined physiochemical conditions.

Biofilm Sampling

Biofilm samplers have been described that are composed of a series of removable plugs that fit flush to the internal wall of a length of tubing, through which an aqueous medium flows (69, 70). They provide replicate surfaces for microbial colonization and biofilm development on which subsequent physiological studies may be performed. The surface of the plug may be fabricated from a variety of metal and plastic materials to mimic those that exist in a variety of environments where biofouling occurs. Nickel et al (77) evaluated the resistance of cells of *Pseudomonas aeruginosa* in a biofilm to the antibiotic tobramycin in a system containing artificial urine. Ruseska et al (98) used a biofilm sampler to compare biocide resistance of biofilm populations to planktonic populations. One problem encountered with removable plug samplers is that a clean break is not always achieved in the biofilm at the point where the test surface of the plug meets the surrounding tubing surface. This hinders collection of biofilm from a definable area of exposed surface. Quantitative sampling of biofilms on surfaces remains a major challenge to microbiologists.

Utilization of Nutrients by Surface-Associated Bacteria

NUTRIENTS ADSORBED TO SURFACES Although almost 50 years have passed since ZoBell (123) recognized the possibility that nutrients from the bulk fluid adsorbed to submerged inert surfaces, only recently was a method developed to demonstrate that bacteria could utilize these nutrients for growth and replication. Kefford et al (55) used a scavenging model system containing

cylinders coated with radioactive stearic acid. They determined that a bacterium with a hydrophobic surface promoted more efficient utilization of the surface-bound fatty acid by allowing firmer adhesion and greater interaction with the surface than reversibly adhering, hydrophilic *Leptospira* sp. and irreversibly adhering, hydrophilic *Serratia marcescens*. The technique provided a reproducible and stable coating of insoluble long-chain fatty acids at a solid-liquid interface. The system could be used as a model oligotrophic aquatic environment to quantitate removal of nutrients from a solid-liquid interface. Kefford et al (55) determined that the scavenging ability of a bacterium depends on its ability to interact with and take up surface localized nutrients.

The scavenging model system was later modified to permit microscopic examination of the surface on which the utilizable nutrient was adsorbed. Hermansson & Marshall (49) used a transparent cellulose dialysis membrane mounted in a dialysis microculture chamber as the surface to which radiolabeled stearic acid was adsorbed. Using this combination of techniques, Hermansson & Marshall (49) found that a marine vibrio, which exhibited only reversible attachment to the stearate-associated membrane, utilized the stearate for growth at the surface before detaching from the surface. Power & Marshall (88) used this approach to show that surface-associated stearate could serve as the sole energy source needed for growth and reproduction of adherent cells. Daughter cells of a *Pseudomonas* sp. remained bound to and slowly spread over the surface during utilization of the surface-associated stearate. The rate of migration was found to be slow (0.04–0.19 μm/min) and the mechanism of movement was not determined. After the surface-associated stearate was depleted, and no other source of nutrients was available, the bacteria detached from the membrane surface. Detachment of the cells was thought to result from modification of the surface properties due to utilization of the stearate.

Granular activated carbon (GAC) was used as a surface to determine the effect of adsorbed nutrients on the growth rate of attached bacteria. The growth rate of *Klebsiella oxytoca* on GAC was evaluated by monitoring changes in colony forming units and by observing the uptake of ^{3}H-thymidine and ^{3}H-uracil following dispersal of cells from the GAC by a wash treatment (16). In the presence of glutamic acid, an amino acid that adsorbed to the GAC surface, the growth rate of the attached cell population was greater than that of the free-living cell population. Differential filtration studies revealed that attached cells were larger than suspended cells in the presence of this amino acid. No difference in growth rate was observed between the attached and free-living bacterial cell populations in the presence of glucose, which does not adsorb to the surface. These data demonstrate that attachment to surfaces affords bacteria an opportunity to remain active in environments

where nutrient levels in the bulk fluid would not support planktonic bacterial activity.

THE SURFACE AS A NUTRIENT SOURCE Surfaces composed of organic matter have long been known to be susceptible to the degradative action of adherent microorganisms (47). Electron microscopic techniques have provided a useful approach for demonstrating the localized action of exoenzymes such as amylase, cellulase, and chitinase produced by bacteria colonizing starch, cellulose, and chitin particles, respectively. Using transmission electron microscopy (TEM), Akin & Amos (1) and later Costerton (13) and Cheng et al (11) demonstrated pitting of plant cell walls by adherent cellulose-degrading bacteria from the bovine rumen. Wyndham & Costerton (122) used TEM to reveal pits in bitumen particles colonized by oil-degrading bacteria. TEM was used to demonstrate that many bacteria in biofilms that covered cobble surfaces in a mountain stream were, in fact, attached to sessile algal cells (38). The bacteria appeared to degrade the cell walls of the algae to which they were attached. This phenomenon was suggested to explain the increase in sessile bacterial biomass that occurred as algal biomass declined during summer months. Additional information on the use of TEM in demonstrating microbial degradation of organic surfaces can be found elsewhere (13).

