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RESEARCH NOTE

BIOFILM STRUCTURAL HETEROGENEITY VISUALIZED BY THREE MICROSCOPIC METHODS

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Abstract—The structural heterogeneity of a microbial biofilm was demonstrated by scanning electron microscopy, confocal scanning laser microscopy (CSLM), and cryoembedding followed by sectioning and microscopic examination. Biofilm was composed of a binary population of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* grown in a continuous flow annular reactor. The three microscopic methods provided a consistent picture of the biofilm as a non-uniform structure characterized by variable thickness and variable local cell and polymer densities. Significant changes in these parameters occurred in the biofilm over distances of 10 μm or less. Though the biofilm was several hundred microns thick in places, areas of bare substratum were also observed on the same sample coupon. Cell-free pores and channels in the biofilm interior were evident. Specific staining of cellular nucleic acids with ethidium bromide and extracellular polymeric substances (EPS) with calcofluor showed that cell and EPS distributions did not always overlap. The ethidium bromide-stained region was contained within the larger region of calcofluor staining; thus, some cell-free areas actually were filled with EPS. CSLM and cryoembedding approaches are superior to SEM in their ability to image the biofilm interior and in their potential to provide quantitative information.

Key words—biofilm, structure, thickness, heterogeneity, scanning electron microcopy, confocal scanning laser microscopy, cryoembedding, extracellular polymeric substance

INTRODUCTION

Microscale heterogeneity in the physical structure of microbial biofilms has begun to receive attention as a potentially important aspect of transport phenomena in biofilms (Costerton *et al.*, 1994; de Beer *et al.*, 1994a; de Beer *et al.* 1994b; Drury *et al.*, 1993; Jih and Huang, 1994; Siegrist and Gujer, 1987). Microscale perturbation of mass and momentum transport processes arising from biofilm structural heterogeneity could help to explain such phenomena as hydrodynamic fouling and microbially induced corrosion. One of the challenges faced in discerning the practical significance of structural heterogeneity on biofilm function is the development of conceptual and mathematical descriptions of biofilm topography and internal structure. Such descriptions invariably originate from visual observations. A wide variety of microscopic techniques, reviewed elsewhere (Sutton *et al.*, 1994; Murga *et al.*, 1994), have been applied to investigate biofilm structure. These methods include light, scanning electron, transmission electron, confocal scanning laser, and atomic force microscopy. The purpose of the research reported in this article was to compare three different microscopic methods with respect to the information they provide concerning biofilm structural heterogeneity. The ability to image the interior of the biofilm and the potential to extract quantitative information from images were of particular interest.

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MATERIALS AND METHODS

Bacteria and growth conditions

Environmental isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were grown on a minimal salts, phosphate-buffered medium with glucose as the sole carbon and energy source. These bacteria were selected because they are both commonly encountered in fouling biofilms. The two species were grown in co-culture because together they produce a thicker biofilm than either one alone (Murga *et al.*, 1994; Siebel and Characklis, 1991). The formulation of the medium is given elsewhere (Chen *et al.*, 1993). The

medium glucose concentration of 20 g/m^3 was low enough to ensure that glucose, not oxygen, was always the growth limiting substrate based on the stoichiometry (Siebel and Characklis, 1991) and diffusion coefficients of the two substrates (Perry and Chilton, 1973). Medium components were autoclaved or filter sterilized (Chen *et al.*, 1993). Experiments were conducted at 25 ± 1°C.

Reactor system

Binary population biofilms were developed on stainless steel slides in a continuous flow annular reactor (Chen *et al.*, 1993; Siebel and Characklis, 1991). The polycarbonate reactor consisted of a stationary outer and rotating (150 rpm) inner cylinder separated by an 8 mm gap. The outer cylinder held 12 removable stainless steel (SS316) slides in recessed grooves. The dilution rate in the system, which can be modeled as a continuous flow stirred tank reactor, was 2.9 h^{-1}. This dilution rate was high enough to ensure that biofilm growth far outweighed planktonic growth. Mixing characteristics and non-idealities in this reactor have recently been described (Gjaltema *et al.*, 1994). The reactor assembly was sterilized by autoclaving.

