Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 68 (2007) 577–587
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Enhanced visualization of microbial biofilms by staining and environmental scanning electron microscopy

John H. Priester [a], Allison M. Horst [a], Laurie C. Van De Werfhorst [a], José L. Saleta [a], Leal A.K. Mertes [b], Patricia A. Holden [a,*]

[a] *Donald Bren School of Environmental Science and Management, University of California, Santa Barbara, CA 93106-5131, USA*
[b] *Department of Geography, University of California, Santa Barbara, CA 93106-5131, USA*

Received 27 October 2006; accepted 28 October 2006
Available online 29 December 2006

Abstract

Bacterial biofilms, i.e. surface-associated cells covered in hydrated extracellular polymeric substances (EPS), are often studied with high-resolution electron microscopy (EM). However, conventional desiccation and high vacuum EM protocols collapse EPS matrices which, in turn, deform biofilm appearances. Alternatively, wet-mode environmental scanning electron microscopy (ESEM) is performed under a moderate vacuum and without biofilm drying. If completely untreated, however, EPS is not electron dense and thus is not resolved well in ESEM. Therefore, this study was towards adapting several conventional SEM staining protocols for improved resolution of biofilms and EPS using ESEM. Three different biofilm types were used: 1) *Pseudomonas aeruginosa* unsaturated biofilms cultured on membranes, 2) *P. aeruginosa* cultured in moist sand, and 3) mixed community biofilms cultured on substrates in an estuary. Working with the first specimen type, a staining protocol using ruthenium red, glutaraldehyde, osmium tetroxide and lysine was optimized for best topographic resolution. A quantitative image analysis tool that maps relief, newly adopted here for studying biofilms, was used to compare micrographs. When the optimized staining and ESEM protocols were applied to moist sand cultures and aquatic biofilms, the smoothening effect that bacterial biofilms have on rough sand, and the roughening that aquatic biofilms impart on initially smooth coupons, were each quantifiable. This study thus provides transferable staining and ESEM imaging protocols suitable for a wide range of biofilms, plus a novel tool for quantifying biofilm image data.

Keywords: Biofilms; Ruthenium red; Image analysis; ESEM

1. Introduction

Bacterial biofilms are three-dimensional sessile structures consisting of layered cells encapsulated in hydrated extracellular polymeric substances (EPS) on a substratum (Characklis and Marshall, 1990). Bacterial biofilms contribute to human infections (Marsh, 1995; Donlan and Costerton, 2002) and enable antibiotic resistance (Davies, 2003). In industry, biofilms inhibit water purification (Baker and Dudley, 1998; Hallam et al., 2001; Lee and Kim, 2003) and cause corrosion (Islander et al., 1991; Hamilton, 1995; Laurent et al., 2001; Diosi et al., 2003) but they also facilitate engineered water, wastewater (Lazarova and Manem, 2000) and hazardous waste (Blenkinsopp and Costerton, 1991; Rittmann et al., 2000) treatment. Across such diverse settings and concerns, biofilm detection and study are facilitated by microscopy.

Scanning electron microscopy (SEM) has been important for high resolution visualization of bacterial biofilms (Walker et al., 2001). In SEM, biofilm specimens are prepared by fixation, staining, drying and conductively coating prior to imaging under high vacuum. While any pretreatment can alter specimen morphology, drying appears to significantly alter biofilms due to EPS polymers collapsing (Little et al., 1991; Fassel and Edmiston, 1999a; Kachlany et al., 2001). Although similar preparation is used in transmission electron microscopy (TEM), specimens for TEM are embedded in a resin which physically stabilizes the EPS matrix (Walker et al., 2001). However, TEM is not applicable for observing the extent and form of surface-associated growth which is often of interest. Alternatively,

* Corresponding author. Tel.: +1 805 893 3195.
E-mail address: holden@bren.ucsb.edu (P.A. Holden).

0167-7012/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2006.10.018

environmental SEM, or ESEM, minimizes biofilm dehydration and thus preserves native morphologies including surface structures (Walker et al., 2001).

In ESEM, specimens are imaged at a moderate vacuum without a conductive coating and in moist atmospheric conditions which better preserve soft matter structures over conventional SEM (Little et al., 1991; Danilatos, 1993). ESEM allows for examining dynamic hydration-, chemical-, temperature- and mechanically-dependent processes at small scales (Manero et al., 2003). Compared to SEM, ESEM produces different, perhaps complementary, information for biological specimens (Surman et al., 1996; Doucet et al., 2005). Cell structures are visible with SEM, but external polymers around cells are more apparent in ESEM (Douglas and Douglas, 2001; Callow et al., 2003; Doucet et al., 2005). Biofilm EPS is particularly evident in ESEM images of metal-exposed biofilms such as those involved in sulfur cycling (Darkin et al., 2001; Douglas and Douglas, 2001) or corrosion (Beech et al., 1996; Ray et al., 1997). In contrast, EPS is less visible with ESEM of biofilms cultured in the absence of concentrated metals (Allison et al., 1998). EPS is known to bind metals (Kachlany et al., 2001), thus ESEM images from untreated biofilms may vary significantly across specimens depending on the metal content of their environments. To overcome, biofilms could routinely be metal-stained prior to ESEM which would "equalize" specimens prior to microscopy.