Although electron microscopy has not provided quantitative information on the activities of surface-associated microorganisms, it has revealed spatial and structural information that is important for the development of quantitative activity assays. Electron microscopy has demonstrated that physical attachment of the bacteria to the surface is required and that the enzymes excreted by these bacteria need to be concentrated at the surface of the insoluble substrate to effect surface degradation and solubilization. Electron microscopic studies also suggest that determination of the rates of exoenzyme activity by attached microbial populations will depend on the development of assays that preserve the structural integrity of the bacterial biofilm and the relationship it maintains with respect to the surface.

UTILIZATION OF NUTRIENTS PRESENT IN THE BULK FLUID Biofilm organisms remove dissolved substances from the bulk aqueous phase through a combination of mass transfer and metabolic processes. An important reaction relating these two processes is the transport of the dissolved material into the bacterial cell. Methods developed previously to study the accumulation of bulk phase nutrients by free-living microorganisms have been adapted to evaluate similar processes in surface-associated cells. Novel techniques, developed to quantitatively sample microorganisms attached to surfaces, have enabled direct comparison of the level of activity of this population with that

of the free-living population within the same experimental system. Costerton et al (15) reviewed the methods that have been used to compare the activities of free-living and surface-associated bacterial populations. In most cases, coupons of a test material are exposed to a population of thigmotactic microorganisms for a sufficient period of time to permit the accumulation of a firmly attached population. The surface is then submerged in an aqueous medium containing a radiolabeled compound. After a period of incubation, the surface is removed from the solution, rinsed to remove unreacted labeled material, and the radioactivity associated with the attached cells evaluated. Fletcher (29) used autoradiography to compare amino acid uptake by surface-associated cells of a marine *Pseudomonas* sp. with that of free-living cells in the same culture. Amino acids from the bulk liquid were accumulated in a larger fraction of the bacteria attached to disks composed of glass (77–99%) and polyethylene (73–96%) than in bacteria attached to polystyrene (53–76%) or in the suspended bacteria (53–82%). When a similar study was performed with glucose as the nutrient source and *P. fluorescens* as the test organism, the amount of glucose taken up by cells attached to a surface was the same regardless of whether the surface was composed of polyvinylidene fluoride, polyethylene, or glass (30). By evaluating replicate surfaces in the manner described above, the effects of surface properties on the nutrient uptake by attached cells were determined.

In general, dissolved organic carbon uptake by attached bacteria differs from that of planktonic bacteria. Using submerged glass microscope coverslips, Geesey et al (39) showed that adherent cells of *Enterobacter cloacae* exhibited a more rapid rate of glucose uptake than suspended cells under anaerobic conditions. The two populations of cells also displayed different glucose uptake kinetics. It was proposed that physiological changes occur in the cells that enhance their metabolic activity soon after they become attached to the surface. Using a similar sampling approach, Fletcher (30) reported that assimilation of glucose by attached cells of *P. fluorescens* exceeded that of freely suspended cells by a factor of two to five. Glucose uptake by cells that became detached from the surface was greater than that of attached cells. Using replicate surfaces submerged in a bacterial cell suspension containing ^{14}C-labeled amino acids, Bright & Fletcher (5) showed that amino acid assimilation by attached cells of a marine *Pseudomonas* sp. was generally greater than, and respiration less than, that of free-living cells. The half-saturation constant of amino acid uptake by the attached cells was greater than that displayed by free-living cells, whereas the maximum velocity of uptake was the same for both populations.

Samplers have been constructed to evaluate nutrient transport reactions in relatively undisturbed biofilms. Eighmy & Bishop (21) used a biofilm sampling device similar to that described by McCoy et al (69) to study uptake

kinetics by bacteria in wastewater biofilms. Like free-living cells, the attached bacteria exhibited multiple transport systems for dissolved nutrients such as aspartate. Two systems with different affinities, specificities, and mechanisms of energy coupling were identified. The biofilm sampler was also used in a tubular reactor to study the effects of turbulent flow on aspartate transport by biofilm populations established on the walls of the tubing under low wastewater flow conditions (22). One of the two systems mediating aspartate transport in the attached cell population was inactivated by the increased shear stress created by the increased flow rate. The results demonstrated that high shear stress can produce a sublethal effect on the biofilm bacteria and that these effects are exerted prior to displacement of the biofilm from the surface. A limitation of the study is that the transport assay was performed under a set of hydrodynamic conditions different from that used during biofilm development as well as that which caused the sublethal effects. The test system could be modified, however, to conduct the uptake assay under the desired hydrodynamic conditions.