To begin an experiment, a sterile annular reactor was filled with medium and inoculated with 1 ml of stock culture (10^8 cfu/ml) of each microorganism. The reactor was operated in batch mode, with the inner cylinder spinning to provide mixing, for 24 h. Pumps were then turned on to feed a steady flow of medium to the reactor (Chen *et al.*, 1993). Reactor effluent was monitored daily for growth and contamination by plating on R2A agar. Both *P. aeruginosa* and *K. pneumoniae*, as identified by colony shading and morphology, were always present in the reactor effluent. If contaminating microorganisms were detected, the reactor was discontinued and no data was collected. The reactor was operated in continuous mode for 7 days before biofilm was sampled.

Scanning electron microscopy

A biofilm covered slide was removed from the reactor and immersed in 2.5% glutaraldehyde for 1 h. The fixed specimen was dehydrated in an ethanol series (20 min each in 15, 25, 40, 55, 70, 85, 95, and 100%) then air dried for 20 min. The sample was sputter-coated with gold using a Hummer VII instrument (Anatech Ltd) and examined with a Jeol JSM-6100.

Confocal scanning laser microscopy

Sample slides were removed from the reactor and placed in a staining chamber containing 14 ml of phosphate buffered saline. Specimens were stained for 20 min with calcofluor (Sigma, fluorescent brighter 28, 75 mg/l). The chamber containing the stained biofilm was transferred to the stage of a confocal scanning laser (Kr/Ar) microscope (MRC600, Bio-Rad). The specimen was excited with light at 488 nm and fluorescent light was imaged using the K1/K2 filter block combination.

Embedding and sectioning

A sample slide was fixed in 2.5% glutaraldehyde for 45 min then stained simultaneously with calcofluor (75 mg/l) and ethidium bromide (1 mg/l) for 20 min. The specimen was cryoembedded and 5 μm thick frozen sections were cut as described elsewhere (Murga *et al.*, 1994; Yu *et al.*, 1994). The procedure involved covering the biofilm with a commercial tissue embedding medium (OCT, Miles Inc.), freezing it on dry ice, snapping the frozen specimen off the metal slide, embedding the other side of the biofilm, and cutting sections in a refrigerated microtome (Reichert-Jung Cryocut 1800, Leica). The specimen was oriented such that the plane of the section is normal to the plane of the substratum. Sections were deposited on polylysine coated glass slides and examined with an Olympus BH2 microscope under epifluorescent illumination. The two stains could be discriminated with appropriate filters because they fluoresce at different wavelengths; the peak emission wavelength for ethidium bromide is 595 nm (orange) whereas for calcofluor it is 430 nm (blue). Photomicrographs were made using filter block G and an O590 barrier filter (ethidium bromide) or using the U excitation filter cubic unit with a UG-1 excitation filter, DM 400 dichroic mirror, and L420 barrier filter (calcofluor).

RESULTS AND DISCUSSION

Biofilm samples collected from the same reactor were examined by three different microscopic techniques with the intent of comparing observations of structural heterogeneity of the biofilm. As assayed by scraping and plate counting on R2A agar (Chen *et al.*, 1993), the biofilm viable cell areal density was determined to be 1.1×10^{13} cfu/m^2. *K. pneumoniae* constituted 66% and *P. aeruginosa* 34% of the viable cells in the biofilm. Non-uniform distribution of biomass over the sample slide was apparent with the naked eye. Though the biofilm was several hundred microns thick in places, areas of bare substratum were also observed on the same sample coupon.

Confocal laser scanning microscopic examination of biofilm stained with calcofluor, which stains polysaccharides specifically (Wood, 1980), revealed rounded clusters of biomass abutting one another with open interstices (Fig. 1). Examination of a series of images in confocal planes at increasing distances from the substratum confirmed the impression conveyed by Fig. 1 of globular structures (images not shown). This illustrates a key advantage of CSLM: it enables the internal structure of the biofilm to be visualized. The overall structure of the biofilm suggested by CSLM was similar to that described in the literature for this technique applied to similar

