The preservation of biofilms on macroalgae by osmium vapour

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Abstract

The aims of this study were to determine if osmium vapour treatment prior to glutaraldehyde fixation could preserve the biofilms found on macroalgae and to gain insight into the structure of the extracellular polymeric substance (EPS or slime layer) of the biofilm. The microscopic surface features of twelve different species of macroalgae from Palm Beach, KwaZulu-Natal, South Africa were compared after being subjected to the different fixation procedures. Treating the seaweed samples with osmium tetroxide (OsO₄) prior to fixation with glutaraldehyde significantly enhanced the preservation of the EPS of the biofilms. The EPS was found to be complex and multi-layered with two types of EPS being distinguished, a fluffy or downy variety, and a flat sheet-like type, of which the latter varied in thickness.

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1. Introduction

Biofilms are ubiquitous, being found in habitats as diverse as the oceans and on mammalian teeth, and consist mostly of various types of bacteria. At maturity, some biofilms often incorporate other life forms, e.g. algae, fungi and/or invertebrates depending on the habitat. These complex communities contain symbionts that work together to utilise resources optimally and even protect each other from antimicrobial agents (Mayer et al., 1999; Flemming et al., 2000). This is achieved by the formation of a slime layer or extracellular polymeric substance (EPS) that consists mainly of water, polysaccharides and proteins, with DNA, RNA, ions and lipids as minor components (Marsh and Bowen, 2000; Sutherland, 2001; Lawrence et al., 2003).

Interactions between the carbohydrates, proteins and nucleic acids in the EPS are thought to maintain its cohesiveness and integrity. Sutherland (2001) states that lipids function as biosurfactants and that their presence is considered to cause a loss of material from the EPS. Other workers mention the presence of lipoproteins, but not their possible function (Lawrence et al., 2003). However, McKeekin et al. (1979) found that biofilms on chicken skin were better preserved by a lipid-stabilising pre-treatment of osmium tetroxide (OsO₄) vapour before the specimens were fixed and dehydrated for scanning electron microscopy (SEM) viewing (Komorowska et al., 1982).

Fixation in glutaraldehyde (without OsO₄ fixation) and drying damages the biofilm by removing the EPS and the underlying cells (Richards and Turner, 1984). Freezing hydrated samples prior to SEM viewing enables one to bypass

Table 1

| Species             | Family          | Genus and species                              |
|---------------------|-----------------|-----------------------------------------------|
| Heterokonta         | Bacillariophyceae| Nitzchia martiana                            |
| Chlorophyceae       | Codiaeae        | Codium duthiae                                |
|                     |                 | Halimeda cuneata                              |
| Rhodophyta          | Caulerpaceae    | Cauderpa filiformis                           |
|                     | Corallinaceae   | Amphiprora bowervankii                        |
|                     |                 | A. ephedrea                                   |
|                     |                 | Cheilosporum multifidum                       |
|                     | Gelidiaceae     | Gelidium abbottianum                          |
|                     | Hypneaceae      | Hypnea rosea                                  |
|                     |                 | H. spicifera                                  |
|                     | Ceramiaceae     | Spyridia hypnoides                            |
|                     | Rhodomelaceae   | Osmundaria serrata                            |

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this problem, but with such cryotechniques only the surface of the biofilm is visible giving no indication of what lies beneath. More recently, non-destructive techniques such as confocal laser scanning microscopy and scanning transmission X-ray microscopy have been used to analyse the interior of biofilms in much greater detail (Lawrence et al., 2003; Larson and Passy, 2005). With these techniques it is possible to view the locations of labelled biochemicals in situ and in vivo, and thus gain a greater understanding of biofilms.

The aim of this study was to determine if an osmium vapour treatment prior to glutaraldehyde fixation could preserve the biofilms found on macroalgae and to gain insight into the structure of the EPS.

2. Materials and methods

All seaweeds samples collected from Palm Beach, KwaZulu-Natal, South Africa (30° 59′ 30″ S, 30° 16′ 30″ E) in June 2002...
Fig. 4. Micrograph of the surface of *Gelidium abbottiorum* showing the remnants of the wispy sheet-like type of extracellular polymeric substance (EPS, arrows).

Fig. 6. Micrograph of the surface of *Osmundaria serrata* showing bacteria under the remnants of the wispy sheet-like type of extracellular polymeric substance (EPS, arrows). (Enlargement of the box in Fig. 5).

Fig. 5. Micrograph of the surface of *Osmundaria serrata* showing the remnants of the wispy sheet-like type of extracellular polymeric substance (EPS) running diagonally across the image (arrows). An enlargement of the area within the box is shown in Fig. 6.

Fig. 7. Micrograph of the surface of *Hypnea rosea* showing an example of the thick sheet-like type of extracellular polymeric substance (EPS, arrows).

Fig. 8. Micrograph of the surface of *Spyridia cuppressina* showing both the fluffy and the sheet-like types of extracellular polymeric substance (EPS, arrows).

