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Correlation between Localized Anodic Areas and *Oceanospirillum* Biofilms on Copper*

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The marine bacterium *Oceanospirillum* produces copious amounts of exopolymer when grown on copper surfaces and binds Cu^{+2} from the substratum. The organism and associated exopolymers result in local anodic regions that can be detected by scanning vibrating electrode microscopy. *Oceanospirillum* was grown in small laminar flow cells with two carbon sources on copper and 316 stainless steel as substrata. The chemical composition of the exopolymer varied with growth medium, but not with substratum. Exopolymers from cells grown in glutamic acid medium on both substrata contained glucose with no other sugar monomers or uronic acids. The quantity of exopolymer did vary with substratum and copper promoted greater polymer production that stainless steel. Published by Elsevier Science Limited

INTRODUCTION

Copper and copper alloys have long been successfully used in marine applications because of good mechanical strength and workability, corrosion resistance, electrical and thermal conductivity and resistance to macrofouling. Applications include distribution and piping systems, heat exchangers, intake screens and sheathing for splash zones, as well as offshore structures (Blunn, 1987; Moreton and Glover, 1980; Kirk, 1987). The corrosion product cuprous oxide, Cu_2O, forms on all copper surfaces in oxygenated seawater (North and Pryor, 1970). Copper ions and electrons pass through this layer, dissolve and precipitate as a layer of $Cu_2(OH)_3Cl$ that retards anodic dissolution and the rate of oxygen reduction (Schiffrin and deSanchez, 1985). Corrosion failures in copper alloys are associated with the breakdown of $Cu_2(OH)_3Cl$ by mechanical (Sato and Nagata, 1978), chemical (Syrett *et al.*, 1979), or biological means (Fischer *et al.*, 1981).

Biofilms develop on all surfaces exposed to natural marine environments. Cuprous oxide is commonly used in marine antifouling coatings to retard macrofouling. However, bacteria, microalgae, protozoa and their exudates readily form slime layers on copper-containing surfaces (Gerchakov & Udey, 1981). Although Leifson *et al.* (1964) described "large spirilla with characteristic curved soma and lophotrichous flagella" as rare in seawater, *Oceanospirillum* has been invariably isolated from United States Navy platforms in locations throughout the world. The platforms had in common that they were all coated with copper-containing antifouling coatings. *Oceanospirillum* (Fig. 1) is an aerobic, heterotrophic, motile, helical bacterium with rigid cell walls and requires sodium chloride for growth (Bergeys Manual, 1975). It has been demonstrated that *Oceanospirillum* not only colonizes copper-containing surfaces, but also detoxifies copper ions from the substrata so that non-copper tolerant bacteria settle on surfaces previously colonized by *Oceanospirillum*. Wagner *et al.* (1991) demonstrated that the organism produces copious amounts of exopolymer when grown on copper and that the organism and associated polymers bind copper ions from solution or substrata. They used electrochemical techniques to demonstrate that the organism increased the corrosion rate of 99% copper in a seawater medium.

Daniel *et al.* (1980), Chamberlain *et al.* (1988), and Geesey *et al.* (1986) investigated the

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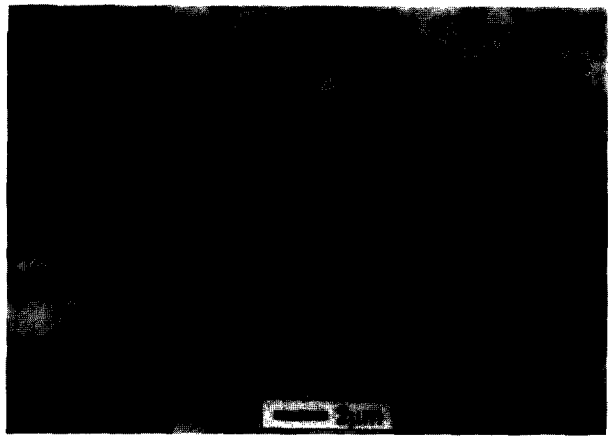

Fig. 1. *Oceanospirillum* grown on copper surface.

relationship between microbial exopolymers and copper corrosion in fresh water systems. Each group emphasized the role of exopolymers in binding copper and the formation of copper concentration cells. The experiments described in this paper were designed to determine the nature of the *Oceanospirillum* exopolymer and the spatial relationship between *Oceanospirillum* biofilms and anodic currents.

MATERIALS AND METHODS

Bacterium and medium

Oceanospirillum was isolated at the Naval Research Laboratory, Stennis Space Center, MS, and maintained by weekly transfers on marine agar and incubation at 25°C. Liquid medium was used for all experiments and contained the following (g/l): sea salts 40, glutamic acid 1.81, ammonium chloride 0.0064, sodium phosphate monobasic 0.00138.

Exopolymer characterization

Exopolymer for characterization was generated by growing *Oceanospirillum* on 99% copper or 316 stainless steel coupons in laminar flow cells described previously by Mittelman *et al.* (1992). Flow cells filled with 10 cm^3 of medium were inoculated and cells grown overnight. Biofilms were allowed to grow for 168 h before being harvested by sonication (Arrage *et al.*, 1995). Biofilms were resuspended in sterile seawater and treated with 0.8 cm^3 of 5 M sodium chloride and 0.5 M EDTA to aid removal of exopolymer from bacteria. Bacterial cells were then removed by centrifugation. The supernatant was treated with 3 volumes of propan-1-ol at 4°C to precipitate exopolymer which was then dialyzed against running tap water overnight followed by dialysis against 18 MΩ water. Any glucose extracted from the cells during sonication would be removed during dialysis. Purified exopolymer was then lyophilized and stored at 4°C. Monomer characterization was carried out as described previously (Franklin *et al.*, 1994). In summary, exopolymer was hydrolyzed using 4 M trifluoroacetic acid at 100°C for 2 h. Lipids were removed by passing through a C_{18} column before high pressure anion exchange chromatography separations of 25 μl samples were performed with a pellicular CarboPac PA1 anion exchange column on a Dionex (Dionex Corp., Sunnyvale, CA) series 4500 high-pressure liquid chromatography system (1000 molecular weight cut-off). X-ray absorption near edge structure (XANES) was used to determine the speciation of copper within exopolymers (Bianconi & Marcelli, 1992).

