MANIPULATION OF BIOFILM MICROBIAL ECOLOGY

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Abstract:

The biofilm mode of growth provides such significant advantages to the members of the consortium that most organisms in important habitats are found in biofilms. The study of factors that allow manipulation of biofilm microbes in the biofilm growth state requires that reproducible biofilms be generated. The most effective monitoring of biofilm formation, succession and desquamation is with on-line monitoring of microbial biofilms with flowcell for direct observation. The biofilm growth state incorporates a second important factor, the heterogeneity in distribution in time and space of the component members of the biofilm consortium. This heterogeneity is reflected not only in the cellular distribution but in the metabolic activity within a population of cells. Activity and cellular distribution can be mapped in four dimensions with confocal microscopy, and function can be ascertained by genetically manipulated reporter functions for specific genes or by vital stains. The methodology for understanding the microbial ecology of biofilms is now much more readily available and the capacity to manipulate biofilms is becoming an important feature of biotechnology.

Introduction

Biofilms are localized concentrations of microorganisms attached to a substratum. The biofilm can consist of a population of a single species or more often a multi-species community. Within the biofilm population or community, heterogeneity in the distribution of organisms and in their metabolic activities are common. At the Eighth International Symposium on Microbial Ecology, we were treated to a series of presentations and posters which illustrate significant progress in understanding the biofilm state and how this state can be manipulated.

With the advent of biomarker methods for detecting microbial biomass that are independent of the ability to isolate and culture specific organisms, it has become abundantly clear that microbes readily attach to surfaces to form biofilms. The importance of microbial biofilms has been established in aquatic, soil, and clinical environments [8, 11, 17]. Biofilm growth can allow microbes to capitalize on substratum activity differences and enhance metabolic prowess as is demonstrated by the localized nature of microbially influenced corrosion (MIC) which is a multibillion dollar problem [23]. To understand biofilm microbial ecology, biofilms need to be generated reproducibly so they can be manipulated rationally.

Methods

Biofilm formation

Biofilm generation requires a substratum over which a bulk phase with sufficiently dilute nutrients flows so that only attached microbes can survive and grow. Effects of different substrata or coatings can be tested with flush mounted coupons mounted in the

laminar flow apparatus [5]. Anti-Fouling (AF) and fouling release (FR) can be tested quantitatively by manipulating the inoculum "spike" and the flow rates [5].

On-line monitoring

Biofilm formation, succession, stability, and sub-lethal toxicity can be monitored with in-line, non-destructive techniques in the flow-through apparatus [22]. Natural bioluminescence of attached biofilms or genetically engineered bacteria with the *lux* gene cassette or green fluorescent protein (GFP) can be monitored in flow-through chambers [19, 25] or mapped with a photon-counting microscope [3, 24, 25]. It has proved possible to examine the shifts in the chemistry of biofilms by monitoring the changes in infrared spectra. An attenuated-total-reflectance Fourier-transform infrared spectrometer (ATR-FTIR) in which three channels can be monitored simultaneously has been utilized to examine biofilm formation and shifts in biochemical composition [20]. This apparatus has been utilized in conjunction with a fiberoptic bioluminescence detector to monitor specific biodegradation of solvents in a biofilm [26]. One of the most exciting new technologies for on-line observation of microbes in biofilms is confocal laser microscopy (CLM). Combined with computer analysis, CLM enables generation of revolutionary three-dimensional images of biofilms [6].

High resolution destructive monitoring

At the completion of experiments, biofilms can be analyzed for viable microbes by plating onto appropriate media, direct microscopic counting after recovery from the

biofilms, or by signature biomarker analysis. Biomarkers often utilized include nucleic acids and membrane lipids. The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane which contains PLFA. The remaining lipid is diglyceride (DG). The resulting DG contains the same signature fatty acids as the phospholipids, allowing for a comparison of the ratio of phospholipid fatty acids to diglyceride fatty acids (viable to non-viable microbes). The signature lipid analysis also provides insight into the physiological status of the microbial community as exposure to toxic environments can lead to minicell formation and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomers [29].

Results and Discussion

Development of a test system

The reproducible generation of biofilms requires control of the three major components that effect biofilm ecology: the bulk fluid, the substratum and the inoculum. The bulk fluid should have a chemical composition of sufficiently dilute nutrients that pelagic growth is not efficient. Individual inocula that will go on to form a biofilm are maintained in continuous culture vessels in media containing sufficient nutrients to maintain pelagic growth. These are then used as a pulsed inoculum into the flow chamber where the biofilm is maintained.