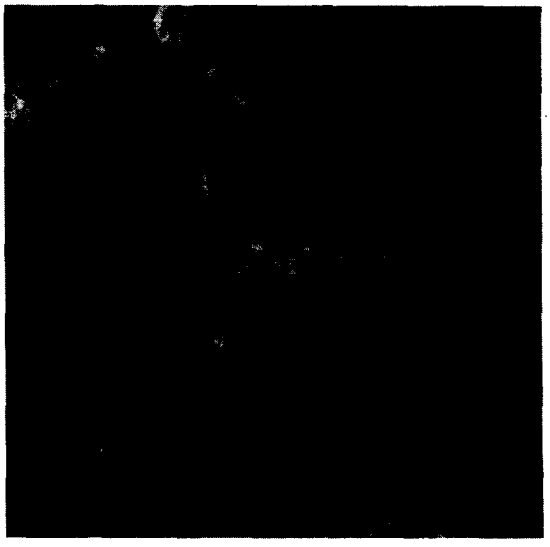

Fig. 1. Confocal microscopic image of biofilm. Biofilm stained with calcofluor was imaged in a focal plane 100 μm above the substratum. Cell clusters (light) are separated by unstained interstices (dark). The scale bar is 100 μm.

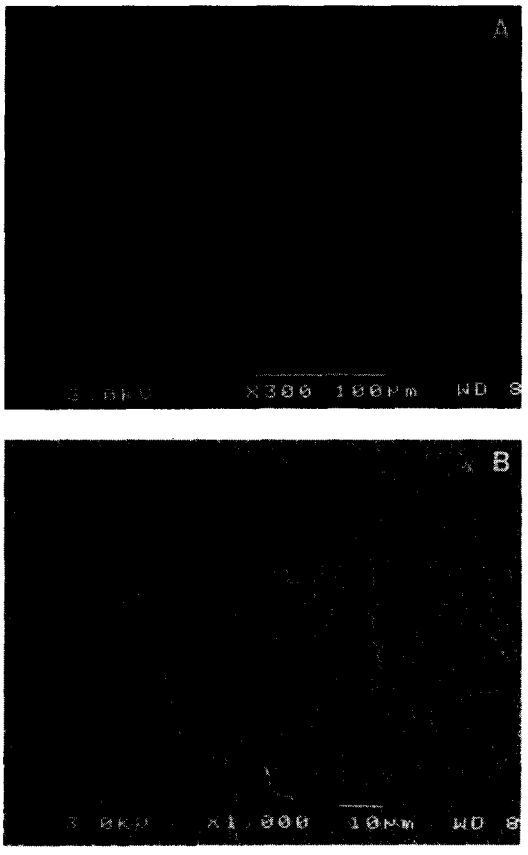

Fig. 2. Scanning electron microscopic images of biofilm (plan view).

systems (de Beer *et al.*, 1994a, Lawrence *et al.*, 1991). The structure observed with CSLM was also similar to that reported for biofilms examined by an Electroscan wet-SEM device that permits direct electron microscopic examination of hydrated samples (Sutton *et al.*, 1994).

The nature of the information about biofilm structure that each microscopic method yields and the artefacts and limitations inherent with the three approaches differ. CSLM involves the least sample processing of the three methods applied and probably provides the most accurate picture of biofilm structure. The biofilm remains fully hydrated and attached to the substratum with this technique. CSLM provides digital images in two dimensional sections; quantitative data are readily extracted. CSLM allows the interior of the biofilm to be analyzed. A unique feature of CSLM is its ability to image biofilms *in situ* and in real time. The only disadvantage of CSLM, aside from the expense of the equipment, is that it may not be applicable to thick biofilms or those containing abiotic particulate matter if they are too opaque.

Conventional SEM imaging of biofilms reveals uneven outer surface topography of the biofilm [Fig. 2(A)]. In contrast to the CSLM results, globules and interstices are not evident in the SEM imaged biofilm. This is probably an artefact of air drying, which causes severe shrinkage. Other conventional SEM specimen preparation protocols also lead to shrinkage and loss of EPS matrix (Chang and Rittmann, 1986; Little *et al.*, 1991; Richards and Turner, 1984; Sutton *et al.*, 1994). SEM does afford high resolution. At higher magnifications, individual cells are readily distinguished [Fig. 2(B)]. In spots where the substratum is bare, a scratch and grain boundaries on the steel surface are evident [Fig. 2(B)]. The observation of bare substratum may be an artefact of biofilm removal during sample preparation. SEM does not allow observation of the biofilm interior.