Fig. 9. Micrograph of the surface of *Osmundaria serrata* showing both the fluffy and the sheet-like types of extracellular polymeric substance (EPS) where the fluffy one is on top of the thick sheet-like type of EPS (arrows).
were divided into two groups and sectioned into pieces about 5 mm in length. Half were fixed in glutaraldehyde (4% in sterile seawater) for 3 h, while the other half were exposed to OsO₄ vapours for 24 h before fixation in glutaraldehyde in the same way. All samples were dehydrated using an ethanol series at ±4 °C. The samples were stored in 100% ethanol at ±4 °C before being critical point dried and exposed to ruthenium vapour for three hours (van der Merwe and Peacock, 1999). At least two replicates were then mounted onto aluminium stubs for viewing with a JEOL JSM-840 SEM at an acceleration voltage of 5 kV.

3. Results and discussion

All species sampled, except for the Corallinaceae, showed better preservation of the EPS from the osmium vapour pretreatment and is consistent with the results of McKeekin et al. (1979). The most dramatic difference between the treatments was seen in *Spyridia hypnoides* (Fig. 1) where the improved preservation of the biofilm and associated EPS by OsO₄ is clearly evident. The mucilage containing EPS covering of *Caulerpa filiformis* was completely lost from the untreated samples (data not shown), but preserved on the treated ones (Fig. 2). The loss of some of the biofilm cells with the damage to the mucilage layer indicates that they were bound together. This exemplifies the intimate association of biofilm and seaweed. The biofilm on members of the Corallinaceae was mostly confined to the joints between the calcified thalli and no differences between the osmium-treated and untreated samples were observed (data not shown). There were no cases where the biofilms on untreated samples were better preserved than the treated ones.

The results obtained with the osmium vapour treatment were similar to the freeze-dried samples of Richards and Turner (1984) where sheets of EPS were seen peeling from a bed of underlying bacteria. They found that glutaraldehyde treatments removed much of the EPS and created fibrillate artefacts. These fibrils were also seen on some of the OsO₄ treated and untreated samples (data not shown). The osmium treatment did not completely prevent damage, but limited the damage and enabled one to distinguish different layers of the EPS. These
results confirm the speculation by Cribb et al. (2004) that SEM preparations may be improved by using OsO₄ and glutaraldehyde simultaneously as fixatives (although we used OsO₄ as a pre-treatment). At least two types of EPS were observed in the treated samples, a fluffy or downy type seen on S. hypnoides and Gelidium abbottiorum (Figs. 1 and 3) and a flat, sheet-like one of varying thickness on G. abbottiorum, Osmundaria serrata and Hypnea rosea (Figs. 4, 5, 6, 7). The latter ranged from gossamer (Figs. 4, 5, 6) to a relatively thick carpet (Fig. 7). A folding back of a sheet-like EPS exposed parts of some bacteria (Fig. 6). The fluffy type was most common, with the thick sheets being rarest. In some instances, the two forms were seen on the same sample with the fluffy type appearing to lie on top of the sheet-like one (Figs. 8 and 9). This complex multilayered EPS structure as opposed to a simple more homogeneous structure is consistent with the production of multi-layered capsules found in some bacteria (Omar et al., 1983). It may indicate different viscosities of the EPS that were never before thought possible to distinguish (Sutherland, 2001). Thus, the term “slime layer” should rather be in the plural, “slime layers”.

A diversity of differently shaped bacterial cells was seen on the macroalgae. Cocci, rod-shaped and filamentous cells dominated, but C-shaped bacteria were also seen on some seaweeds (Fig. 3). The latter were most likely Cyclobacterium marinus, because they grow on macroalgae, form coils and have rounded ends (Holt et al., 1994). The other cells are almost impossible to identify, for example filamentous cells may be Leucothrix mucor or Erythrobacter longus which are commonly found on seaweeds (Harold and Stanier, 1955; Holt et al., 1994). However, some bacteria are rod-shaped in normal cultural conditions, but form filaments up to 1 mm long in response to toxicants at concentrations below those found to inhibit growth (Beveridge et al., 1991). Since many algae release toxic chemicals (Hellebust, 1974), some of the filamentous bacteria seen on the macroalgae may grow as different shaped cells in culture, assuming that they could be isolated at all. Thicker filaments resembling fungal hyphae were also seen (Fig. 9).

Diatoms of the genera Campyloneis, Plagiogramma and Thalassiosira were seen on some of the samples (data not shown) and there was also an unidentified filter-feeding animal on Spyridia cuppressina (Fig. 10). The epidermis of this animal was nano-rough and only the tips of bacteria were attached to it (Fig. 11). These nano-structures sensu Baum et al. (2002) are similar to those found on the pilot whale (Globicephala melas) and are thought to keep their surfaces relatively clean by preventing the attachment of bacteria (Baum et al., 2002). The staff at the Electron Microscopy and Microanalysis Unit at the University of Pretoria (UP) for their help and Dr S Sym, School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, for identifying the diatoms.

In conclusion, the stabilisation of lipids in the EPS using OsO₄ better preserved the biofilm and indicates that lipids play a role in maintaining its cohesiveness and integrity. Confirmation of the multilayered nature of the EPS of biofilms is needed. Diverse and complex biofilm communities were seen on the macroalgae.

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