I.*Effects of the inoculum*

In experiments involving *Pseudomonas fluorescens*, *Hafnia alvei*, *Desulfovibrio gigas*, and *Bacillus subtilis*, the order of the inoculation from the continuous cultures effected the composition and viable biomass of the resulting biofilm as determined at harvest after 5 days [5,18]. *Pseudomanas fluorescens* dominated the biofilm and when utilized as the initial inoculant, resulted in the highest biomass biofilm (~10^8 cells/cm^2). In a further example, adding monocultures, bicultures and the tri-culture of a *Bacillus* sp., *Hafnia alvei*, and *Desulfovibrio gigas* resulted in different biomasses of attached microbes which showed signature fatty acids in different ratios [16] . These different biofilms induced different rates of localized corrosion as measured by electrochemical impedance spectroscopy and the microbial influenced corrosion generated was not directly proportional to the viable biomass in the biofilms. [16]. The rate of corrosion did not depend on the ratio of heterotrophic to sulfate-reducing bacteria (SRB) or the absolute number of SRB. The PLFA analysis showed the microbes recovered from the biofilm were more metabolically stressed than those recovered from the bulk phase for the inocula.

The biomass and community composition of biofilms of *Bacillus* spp., *Pseudomonas* spp., and *Acidovorax* spp. recovered from drinking water biofilms also depended on the order of inoculation [5]. However, a reproducible biofilm was generated by mixing equivalent cell numbers of the three monocultures as the inoculum. The triculture of these bacteria formed a reproducible biofilm in the laminar flow apparatus described above. The system was operated in triplicate and was exposed to 1 and 5 ppm of chlorine. The triculture biofilm showed effects of exposure to chlorine. After 96 hours the control showed 43 ± 8 x 10^8 cells/cm^2, 9 ± 6 x 10^8

cells/cm^2 (1 ppm), and 2.8 ± 2 x 10^8 cells/cm^2, (5 ppm) of which about 10-20 % were detectable with viable plate counts. Into this background pathogens and pathogens-surrogates were tested.

Mycobacterium smegmatis that was engineered to contain green fluorescent protein (GFP) was used as a pathogen in monoculture and triculture systems. The monoculture system was inoculated with 10^8 cells over 5 minutes from a continuous culture forming a thin biofilm. Addition of chlorine resulted in an increase in DGFA/PLFA to ~0.55 in contrast to the ratio of 0.1 detected in the non chlorine treated control and when the *M. smegmatis* formed part of chlorine treated triculture biofilm. Further evidence of toxicity exposure was displayed in the *M. smegmatis* monoculture with *trans/cis* ratios of 0.6 to 0.8 in contrast to the ratios of < 0.1 in the in the control and the chorine treated tricultures.

In a similar study,a triculture biofilm containing an *E. coli* engineered to contain GFP was utilized. Comparison of the viable count to the total count showed that the ratio dropped from 1.2 in non chlorine treated controls to 0.1 when exposed to 1 ppm chlorine (1 or 5 ppm) for 15 minutes. When the *E. coli* formed a biofilm, the cells maintained a ratio of viable to total count of 0.05 for 4 days, although following exposure to 5 ppm chlorine, the biofilm contained no viable cells after 4 days. When the *E. coli* was protected for 5 days in the triculture biofilm, the ratio's of viable to total counts were 0.3 (1 ppm chlorine) and 0.2 (5 ppm chlorine). Relative to liquid culture, the triculture biofilm increased the survival of *E. coli* more than 2-fold in chlorine. Lipid analyses paralleled the viable count data. Exposure to chlorine increased the DGFA/PLFA ratio from 0.0045 in the control to 0.007 (1 ppm chlorine) and 0.01 (5 ppm

chlorine). In contrast, in the non chlorine treated control and the tricultures (both 1 and 5 pmm chlorine exposed), the ratio was 0.005-0.006. The toxicity estimated as the ratio of *trans/cis* PLFA was 0.005 for the control and 1 ppm chlorine and 0.035 for 5 ppm chlorine in the *E. coli* monoculture biofilm, in contrast to the ratio of < 0.005 for the control and both chlorine exposured triculture biofilms. These SLB data clearly indicated the protective effects of the triculture biofilm on the lysis, toxicity exposure, and nutritional status of both the *E. coli* and the *M. smegmatis.*

II *Effects of the Substratum*

The chemistry, topology, and heterogeneity of the substratum markedly effect biofilm formation, succession and desquamation. The biofilm system described above is ideal for testing coatings performance. Utilizing the naturally bioluminescent bacteria *Vibrio harveyi* as a test organism, the ratio between biomass measured as tryptophan fluorescence and metabolic activity measured as bioluminescence provides a means of assessing both the formation of the fouling biofilm and the response of the bacteria to sublethal toxicity. This once-through device utilizes quartz windows which may be changed aseptically for viewing the test substratum and up-stream and down-stream control surfaces with fiberoptic probes. The biomass of the biofilm can be monitored non-destructively with tryptophan fluorescence [1], and the bioluminescence, a measure of metabolic activity, can be ratioed to the biomass as a measure of sublethal toxicity [5]. In this system the shear gradients can be controlled which allows the study of both antifouling and fouling-release effectiveness of various surface treatments as well as the effects

of adding other bacteria to the system [5]. This system provides an excellent place to examine mechanisms of cellular injury. Zosteric acid, a component discovered in the seagrass *Zostera marina* [27], is bacterostatic in that it appears to prevent attachment but not biofilm growth in attached biofilm bacteria with initial studies indicating it could be an excellent non-polluting AF component to add to coatings.