SEM methods for examining biofilms are familiar and afford high resolution, but these attributes may be beguiling. With the exception of the environmental SEM manufactured by the Electroscan Corporation (Little *et al.*, 1991; Sutton *et al.*, 1994), SEM preparatory techniques are prone to artefact (Chang and Rittmann, 1986; Little *et al.*, 1991; Richards and Turner, 1984; Sutton *et al.*, 1994). Even the Electroscan instrument has limitations for studying biofilm structure: the interior of the biofilm is still not accessible. In addition, quantitative analysis of SEM images is difficult because they condense three dimensional information into two dimensions.

Embedding and sectioning techniques do allow internal biofilm structure to be studied (Kristensen

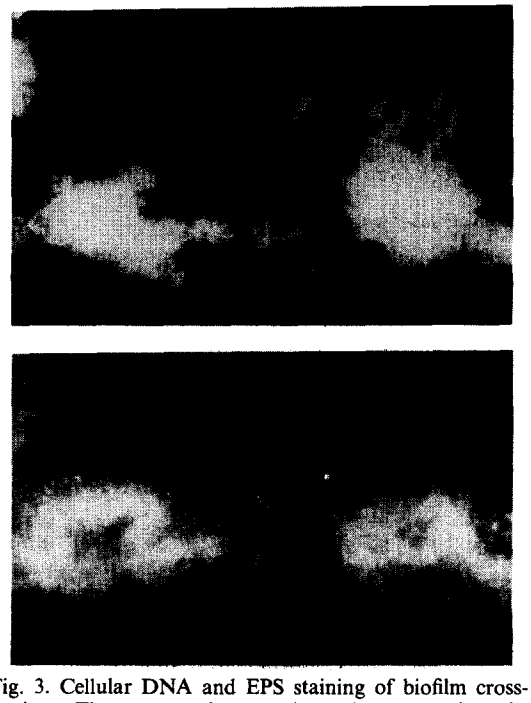

Fig. 3. Cellular DNA and EPS staining of biofilm cross-sections. The cross sections are in a plane normal to the substratum. Staining with ethidium bromide (a) reveals the distribution of nucleic acids; staining with calcofluor (b) indicates the distribution of EPS. The substratum was at the bottom and the bulk fluid at the top in both images. Arrows indicate a region that contains polysaccharide but no cells. The scale bar is 100 μm.

et al., 1982; Murga *et al.*, 1994; Yu *et al.*, 1994). As reported elsewhere, binary population biofilms of *K. pneumoniae* and *P. aeruginosa* grown in annular reactors exhibit variable thickness and cell-free voids (Murga *et al.*, 1994). The appearance of stained cross-sections is consistent with the structure consisting of cell clusters and voids suggested by CSLM (Fig. 3). Staining of the same frozen section with ethidium bromide and calcofluor indicates that nucleic acid and polysaccharide are distributed differently. In this and other sections examined, the ethidium bromide-stained region [Fig. 3(A)] was contained within the larger region of calcofluor staining [Fig. 3(B)]; thus, some cell-free areas actually were filled with EPS. The region occupied by EPS could be even larger than visualized if glutaraldehyde fixation removed polysaccharides or if calcofluor failed to stain all types of polysaccharides.

Cryoembedding and sectioning methods are simple and generally applicable to biofilms from a wide variety of systems. Internal biofilm structure is easily studied with this approach, even in biofilms that are quite thick or that do not transmit light well. Images captured from frozen sections are readily amenable to quantitative image analysis (Huang *et al.*, 1994; Murga *et al.*, 1994) with excellent resolution in the direction normal to the plane of the substratum. Cryoembedding can be performed without any fixation or dehydration (Murga *et al.*, 1994). The extent to which cryoembedding accurately preserves biofilm structure has not yet been fully demonstrated. The similarity of features observed by cryoembedding and by CSLM suggest that frozen sections do preserve much of the native biofilm structure.

In summary, confocal scanning laser microscopy, scanning electron microscopy, and cryoembedding and sectioning methods provided a consistent picture of the biofilm as a non-uniform structure characterized by variable thickness and variable local cell and polymer densities. Significant changes in these parameters occurred in the biofilm over distances of 10 μm or less. CSLM and cryoembedding approaches are superior to SEM in their ability to image the biofilm interior and in their potential to provide quantitative information.

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