Scanning vibrating electrode microscopy (SVEM) studies

Coupons colonized by *Oceanospirillum* and controls in sterile media were examined with the SVEM system (Angell *et al.*, 1994). The SVEM system consisted of a 20 μm stainless steel microprobe electroplated with platinum black that was vibrated in two orthogonal directions while scanned over the coupon surface using computer control. The probe is capable of capacitively measuring local current density at each point of the scan, allowing the computer to generate maps of the local current densities over a surface. The vibrating probe system is mounted under a microscope fitted with reflected light Nomarski and an epifluorescence system equipped with a 40¥, 2 mm working distance water objective and an imaging system. Current density scans can be concurrently mapped with images of the surface. The SVEM maps any localized currents. Cathodic currents are dissipated over the surface and not localized. The 13 mm diameter coupon was scanned in a central 9 mm^2 area to eliminate edge or crevice effects.

RESULTS AND DISCUSSION

Biofilm generation and characterization

Biofilms formed readily on both copper and stainless steel coupons. The color of extracted

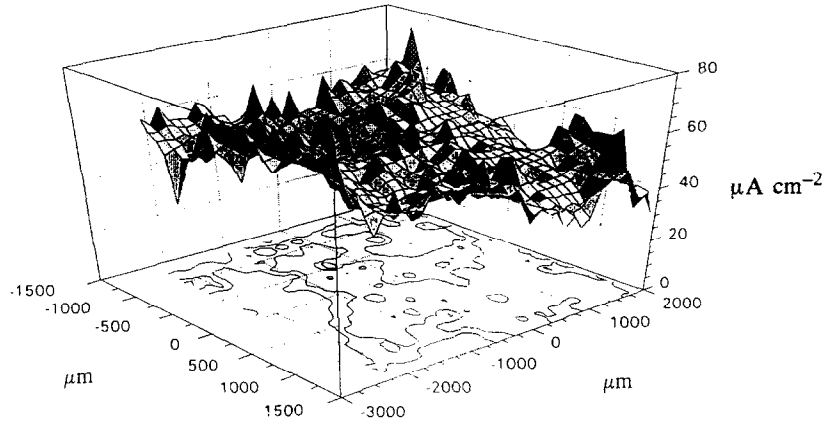

Fig. 2. Current density scan over a copper coupon exposed to *Oceanospirillum* for 160 h showing several anodic peaks with a large anodic area in the back corner.

biofilm was variable, particularly in the case of the copper. When the medium was recycled through the system containing copper coupons, it turned blue due to the interaction of copper ions with functional groups in the medium. Extracted biofilms were also blue, especially in areas where air had become trapped in pockets over parts of the coupon surface.

Following extraction and purification, exopolymer from each system was weighed. Exopolymer recovered from copper and stainless steel coupons weighed 9.6 and 4.3 mg, respectively. The recoveries corresponded to 0.74 mg cm^{-2} on copper and 0.33 mg cm^{-2} on stainless steel, indicating that roughly twice as much polymer was generated under similar conditions on copper compared with stainless steel.

Analysis of polymer recovered from both surfaces revealed the presence of glucose units. No other monosaccharides were detected using the Dionex system that has a sensitivity of approximately 10, 50–100, 1–5 picomolar for neutral, acidic and amino sugars, respectively. No uronic acid residues were detected in any of the exopolymers. Their absence was confirmed using gas chromatography mass spectrometry. XANES spectra of exopolymers indicated the presence of bound Cu^{+2}.

SVEM observations

Sterile control

SVEM studies of copper exposed to sterile medium showed no localized anodic activity after 200 h when medium volume levels were maintained. Under normal operating conditions no coloration of the medium in SVEM experiments was noted unless fluid levels were reduced, forming a thin film of liquid over the copper coupons, in which case a heavy blue coloration was noted. Occasionally a thin gel formed. Scans conducted immediately after the liquid level was restored showed strong anodic activity which persisted for up to 18 h.

Oceanospirillum *culture*

When copper coupons were inoculated with *Oceanospirillum* several localized anodic regions were observed after about 48 h. Anodic regions persisted until termination of the experiment at 180 h. Anodic currents were always present but their intensities varied. One anodic region was stronger and more persistent than others (Fig. 2).

At the conclusion of the experiment, coupons were stained with a fluorescent stain (Live/Dead Backlight Viability Kit, Molecular Probes, Eugene, OR) for detection of viable bacterial cells. All bacteria on the surface were alive. Scanning the sample using the 40Ɣ water immersion lens demonstrated that bacterial colonization was not uniform. Instead, micro- and macro-colonies had formed. Areas with higher densities of bacteria correlated with anodic regions.

CONCLUSIONS

The marine bacterium *Oceanospirillum* produces copious amounts of exopolymer when grown on copper surfaces, binds Cu^{+2} from the substratum and produces local anodic regions on copper surfaces detected with SVEM. Analysis of the exopolymer produced in biofilms formed by this bacterium on copper surfaces indicated the

presence of glucose. No other sugar monomers or uronic acids were detected.

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