Metal stains are used in conventional TEM and SEM to increase the electron density of EPS polymers and improve their resolution (Fassel and Edmiston, 1999a,b; Erlandsen et al., 2004). Glutaraldehyde and osmium tetroxide fixatives are used to preserve delicate extracellular structures (Dawes, 1971; Carr and Toner, 1982; Gerhardt et al., 1994; Erlandsen et al., 2004) in conventional EM. In ESEM, either glutaraldehyde (Ray et al., 1997; Larson et al., 1998) or permanganate and osmium tetroxide (Collins et al., 1993) enhance the contrast of microbial structures. But fixatives alone don't fully preserve or stain bacterial EPS (Jones et al., 1969; Fassel et al., 1992; Fassel and Edmiston, 1999a,b; Erlandsen et al., 2004). Rather, ruthenium red ($H_{42}Cl_6N_{14}O_2Ru_3{\cdot}4H_2O$, F.W. 858.36), a histological stain used with light microscopy of microbes since the 1890's (Fassel and Edmiston, 1999a,b), greatly improves the resolution of EPS. Ruthenium red is electron dense and, as a cation, binds to the many polyanionic (Sutherland, 2001; Erlandsen et al., 2004) constituents of EPS. Ruthenium red for visualizing extracellular structures with EM was pioneered by Luft (1964), thus the terminology of "Luft-based" used in Fassel and Edmiston's review (Fassel and Edmiston, 1999a). SEM resolution of EPS on planktonic *Methylomonas* spp., *Methylosinus* spp., *Methylocystis* spp. (Fassel et al., 1992), *Staphylococcus* spp. (Fassel et al., 1992; Fassel and Edmiston, 1999a,b), *Pseudomonas* spp. (Carr et al., 1996), *Enterococcus faecalis* (Erlandsen et al., 2004), *Klebsiella pneumoniae* (Erlandsen et al., 2004), and a mixed bacterial colony (Jones et al., 1969) was enhanced by ruthenium red staining. Ruthenium red also enhanced EPS in SEM of infected tissue (Fulcher et al., 2001) and in TEM of biofilms (Fassel and Edmiston, 1999a,b). More recent applications of ruthenium red-based EPS staining used lysine to improve staining (Jacques and Graham, 1989; Fassel et al., 1992; Fassel and Edmiston, 1999a,b; Erlandsen et al., 2004). However, ruthenium red with ESEM of biofilms has not been reported.

Because ruthenium red binds strongly to negatively charged polysaccharides (Fassel and Edmiston, 1999a), we hypothesized that it would routinely improve the resolution of biofilms and EPS during ESEM, thus equalizing ESEM performance across various biofilm types. Our preliminary experience with ESEM imaging of bacterial biofilms in moist silica sand provided additional motivation because of the difficulties in resolving either cells or EPS of untreated biofilms (unpublished data). Here, documented "Luft-based" ruthenium red staining and fixation protocols (Fassel and Edmiston, 1999a) were modified for ESEM visualization of *P. aeruginosa* biofilms grown on polyester membranes. Geostatistical analyses of ESEM images were used to determine which staining regimes enhanced the subtle topography of the smooth biofilm surfaces. When applied to aquatic biofilms growing on smooth substrata and unsaturated biofilms growing on rough sand, these methods revealed how biofilms physically alter substrata relief.

2. Methods

2.1. Biofilm cultivation on membranes

P. aeruginosa strain PG201 (Urs Ochsner, University of Colorado) was maintained at −80 °C in 70% Luria–Bertani (LB) broth plus 30% glycerol. Solid media for biofilm culturing were prepared from an aqueous mineral stock (Holden et al., 2002) containing per liter of Nanopure water 0.5 g NH_4Cl, 1.725 g $Na_2HPO_4{\cdot}7H_20$, 1.38 g KH_2PO_4 and 1% v/v Hutner's mineral solution (Smibert and Krieg, 1981). All chemicals (Fisher Scientific, Pittsburgh, PA) were reagent grade or better. Bacto-Agar (Difco, Fisher Scientific) was amended (1.5% w/v) to the mineral stock prior to autoclaving. Four different levels of carbon amendment were used to simulate C:N conditions of 7, 42, 70, and 140 which represented a nutritional gradient from C-deficient to highly C-enriched. C enrichment is known to increase bacterial EPS production (Sutherland, 1977), and thus these conditions were assayed for their effects on EPS chemistry prior to cultivating biofilms for ESEM optimization experiments. Separate glucose solutions for the 7 (25 g/l) and other (500 g/l) C:N conditions were prepared in aqueous mineral stock. The solid media was prepared by separately dispensing 23 ml of the molten agar solution, appropriate amounts (2 ml for each of the 7 and 140 C:N media; 600 µl and 1 ml for the C:N conditions of 42 and 70, respectively) of filter-sterilized (0.2 µm) glucose solution, and additional sterile mineral stock (1.4 ml and 1 ml for the C:N conditions of 42 and 70, respectively) into standard Petri dishes then swirling to mix prior to cooling.

The methods for culturing unsaturated biofilms on membranes were similar to before (Auerbach et al., 2000; Steinberger et al., 2002). Nuclepore® polyester membranes (13 mm diameter, 0.1 µm pore, 6 µm thick, Whatman, Clifton, NJ) were sterilized by immersion in 70% ethanol for 2 min, then air-dried and transferred to the solid media surface. For biofilms used in