Possibly the most concrete example of the effects of heterogeneity in substratum chemistry and topology is in the effect on localized microbially influenced corrosion (MIC). Localized concentrations of microbes can lead to localized corrosion [15]. With application of a scanning vibrating electrode across a coupon, the charge density can be mapped and localized anodic areas detected in time and space [12]. The scanning vibrating electrode can be utilized in a epi-illuminated microscope with a photon counting camera and bioluminescent organisms to establish the congruity between the localization of microbes in microcolonies, their metabolic activity as indicated by their bioluminescence and the development of an anode detected in the charge density field [3]. Inhomogeneity in the substratum induces differences in microbial biofilm distribution, therefore weldments are especially vulnerable to MIC activity [10, 13]. To reproducibly localize MIC to a specific area, a concentric electrode system was developed in which a small circular metal region was separated by a Teflon ring from a larger circumferential metal region of 100 times the area of the inner region. The small central area was then driven electrochemically as an anode compared to the large circumferential area in an anaerobic flow-through system. The system was inoculated and the potential between the anode and cathode

was shut off. The current flow between the electrodes was then monitored with a zero resistance ammeter [14]. The presence of bacteria including SRB resulted in stabilization of a corrosion current between the anode and cathode [7]. With this technology it proved possible to reproducibly induce MIC of 304 stainless steel anaerobically in the anode area [2]. In this system a biculture of an SRB and a *Vibrio* spp. maintained a current of about 3 $\mu A\ cm^2$ for > 200 h after removal of an imposed current (11 $\mu A\ cm^2$ for 72 h). No current was maintained in the sterile control or with a monoculture of either bacterium. With the biculture, charge transfer resistance > $100k\Omega\ cm^2$ was measured with EIS on the cathode, in contrast to 1 $k\Omega\ cm^2$ on the anode. In this system removal of sulfate or the addition of 10 mM azide did not effect the sustainability of the current after an initial 30 hours. Apparently, microbial metabolic activity is necessary for initiation processes. However, once started, the corrosion proceeds independent of microbial metabolic activity [4].

III. *Effects of the Bulk fluid*

Effects of changing the bulk fluid chemistry can be readily detected non-destructively with attenuated-total-reflectance infrared spectroscopy (ATR-FTIR). ATR-FTIR provides on-line, real-time biochemical monitoring of an "inside out" view of the biofilm within the first micrometer at the substratum (within the evanescent wave region). This technique monitors the entire area of the internal reflectance unit. With this technology it was possible to monitor the differences in the biofilm structure of mutants of *P. aeruginosa* with lesions in the alginate biosynthetic pathway [21]. It is possible to combine ATR-FTIR monitoring of biofilm chemistry

with bioluminescence [26]. Recently ATR-FTIR has been combined with microscopy and demonstration of localized microbial heterogeneity on substrata has been possible [27]. The effects of adding chlorine to the bulk fluid on the survival of the pathogens has been alluded to above with the nurturing properties of the drinking water biofilm.

Heterogeneity

One of the most significant aspects of the biofilm world is the heterogeneity in the distribution of species and in metabolic activities. Now that the ability to examine biofilms non-destructively in four dimensions (x, y, z, t) exists, one can explore the "developmental biology" of biofilms on levels from the single cell to organization of large multicellular colonies. The most promising application of this approach lies in the study of multi-species biofilms. Systems such as oral communities, marine snow, and microbial mats are prime candidates for real-time non-destructive study because they are reasonably well defined systems that are still complex enough to support a variety of niches. Application of the wide variety of fluorescent probes developed by cell biologists and neurophysiologists will yield new insight into physiology and community composition within biofilms.

It proved possible to detect differential metabolic activities in biofilm microcolonies of *Pseudomonas fluorescens* genetically engineered to contain the full *lux* gene cassette under control of a toluene-degradation promoter. Microcolonies with 10 to 50 cells showed differential respiratory activity in individual cells using the confocal microscope and the membrane activity stain tetramethylrhodamine methyl ester in the microcolonies [24]. Using photon-counting

microscopy followed by laser confocal microscopy, we could see areas of differential bioluminescent regions within a microcolony that were unrelated to biomas differences or activity differences at the level of the single cell. Clearly, heterogeneity exists in metabolic activity in these microcolonies.

Conclusions

The work summarized herein and the excellent presentations at this Symposium have led to several new principals. First, the biofilm growth state is different from the metabolic state in the same cells during pelagic growth. Second, extensive heterogeneity exists within the biofilm and extensive communication between the various components within the biofilm must take place. Generation of reproducible multi-component biofilms and new non-destructive sensors capable of mapping the biofilm in 4-dimensions allows new and exciting insights.

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