In the laboratory studies cited above, the sampling methods were designed to minimize disturbance to the sessile bacteria and the surface to which they were attached during exposure to the radiolabeled compounds. Protocols that disrupt the structural integrity of attached populations prior to performing the uptake assay run the risk of modifying diffusion characteristics within the biofilm that could alter uptake rates. In some instances, disruption is unavoidable where studies are conducted to evaluate autotrophic and heterotrophic potential of microbial populations colonizing natural surfaces (43, 58). New approaches need to be developed to minimize the disturbance that assay conditions impose in these situations.

Degradation of Metallic Surfaces and Consequent Corrosion

The activities of biofilm populations on submerged metal surfaces have been implicated in metal corrosion (14, 44). While many corrosion monitoring methods exist, few can be carried out without disturbing the biofilm and underlying metal surface. The perturbation-response techniques that involve small amplitude cyclic voltametry or electrochemical impedance spectroscopy (EIS), however, provide on-line, nondestructive monitoring of microbial corrosion activity (120). Laboratory experiments readily demonstrate the effectiveness of EIS in the detection of microbial influenced corrosion (MIC). Mild steel coupons incubated in artificial seawater enriched with diluted nutrients and inoculated with the bacterium *Vibrio natriegens* displayed equivalent corrosion rates when evaluated by destructive DC linear polarization and EIS (17).

EIS also provides indications of local heterogeneities in the corrosion process that become more pronounced as the sweep frequency decreases. This

complication results from the influence of the local (pitting or corrosion-cracking) processes on the average corrosion rates. These complexities are most often detected as deviations from the semicircular response in the Nyquist complex plane analysis at the low-sweep frequencies (18). EIS analysis can provide indications of the microbial influence on the average corrosion rates as well as indications of localized activity that could lead to serious failures.

The spatial relationships between biofilm composition and activity and microbially influenced corrosion can now be examined by a new technique developed by H. S. Isaacs (personal communication) of Brookhaven National Laboratory known as scanning vibrating electrode mapping. This technique allows the mapping of the open cell potential and charge density (and electrochemical impedance) over a working electrode surface on which bacteria are present in a biofilm. Initial studies show the variability in the passivation layer of mild steel in the presence and absence of bacteria (35–37). Techniques such as this should promote a clearer understanding of the distribution of different bacterial processes within biofilms.

CONCLUSIONS

During the past decade, a number of analytical techniques have been developed that detect various products and activities of surface-associated microorganisms under conditions that preserve cell-cell and cell-surface interactions. These techniques have permitted an expansion of our knowledge on sessile microbial populations that would otherwise have been difficult to achieve using methods adapted from those developed for the characterization of free-living microorganisms. As evident from the studies described above, dissection of the activities of specific populations within a biofilm or mat demand an appreciation of spatial order and understanding of the extracellular (but not necessarily bulk phase) environment. Also apparent in the evaluation of the various microbial activities that occur within biofilms is that we are approaching a level of experimentation that necessitates collaboration between individuals and laboratories with different analytical expertise. Success in understanding biofilm processes will require contributions from engineers, surface scientists, and analytical chemists as well as from microbiologists and biochemists.

The benefits of a better understanding of the activities of microorganisms on surfaces include the control of industrial water problems such as biofouling and biologically influenced corrosion and the control of particulates in high-purity water used in the electronics and pharmaceutical manufacturing industries. It should also help to control undesirable slimes in rivers, lakes, and streams while, at the same time, enhance our appreciation of the role sessile

microorganisms have in maintaining a stable ecosystem. There is little doubt that our success in developing more efficient industrial processes with reduced environmental impact will depend on how well we understand the surface-associated microbial activities in these systems.

ACKNOWLEDGMENTS

The authors thank the following agencies for support of the research conducted in their laboratories: National Science Foundation (grants ECE-8701462, DMR-8900417, CTB-8619333, CSI-8650736, DMB-8515584), the International Copper Association (grant 413), the Office of Naval Research and Defense Advance Research Products Agency (grants N00014-87-K00012, N00014-88-K0489, N00014-09-J0095), Department of Energy Subsurface Science Program (grant DE-PG05-00ER60643).

We thank the following individuals for providing reprints and preprints of their research to facilitate the preparation of this review: D. E. Caldwell, K. C. Marshall, S. Kjelleberg, W. A. Hamilton, G. A. McFeters, M. W. Mittelman.

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Annual Review of Microbiology
Volume 44, 1990

CONTENTS

viii (*continued*)